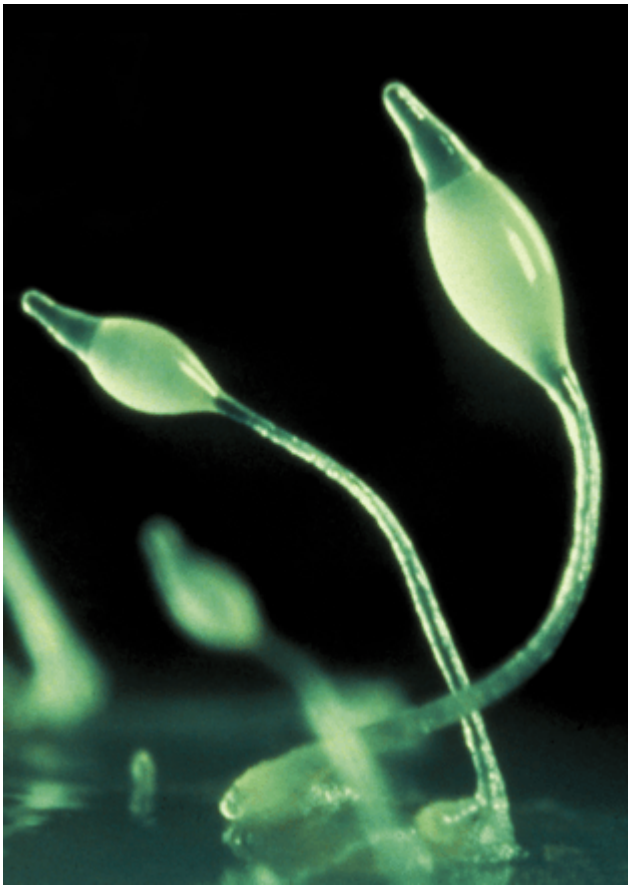
MOLECULAR SYSTEMATICS OF DICTYOSTELIDS: A CASE FOR SINGLE GENUS



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A thesis submitted in fulfilment of the requirements of Anglia Ruskin University for the degree of Doctor of Philosophy.

“My fascination with the experimental analysis of slime molds is based on the idea that one wants to find out about slime molds. Slime molds are a model for the study of slime molds.”

(Bonner 1991)

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ABSTRACT

The cellular slime moulds or dictyostelids, are a major group of edaphic (soil-dwelling) unicellular eukaryotic micro-organisms which feed on bacteria in the humus layer of tropical and temperate soils. The extraordinary behaviour of dictyostelids where solitary, predatory amoebae become social, forming a multi-cellular spore-producing 'organism' when their food supply becomes limited has fascinated generations of researchers. Since their discovery the taxonomy of the cellular slime moulds has been largely based on morphological features, revised many times, but still remains unresolved and controversial. One focus of discussion is the controversial relationship between the two principal genera of *Dictyostelium* and *Polysphondylium,* which by virtue of a common amoeba type are placed in the same family - Dictyosteliaceae. Despite the current distinction between *Dictyostelium* and *Polysphondylium* it is impossible to definitively resolve these genera, or indeed the broader classification of cellular slime moulds, using only morphological features. Given that dictyostelids are both of major ecological importance and often used as a model eukaryotic system, it has become imperative to resolve these taxonomic uncertainties and to finally establish the validity of one or two genera.

This study used two PCR based techniques: (1) direct sequencing of the ITS 1 and ITS 2 regions of the rDNA complex together with (2) ISSR-PCR, a standard molecular technique but rarely applied to the dictyostelids. The latter requiring the development of a working protocol before it could be implemented. The sequence data from 51 different dictyostelid species and isolates was aligned with CLUSTAL and analysed via PAUP. This study clearly demonstrates that molecular markers cannot distinguish *Dictyostelium (sensu stricto)* from *Polysphondylium* (*sensu stricto*): indeed it presents evidence to support the existence of a single genus. The implications of these results are unequivocal: the current systematics of cellular slime moulds, based on morphological characters, must be revised.

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CHAPTER ONE: THE BIOLOGY, ECOLOGY AND PHYLOGENY OF THE DICTYOSTELIDS

1.1 What Are Dictyostelids?

The dictyostelids or cellular slime moulds are unicellular, eukaryotic organisms classified in the group of Mycetozoa. Shown below is the scientific classification of the dictyostelids:

Domain: Eukariota

Kingdom: Amoebozoa

Phylum: Mycetozoa

Class: Myxogastria (acellular slime moulds)

Class: Protostelia (cellular slime moulds)

Class: Dictyostelia (cellular slime moulds)

Order: Dictyosteliida

Family: Dictyosteliidae

Genus: *Dictyostelium*

Genus: *Polysphondylium*

Genus: *Caenonia*

Family: Acytosteliidae

Genus: *Acytostelium*

(adapted from Raper, 1984)

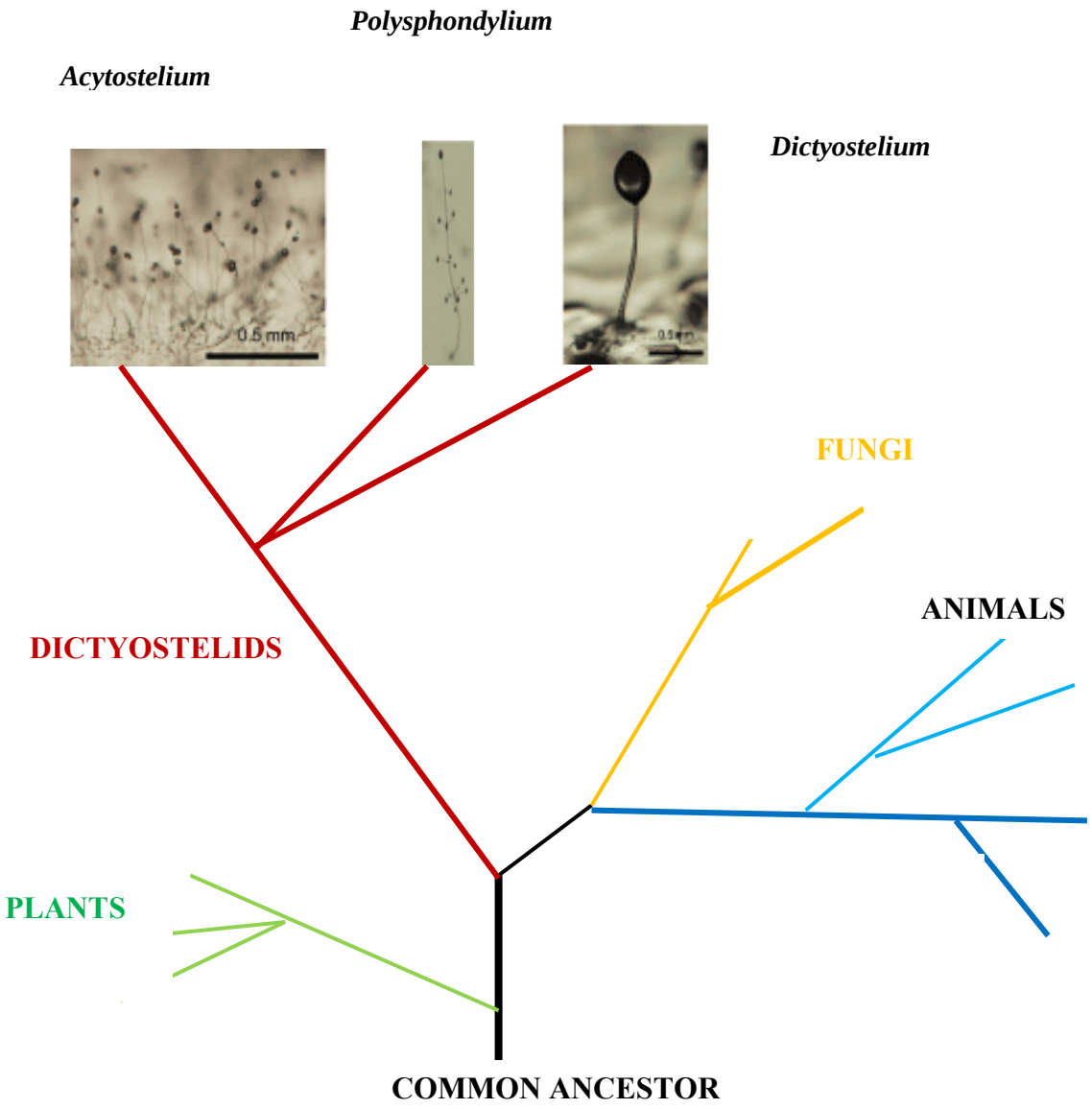


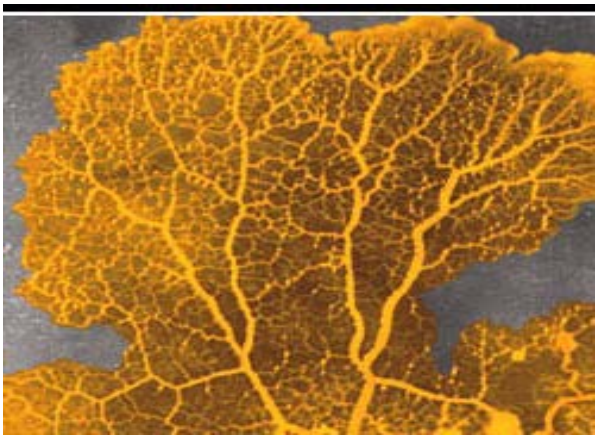
Figure 1.1 TREE OF LIFE - showing the hypothetical phylogenetic position of dictyostelids within the eukariota (adapted from Baldauf *et al.*, 1993 and Eichinger *et al.*, 2005). The three dictyostelid genera (*Dictyostelium*, *Polysphondylium* and *Acytostelium*) form a branch between plants and fungi (see section 1.10).

Amoebozoa are a group of amoeboid protozoa, most of which move by means of cytoplasmic flow. This Kingdom contains the Phylum Mycetozoa, a group of organisms which reproduce via spores, and consists of three classes: Dictyostelia, Myxogastria and Protostelia. Dictyostelids or slime moulds are strictly amoeboid: their life cycle proceeds from unicellular free living amoebae, to the multi-cellular fruiting body bearing spores (the life cycle is described in section 1.4). The myxogastrids, including *Physarum polycephalum* are amoeboflagellates, so- called 'true' or 'plasmodial' slime moulds. The protostelids, a group of primitive Mycetozoans, first described in 1960's, are a morphologically diverse group of organisms exhibiting various combinations of myxogastrid and/or dictyostelid traits as all Mycetozoa share a structurally similar fruiting body. They are believed to be ancestral to both cellular slime moulds and the Myxomycetes. Examples of Mycetozoa are shown in figure 1.2.

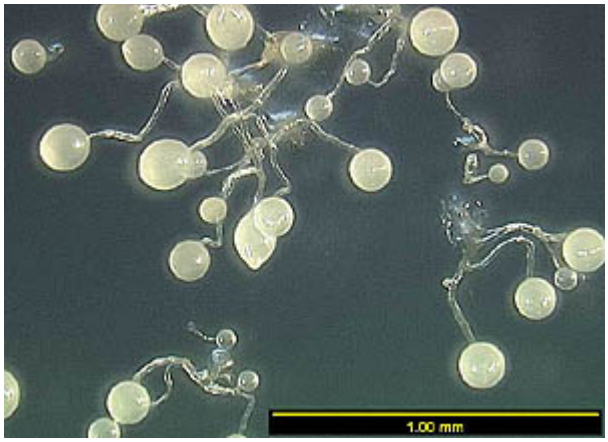
Dictyostelids taxonomy has long been a source of confusion and thus has been revised several times. Some morphological characters e.g. fungal-like plasmodia (myxogastrids), motile animal-like slug (dictyostelids) or plant- like fruiting body have led to them being classified as plants, animals or fungi. Whittaker (1969) for example, placed slime moulds together at the base of the fungi; but it was Olive (1969), who finally classified them as Protists, arguing that they had nothing in common with fungi. The taxonomy and phylogeny of dictyostelids is further described in section 1.7 and 1.8.

The dictyostelids, e.g. *Dictyostelium discoideum* the best studied species, are a group of simple, heterotrophic organisms which feed on a wide range of bacteria in the upper soil layers, particularly amongst decaying leaves (Raper and Smith, 1939). These organisms have fascinated generations of researchers because of their extraordinary behaviour, where during food depravation, predatory individuals become social and form a multi-cellular organism which then undergoes cell differentiation. The first cellular slime mould was isolated by Brefeld (1869) while investigating coprophagous organisms. He named it *Dictyostelium mucoroides* because of the net-like structure of the stalk and the superficial resemblance to the fungus *Mucor*. Although that work started further work on cellular slime moulds, the most significant discovery was made in 1933 when Raper isolated a new species, *D. discoideum* (Raper, 1935).

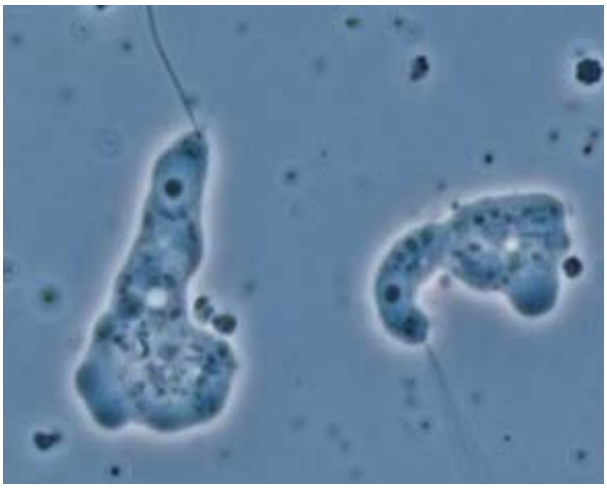
Figure 1.2 Examples of the three Mycetozoa classes



(a) *Physarum polycephalum* (Myxogastrida) – a common species with a crawling plasmodium mass (after Anonymous, 2007); magnification x 40.



(b) *Dictyostelium sp.* with fully developed fruiting bodies (after Landolt, 2008); magnification x 40.



(c) *Planoprotostelium sp.* (a protostelid) showing the flagellate stage. Protostelids may become amoeboid and form a stalk with a distal cyst (after Patterson, 2001); magnification x 200.

The distinct growth habit of this organism made it an ideal candidate for laboratory research with the result that most information available on the life cycle of dictyostelids pertains to this species, which is now a focus of developmental and molecular biology. *Dictyostelium discoideum* is a single primitive eukaryote studied at the cellular, biochemical and genetic level, often used to model the mechanisms of chemotaxis, cell movement and signalling. Moreover, genes involved in pattern formation or cell differentiation, especially programmed cell death (apoptosis), have prompted a great research interest. For instance, around 20% of dictyostelid cells altruistically sacrifice themselves, undergo apoptosis and die during the stalk formation (part of the fruiting body) while the rest become spores, highlighting the role of apoptosis in the formation of a reproductive organ (Gilbert and Scott, 2006). Furthermore, the dictyostelids life cycle is notable for comprising transition from unicellular to a multi-cellular organism making it a valuable model for developmental and physiological processes in other organisms. *D.discoideum* for example, shares a similar cytoskeleton and cellular processes with mammalian cells and may become a host for *Legionella,* the bacterium causing Legionnaire's disease in humans, and therefore a model system to study the infection processes of phagocythosis, endocytosis or chemotaxis (Bruhn *et al.,* 2008). *D. discoideum* is also an interesting example by which to study the evolution of cooperation and 'cheating mechanisms' (Strassman, *et al.,* 2000; Brännström and Dieckmann, 2005).

Studies on *D. discoideum* have prompted several significant discoveries in molecular research. The first, in the 1960s, was the isolation of axenic mutants which can be cultivated on a defined medium in the absence of bacteria, their usual nutrient source (Sussman, 1967). The discovery of these mutants opened the way to new studies in many areas previously closed by the need for bacteria for culturing *D. discoideum*. In the next 1984 paradigm shift, foreign DNA was introduced into *D. discoideum* cells (Nellen *et al*., 1984). More recently restriction enzyme mediated insertion mutagenesis has been used to search for genes regulating development (Kuspa, 1994), founding the *Dictyostelium* Genome Project (Kay and Williams, 1999; Williams, 1999). The international initiative to sequence the *D. discoideum* genome launched in 1998 has resulted in the completion

of the sequencing of chromosomes 1 and 2 of 6 and shed more light on the structure of the *Dictyostelium* genome (Eichinger *et al.,* 2005). The genome comprises 45 Mb of haploid nuclear DNA, 55.5 kb mitochondrial DNA genome and multicopy 88 kb extra-chromosomal rRNA genes. *Dictyostelium* chromosomes are gene dense; coding genes represent 62% of the whole genome encoding ca. 12,500 proteins, many of which contain long, repetitive amino-acid tracts. The dictyostelids genome is A & T rich (78%), especially in the generally short introns, while the coding regions appear skewed towards G & C bases, which relates to codon usage (Eichinger *et al.,* 2005). The genome is also rich in complex repeats, some of which may serve as centromeres, while partial copies of extra-chromosomal rDNA elements were found at the ends of chromosomes suggesting a novel telomere structure (Eichinger *et al*., 2005). Non-coding simple sequence repeats regions are A & T rich (99 %), with dinucleotides and longer 3-6 nucleotide motifs being predominant in these sequences (Eichinger *et al.,* 2005). The Genome Project also resolved the controversial issue regarding the number of chromosomes, confirming the existence of only six and not seven suggested by earlier research (Loomis *et al*., 1995; Loomis *et al.,* 1994; Eichinger *et al*, 2005).

1.2 Nineteenth to 20th Century: Outline History of Dictyostelid Research

Dictyostelids were first described in 1869 when Oskar Brefeld isolated and described *Dictyostelium mucoroides*. Brefeld was examining horse dung for fungi when he found tiny fruiting bodies resembling the sporangia and sporangiophores of a fungus, *Mucor mucedo*, and initially thought that this new organism belonged to the fungal kingdom, but several features indicated otherwise. For instance, this organism's "sporangia" (structure bearing spores) had no enveloping walls and there were no vegetative hyphae. Additionally, spores produced small ameboid cells upon germination and their spore-bearing stalks consisted of packed parenchyma-like cells. The generic name, *Dictyostelium*, was later chosen by Brefeld to indicate the net-like appearance of the organism`s fruiting body.

On the basis of perceived parallels between the behaviour of the free-living amoebae of the newly discovered organisms as well as the early life cycle stage of the acellular or true slime moulds, the Myxomycetes, Brefeld concluded that the new species represented a bridge between the Myxomycetes and the fungus *Mucor.* This was however, only partly true because he had wrongly concluded that when the cells of the new species aggregated, their cytoplasm fused to form a true plasmodium, a structure which taxonomically should be placed with Myxomycetes. He corrected that error in his later work after van Tieghem (1884) had presented a proof that the aggregated *D.discoideum* myxoamoebae never fused so did not form a true plasmodium. He proved it by placing the so called "plasmodium" in a drop of water and observed that the cells readily separated and had thus retained their individuality, which was not the case for a true plasmodium. As a result of van Tiegham's work the genus *Dictyostelium* was removed from the order Myxomycetes and placed in a new order Acrasieae within the Mycetozoa. Later in 1884, Brefeld confirmed van Tieghem's observation regarding the unique character of the aggregating cells and introduced the new term "pseudoplasmodium" to describe that process. Moreover, he also discovered a new species with spore-bearing branches arranged in regular whorls for which he erected a new genus *Polysphondylium* containing the new species, *Polysphondylium violaceum.*

Mycetozoan research progressed slowly but by 1902 there were eleven described species, with only one recorded in England (Olive, 1902). Most of the research was focused on the growth conditions, particularly the influence of different soils and bacteria on growth and development of different species. Furthermore, in 1917, Skupienski initiated studies on the sexuality of slime moulds (Raper, 1984), and ca. 1922 Oehler, working in Wurzburg, Germany isolated *D. mucoroides* from forest soil and compost. The years 1924-1925 brought the seminal work on biology and morphogenesis of *D. mucoroides* published by von Schukmann.

Except for some pioneering work done on the Dictyosteliaceae by Olive (1902), dictyostelids attracted very little attention in America until 1920s, when Charles Harper of Columbia University started to publish on the morphogenesis of *Dictyostelium* and

Polysphondylium species. In 1930 Kenneth Raper, then a young researcher working in the Department of Agriculture (Columbia), inspired by Harper's work, together with Charles Thom, began isolating and studying dictyostelids. Whilst on holidays in North Carolina, he collected soil samples at Little Butts Gap from which he isolated a new species *Dictyostelium discoideum* (Raper, 1935). Its comparative simplicity of structure and ease of culture makes this species an ideal model organism to study intracellular communication and differentiation. Around this time, and parallel to Raper's study, Arndt in Germany was studying the growth and development of *D. mucoroides* (Arndt, 1937). His remarkable work was published posthumously and remains the most comprehensive work ever done on *D.mucoroides.* Other major contributions to the biology of slime moulds include Olive's (1975) monograph on the Mycetozoa with a key section on the structure, life cycle and taxonomy of Dictyostelia, and Hagiwara (1979) who identified 20 new species, mostly from Japan and China and made a detailed study of *Polysphondylium pallidum*.

1.3 Distribution and Ecology of Cellular Slime Moulds

The widespread distribution and extensive occurrence of cellular slime moulds in the world`s soils, was first presented by Raper then Singh (Raper and Thom, 1932; Smith, 1939; Singh, 1946; 1947). Dictyostelids were originally considered to be coprophilous organisms (Olive, 1902) and that herbivore dung was their natural habitat (Raper, 1984), because the first species of *Dictyostelium* and *Polysphondylium* were isolated from dung. It was however later noted that slime moulds could also be isolated from soil. Indeed it is now well established that cellular slime moulds represent a normal component of soil and leaf litter micro-flora (Cavender and Raper, 1965; Raper, 1984; Stephenson and Landolt, 1996), and the major component of bacteriophagic soil micro-flora. Dictyostelids serve as bio-indicators of soil microbial activity (Landolt *et al*., 1992; Swanson, 1992), by stimulating mineralization and decomposition (Stephenson and Cavender, 1996), as well as by controlling and modifying bacterial soil populations (Fenchel, 1987; Stephenson and Cavender, 1996). There are various environmental and nutritional factors which

influence the abundance and diversity of soil dictyostelids. As was suggested by Raper (1984), the most important factors are the soil moisture, pH, temperature and food availability; as well as the availability of the plant cover with its associated decomposed matter and dependent bacterial content. As early as 1947, Singh showed the important influence of soil moisture and the content of the different types of soil bacteria on dictyostelid growth regulation, suggesting a minimal moisture content of 15% to promote growth.

Singh (1947) noted that both the rate of dictyostelids growth and fruiting body production time varied with the bacterial host and the soil or agar- medium type. Although dictyostelids can feed on a wide range of bacteria, his experiments showed that some types are consumed faster than others, with capsulated bacteria being much more difficult to digest, and that bacteria grown on some substrate types can be a much better nutrient source for the amoebae than those grown on others. Gram-negative bacteria such as *Escherichia coli* or *Klebsiella* species are the most suitable food source though other types can be used if they are small and normally produce moist, non-gummy colonies, that can be easily digested by amoebae (Raper, 1984). Later studies on the effect of other factors on dictyostelids growth have shown that a favoured species dependent pH was in the range of pH 5.0 to 8.0, with an optimum of ca. pH 6.0 for both dictyostelids and bacteria (Kitzke, 1951). Maximum growth and development occurs in a nearly moisture saturated atmosphere and an optimum temperature range of 20 to 25°C. Some species, will however grow optimally outside of this range, *D. septentrionalis,* for example grows best at 15 to 17° C (Cavender, 1978) while the *Polysphondylium pallidum* ”Salvador” strain, fruits at 37°C (Raper, 1984).

Both Kitzke (1951) and Raper (1951) have shown that dictyostelids are common inhabitants of forest soils, particularly the surface soils of deciduous forests. Yet, it was Cavender who initiated the comprehensive ecological study of these organisms, initially in Wisconsin from where he extended his research throughout North America. Parallel studies by Hagiwara (1971), Kawabe (1980), Kanda (1981), Traub and Hohl (1976) and Traub, Hohl and Cavender (1981), and other have helped to develop a clear picture of

global dictyostelid distribution, their occurrence in different habitats and at different soil depths, as well as seasonal variations in numbers and/ or species.

Both Cavender's and Raper's work showed that dictyostelid species diversity tends to decrease with soil depth, with 25 cm being the maximal depth from which species have been isolated. Furthermore, species which occur in grasslands and other open environments subject to strong sun and winds are more likely to be smaller taxa. The larger and more diverse species usually occupy temperate, moist habitats in leaf-protected humus near the soil surface. There is also a significant correlation between soil nutrient level and dictyostelid species diversity particularly with respect to high organic matter content but also with low levels of non-organic nutrients such as calcium, potassium and phosphorus (Raper, 1984). This has led to the suggestion that temperate regions, and in particular woodland, with their increased level of organic matter, are preferred dictyostelids habitats (Feest, 1987). This does not however appear to be a universal truth as high diversities and densities have been found in both desert and marshes (Benson and Mahoney, 1977), Alaskan tundra (Stephenson *et al*., 1991), and the jungles of South America (Waddell, 1982**)**. Cavender's summary (1973) suggested another correlation between latitude, altitude and dictyostelid species diversity, noting diversity decreased along a tropical/ arctic cline (Raper, 1984). Many of the known dictyostelid species can be divided into four categories based on their distribution in forest soils (Swanson, *et al*., 1999; Swanson *et al.,* 2002).

Cosmopolitan - occupy most of the temperate deciduous forest soils, including *P. pallidum, P. violaceum, D. mucoroides, D. purpureum* and *D. minutum* as examples.

Disjunct - occur in similar habitats of widely separated regions for example *D. discoideum* in forests of eastern North America and Japan but not in temperate deciduous forests of India. This distribution could suggest a once close relationship between two populations, long-distance dispersal or the existence of a now extinct intermediate population (Swanson *et al*., 1999).

Restricted - endemic to certain specific regions. Such species may be relict by local geographical or environmental criteria (Watson, 1995). Examples include *D. fascicularum* in Switzerland, Germany and Great Britain and *D. brunneum* in Japan.

Pan-tropical - occur in many tropical deciduous and seasonal evergreen forest soils. These species are rarely, if ever, isolated in any parts of the temperate zone. An example is *D. lavandulum* from the Peruvian Amazon.

These biogeographic divisions are based on very limited data and are, to some extent, arbitrary. A few dictyostelid species do not display any clear distribution patterns probably as a result of other as yet unquantified variables (Swanson *et al*., 1999), and thus cannot be grouped into any of the above categories. However, the global distribution of the dictyostelid species, their aforementioned edaphic (soil-dwelling) ecology, simplicity of structure and their lack of inherent long distance dispersal mechanisms, all suggest that these organisms may show an ancient division and have been dispersed by other mechanisms such as continental drift and natural vectors. Dictyostelids spores are slime-coated and so too heavy to be carried by wind (Olive, 1975). There is evidence that edaphic/ hemi-edaphic (leaf litter and soil inhabiting) invertebrates could function as phoretic (a symbiotic dispersal relationship) vectors of dictyostelid species, which either adhere externally to the invertebrates or present in their gut contents as spores. Indeed, dictyostelids have been isolated from field-collected pillbugs (Crustacea) and earthworms (Oligochaeta) gut content (Huss, 1989), even rodent faeces and terrestrial salamanders (Stephenson and Landolt, 1992). It has also been suggested that ground-feeding migratory birds are potential long-distance dictyostelids dispersal vectors as it has been established that amoebae, macrocysts and spores can survive passage through the avian digestive tract and remain *in situ* for long enough to be effectively transported to distant habitats (Feest, 1987). Additional extra-survival mechanisms have been proposed including the production of macrocysts (see section 1.4.2) which have an extensive cellulose coat that could provide protection in unfavourable climatic conditions. Moreover, the maturing zygote feeds on endocytes, which probably helps it to survive subsequent starvation.

1.4 Dictyostelid Life Cycle

As discussed above, the classification of dictyostelids is mainly based on morphological characters of fruiting bodies produced in the final stage of their asexual life cycle. Although asexual life cycle is the most common form of reproduction within dictyostelids, the sexual reproduction by macrocyst formation is also known in some dictyostelid species e.g. *D. discoideum, D. rosarium, D. purpureum, Polysphondylium violaceum* or *P. pallidum* but requires certain environmental conditions such as darkness or excessive humidity. Aspects of dictyostelids sexual reproduction, however, have been sparingly used in their taxonomy (Raper, 1984). Although the best known dictyostelid species *Dictyostelium discoideum* has only two alternative reproduction cycles, (1) asexual fruiting body and (2) sexual macrocyst formation, many other dictyostelid species, e.g. *P.pallidum* have a third asexual cycle, (3) the microcyst cycle, which starts when some myxamoebae fail to enter the aggregation stage, but instead form microcysts (the myxamoebae encysted as individuals). Considering the unique phylogenetic position of cellular slime moulds and the contribution made to the understanding of the mechanisms and evolution of sexual reproduction systems made by the analysis of these systems, the following pages introduce two most studied reproduction systems (asexual fruiting body and sexual macrocyst) in more detail.

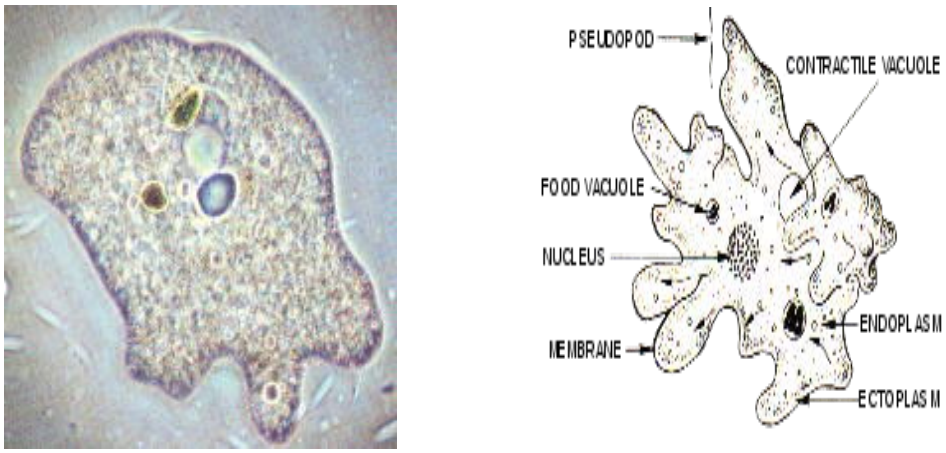
1.4.1 The asexual life cycle

The asexual life cycle of the cellular slime moulds is divided into two temporally distinct phases. In the first trophic stage they exist as a group of amoeboid 'Myxamoebae' cells. The amoebae are ca. 15-25 μm in diameter and have filose pseudopodia, are normally haploid, though diploid amoebae do occur (Wilson and Ross in Newell, 1978). Figure 1.3 shows the amoeba stage and its organelles. When the food supply is exhausted or other unfavourable environmental conditions prevail, myxoamoebal behaviour changes and dictyostelid enter the second phase of a multicellular spore-producing organism. This is achieved by amoebae becoming attracted to each other in great numbers, aggregating and ultimately forming a collective mass called a pseudoplasmodium in which the constituent

cells ultimately become the fruiting body or sorocarp. It is this stage that gives the organism a superficial resemblance to a fungus.

Figure 1.3 The amoeba stage

a) **b)**

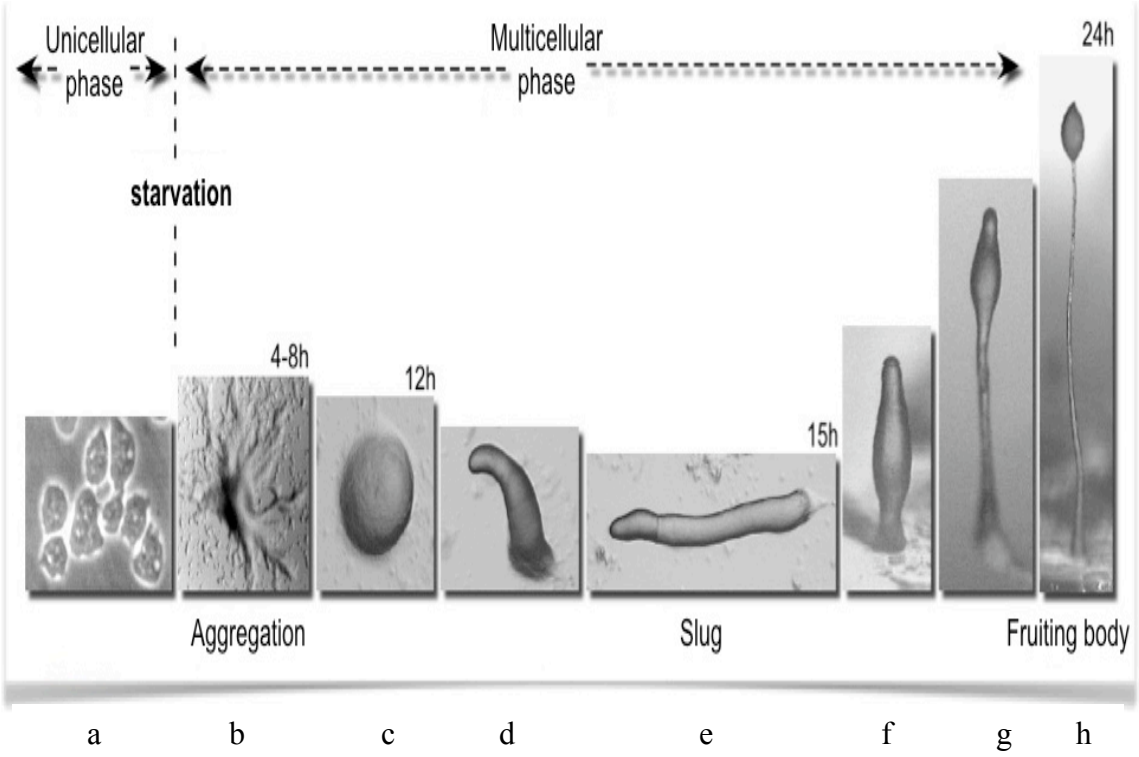


Amoeba stage; (a) habitat; magnification x 200; (b) descriptive morphology (after Anonymous 2009).

These multi-cellular developmental stages are 1) chemotactic aggregation into mounds of < 100,000 cells; 2) transformation of mounds into slug structure surrounded by the slime sheath; 3) an optional migratory phase (depending on the dictyostelid species) and finally 4) the culmination and terminal differentiation phase in which future stalk cells being at the top of the slug start to gastrulate through the mass of pre-spore cells and form a rigid stalk of dead cells supporting the spore-containing sorus (see figure 1.9). Under favourable conditions spores germinate and produce new amoebae being a beginning of a new round of the asexual life cycle. Figure 1.4 shows the developmental stages of dictyostelids. The stage at which the fruiting body is produced is not fixed but depends on the availability of food sources and on environmental conditions including temperature, light and humidity (Bonner and Shaw, 1959; Cavender, 1978; Raper, 1984). These

factors can affect the developmental process of dictyostelids (Francis *et al.,* 1991) and are discussed in more detail below.

Figure 1.4 Dictyostelid developmental stages.



Up to eight hours of starvation triggers in free living amoeba an aggregation of individual cells (a,b); c-f shows several steps of morphogenesis and differentiation of cells which result in culmination and production of fruiting body carrying spores (g-h) (after Anonymous, 2008).

The aggregation stage starts when dictyostelid amoeba are chemotactically attracted towards the folic acid released by bacteria and can ingest ca. 1000 bacteria between cell divisions. As discovered by Bonner (1947), the central role in the process of aggregation is played by the extracellular emission of the chemo-attractant molecule ′acrasin′, designated by Bonner (1947) for its rapid enzymatic degradation by the enzyme

acrasinase. Each species uses a single acrasin and it is thought that there are at least eight different acrasins although, to date, only four have been identified (Bonner, 1982). The most common appears to be cyclic adenosine monophosphate (cAMP), found in *D. dictyostelium, D. mucoroides, D. purpureum* and *D. aureum* (Konijn, 1968), glorin (N-propionyl-y-L-glutamyl-L-ornithine-lactam ethyl ester) secreted by *Polysphondylium violaceum* (Shimomura *et al.,* 1982), a pterin secreted by *D. lacteum* (van Haastert *et al*., 1982), and a form of folic acid secreted by *D. minutum* (DeWit and Konijn, 1983). The chemo-attractant role of cAMP in *D. discoideum* is the most well known where after ca. 4 hours of starvation, cell division stops and some of the population begin to secrete cAMP (Konijn *et al*., 1968; Richards *et al.,* 1990) into the extracellular space. cAMP is the fundamental chemo-attractant which controls the process of aggregation. It starts when cAMP binds to the specific receptors on the cell surface e.g. a serpentine G-proteine-coupled receptor (CAR-1). This leads to the stimulation of guanylyl- and adenylyl-cyclase enzymes and as a result the formation of intracellular cAMP which is then secreted to the extracellular space by the central amoebae where it stimulates nearby amoebae to initiate the aggregation process (see figure 1.5). Gomer and Firtel (1987) believed that the percentage of the population which began to secrete cAMP were those which were at the S-phase in the cell cycle at the onset of starvation. In most species these stimuli are delivered in pulses and they induce the expression of genes needed for the later developmental stages. The pulsing period differs between species, ranges from 5 minutes in *D. purpureum* to 20 minutes in *D. rosarium* (Raper, 1984). Some species such as *D. minutum* deliver the stimulus continuously rather than in pulses, whereas others such as *P. pallidum* and *P. violaceum* alternate between both types of pulsing (Schaap, 1986). cAMP controls all dictyostelids developmental cycle events from the aggregation to the culmination stages. The stimulated cells respond to this extracellular cAMP in that the amoebae of most pulsatile species orient themselves and move up toward the highest gradient of the cAMP, forming streams of cells flowing towards a central aggregation forming a mound.

Figure 1.5 The aggregation stage.

Sorogen a term applied to structure in process of fruiting e.g. erect or semi-erect peg-like column formed by inflowing myxamoebae.

Once there, cells start to produce their own cAMP as a response to the signal, intensifying the cAMP gradient, which then spreads to neighbouring cells bringing them into the aggregation centre. *D. discoideum* aggregations comprise 1000-1,000,000 amoebae (Loomis *et al*., 1977). Non-pulsatile species, e.g. *D. minutum*, which secrete the chemo-attractant in the continuous rather than pulsatile way, also orient themselves relative to the cAMP gradient, but flow together without streaming. The aggregation mound forms the pseudoplasmodium or ′slug′, so called because of its shape and because it is effectively an independently motile organism (see figure 1.6) Although, the cAMP gradient is still steep in the post-aggregative stage, the CAR-3 is now expressed instead of CAR-1, and the process of differentiation starts at the dorsal side. The initial aggregation is dome-shaped and the differentiation is initiated at the top of this dome. However, not all amoeba will survive differentiation, some will sacrifice themselves so that the rest may become dispersive spores and seed new colonies. The slug begins to migrate seeking out light and leaving a slimy trail. Whether the slug starts to migrate (a distance of no more than 1 cm) along light, temperature and humidity gradients prior to fruiting body formation or proceeds directly to the formation of a fruiting body depends

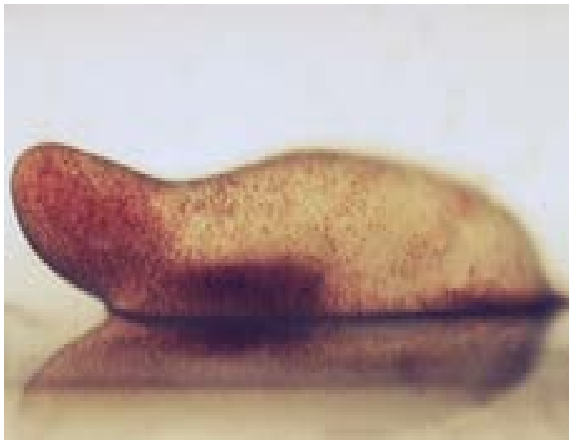
on the environmental conditions (Winckler, 2003). Other species do not form a motile slug but become immediately anchored by the creation of the stalk.

After a period of time the slug stops migration and initiates the culmination stage when cells within the slug begin to differentiate and take on specialized roles, with for example those at the tip of the slug being highly active cAMP producers. *D. discoideum* cells, once influenced by cAMP, start to manifest their differentiation in relation to their position on the mound cAMP gradient. During differentiation the slug develops a clearly defined tip where cells develop into pre-stalk cells whereas those towards the rear develop into pre-spore cells. This differentiation is controlled by differentiation-inducing factor (DIF), another chemical signal with functions similar to cAMP (Kay *et al*., 1989). Finally, a cellulose-containing ring is deposited in the place joining the main mass of the cells and the tip, and the pre-stalk cells migrate posteriorly to form a stalk. These cells thicken their membranes, vacuolate, and then die.

The pre-spore cells posterior of the slug are lifted vertically, as the sorophore extends, undergo encapsulation and become spores forming the sorus at the tip of the sorophore. The culmination stage ends when all the cells which did not enter the stalk become encapsulated (Loomis, 1975; Williams, 1988). Now the structure resembles a small ball balancing on top of a fexible stalk and is known as a fruiting body (see fig 1.9).

Figure 1.6 Dictyostelid slug.

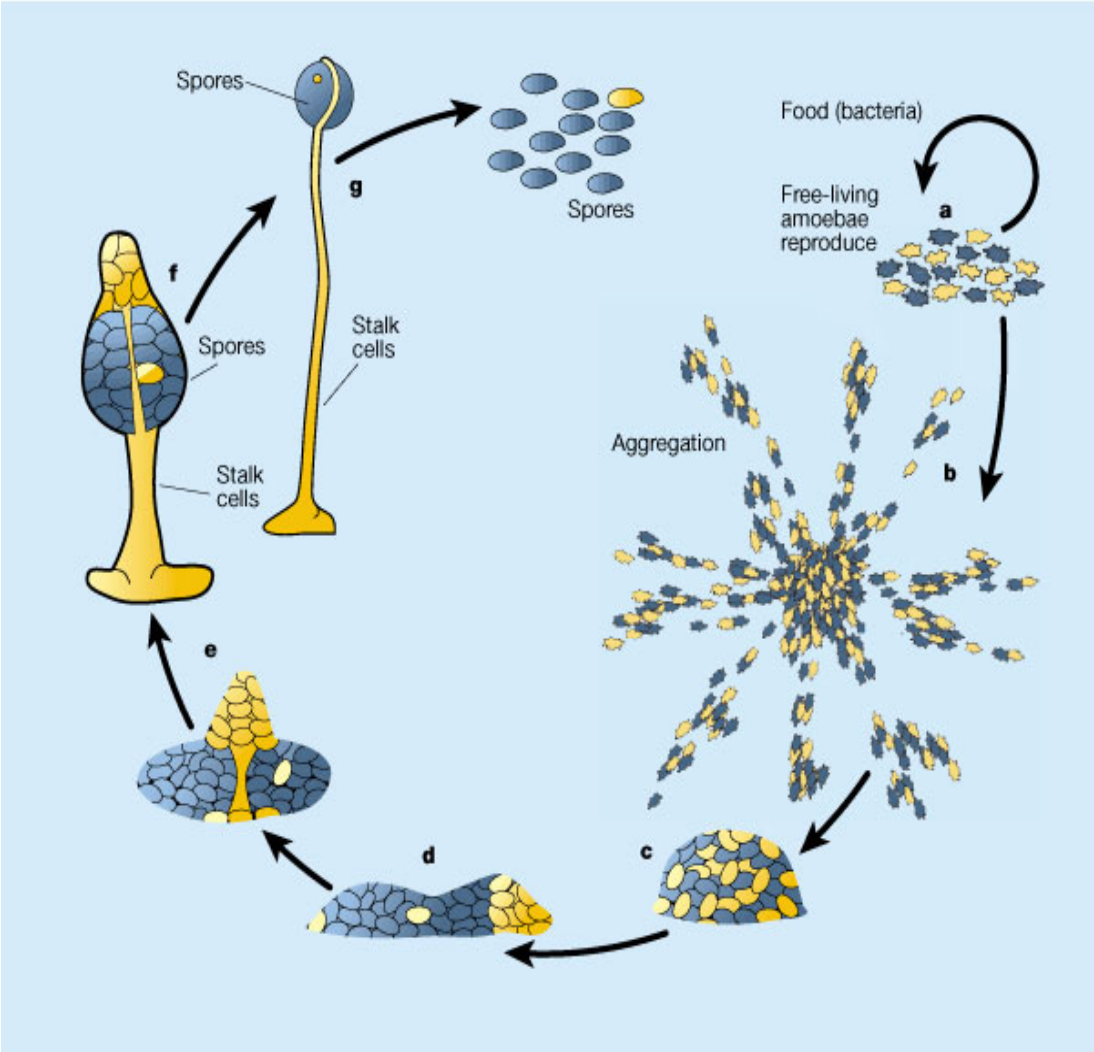
(after Dormann, 2009; magnification x 200).



In the process of fruiting body formation, only 20% of the cells are lost to form the stalk, the remaining 80% becoming spores. Most species, except *D. discoideum*, do not migrate but anchor the stalk soon after slug-formation synthesising the stalk as they migrate, to create a very long sorophore (see fig1.9). Species in the genus *Polysphondylium* segregate the pre-spore mass as it ascends the stalk and each of the separate groups of cells then produce short stalks with apical sori. Typically, several such short stalks will arise from each segregated group, forming characteristic whorls at intervals along the sorophore as well as the apical sori (Loomis, 1975; Kessin, 2001).

Spores are usually 3-10 μm in length and of various species-specific shapes; being spherical, elongate or reniform. The cytoplasmic core is surrounded by a three-layered structure constructed of an outer amorphous layer containing cellulose, sporopollenin (Maeda, 1984), proteins, a fibrous-central layer and an inner, electron dense layer close to the plasma membrane. The spores of *D. discoideum* also contain other materials such as trehalose and glutamine, which serve as germination enhancers and as metabolic material for pre-amoebae (Klein *et al*., 1990). Spores can remain viable for years until conditions are favourable to allow germination (Winckler, 2003). The germinating spores close the life cycle by giving rise to amoebae, which start a new asexual cycle. Figure 1.7 shows the complete dictyostelid life cycle with the process of the differentiation of the slug cells into spore and stalk cells.

Figure 1.7 Dictyostelid asexual life cycle.



(a) and (b) show unicellular stages, starting from spore germination through free living amoeba to the beginning of aggregation, the first multicellular stage, (c) to (g) show stages of fruiting body formation through slug (d) to fully developed fruiting body (g), after Kessin (2009).

1.4.2 The sexual life cycle

The process of sexual mating was first fully characterised between two classic strains of *D.discoideum*, namely NC4 and V12, under well defined environmental conditions of darkness, moisture, low phosphate levels, the presence of calcium and absence of bacterial food supply (Robson and Williams, 1981). Mating begins with the appearance of small amoebae which produce gametes - tiny cells with small nuclei locked in the G1 stage of the cell cycle. Data from comparative studies suggest that the gametes may initiate mating by being the source of a sexual pheromone. When the gametes come into contact, they form a bulge and fuse in pairs to give rise to binucleated cells, then, they undergo changes resulting in the appearance of a zygotic giant cell. This starts secreting cAMP, initiating the chemotactic stage. The function of cAMP here is similar to that during the asexual formation of the fruiting body, attracting non-zygotic amoebae, which aggregate close to the zygote surface to be engulfed and digested by the zygote in a proxy of cannibalistic phagocytosis and the phagosome containing the ingested amoebae called an endocyte. Once all the amoebae surrounding the zygote are digested, the zygote secretes a primary wall of slime to become a precyst. A second cellulose wall is added to form a macrocyst which enters a dormant phase (Newell, 1978). There then follows one cycle of meiosis and several of mitosis. Macrocysts germinate with a low efficiency of ca. 2%, releasing between 20 and 200 haploid amoebae (Francis and Eisenberg, 1993). Macrocysts are formed from a mere ca. 100 cells, in contrast to the fruiting body which is formed from < 100,000, however, the relative cost of production of the macrocyst is very high because most of the cells die. Although the sexual life cycle is well described, it is not common and therefore not used as a taxonomic feature.

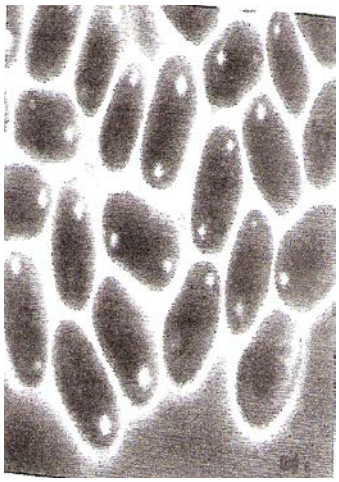
1.5 Morphology of Dictyostelid species

Detailed variation of key morphological features has formed the basis for dictyostelids systematics. This section reviews relevant morphologies.

The first description of dictyostelids, published by Olive (1902), were entirely morphological. These focused on (1) branching pattern, e.g. whether it was regular or irregular, (2) spore shape and length, (3) sorocarp height, (4) soral diameter and colour with only occasional notes made on the habitat. The ″Dictyostelids″ by Raper (1984) gave comprehensive description of all, then known, dictyostelid species. His publication included new physiological characteristics of growth media, optimal growth temperatures, temperatures for the development of sorocarps, slug migration (if observed) as well as microcyst or macrocyst production. Additional information about the type of chemo-attractant, e.g. cAMP or glorin, used in aggregation behaviour or the presence/absence of refractile polar granules was also included. All *Polysphondylium* and a few *Dictyostelium* species contain polar granules (Traub and Hohl, 1976).

In his dictyostelids monograph, Raper, used polar granules as one of taxonomic criteria for their classification. Additionally, Hagiwara's (1989) following his taxonomic study on Japanese dictyostelid species, detailed their description of sorophore and spore morphology, adding more taxonomic features, for example, shape of sorophore tip or spore size. Furthermore, in *Polysphondylium* species, branch length, number of whorls and number of branches per whorl were considered to be important taxonomic criteria.

Figure 1.8 A dictyostelid showing refractile polar granules (white spots).

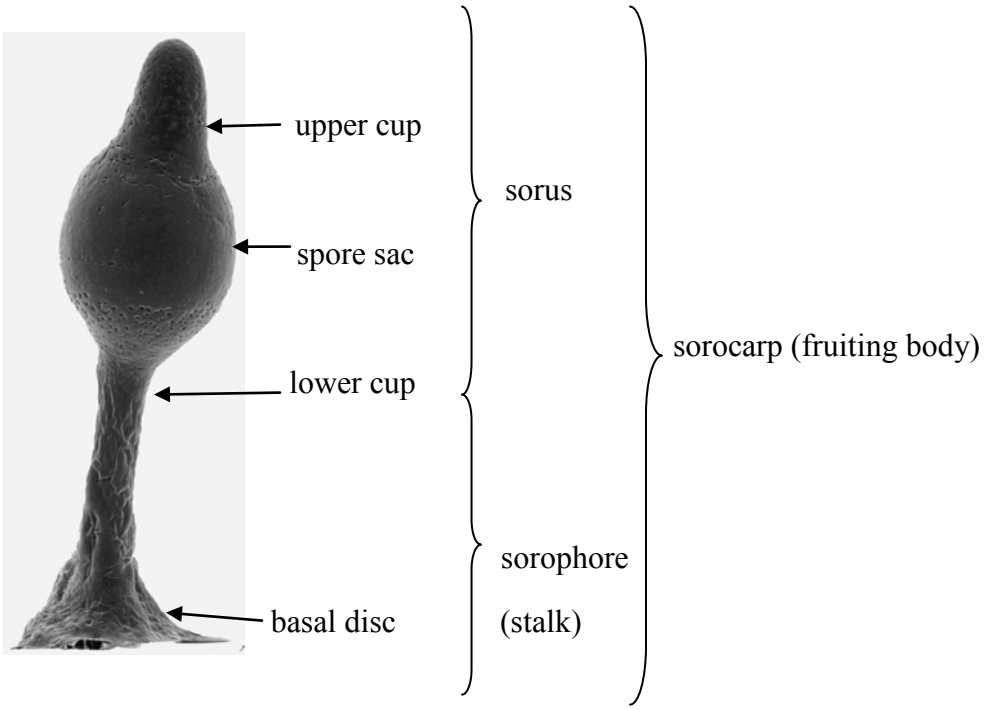


(after Hodgson, 1999); magnification x 150.

1.5.1 Brief introduction to the dictyostelid sorocarp

The Dictyostelid sorocarp is a relatively simple structure, often species diagnostic but its morphology is dependent upon culture conditions. Figure 1.9 describes the basic sorocarp structure and figure 1.10 shows the range of sorocarp morphologies.

Figure 1.9 Fruiting body structure.



(after Anonymous, 2008; magnification x 150).

Sori of some species may be a globose, subglobose, citriform (e.g. figure 1.9) or rounded-apiculate: while some species may exhibit more than one morphotype. Sorus size depends on the stalk (sorophore) height which in turn can be influenced by environmental factors such as humidity, while stalk base and tip shapes are similarly variable. According to Hagiwara (1989) the range of sorophore tip shapes (upper cup) is considered one of the most important species-diagnostic characters. In contrast, the shape of the sorophore base is often difficult to determine due to a thick deposit of mucopolysaccharide material.

Figure 1.10 *Dictyostelium* sorocarp morphologies. (a) – *D. lacteum*; (b) - *D. minutum*; (c) – *D. implicatum*; (d) – *D. mucoroides*; (e) – *D. fasciculatum*; (f) – *D. aureo-stipes*; (g) – *D. polycarpum*; (h) – *D. sphaerocephalum*; (i) – *D. rosarium*; (j) – *D. giganteum*.

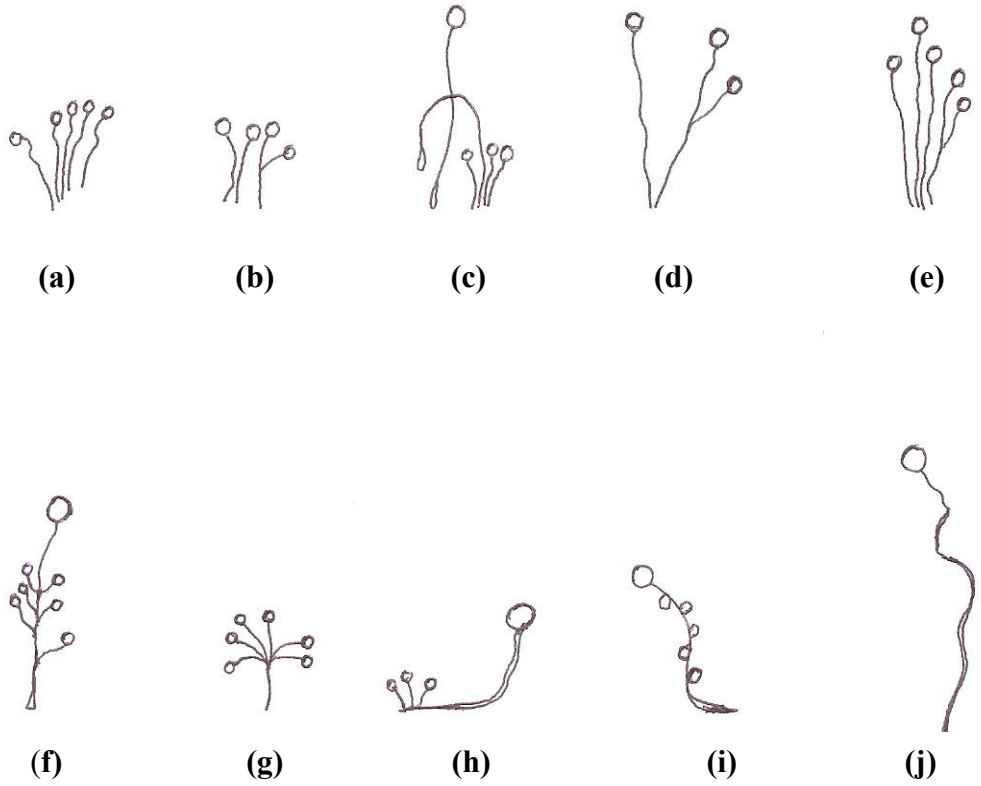
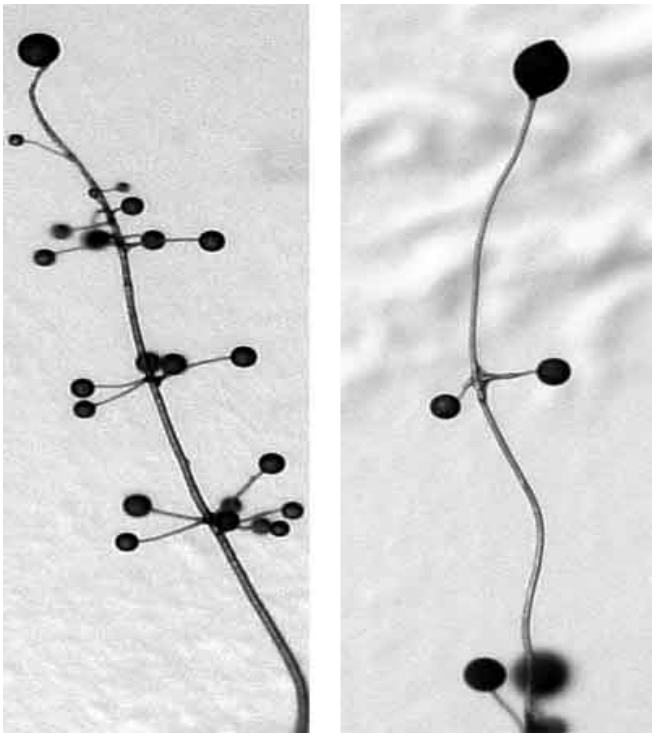


Figure 1.11 *Polysphondylium* sorocarp morphologies. a - left) *Polysphondulium violaceum;* b - right) *Polysphondylium pallidum*



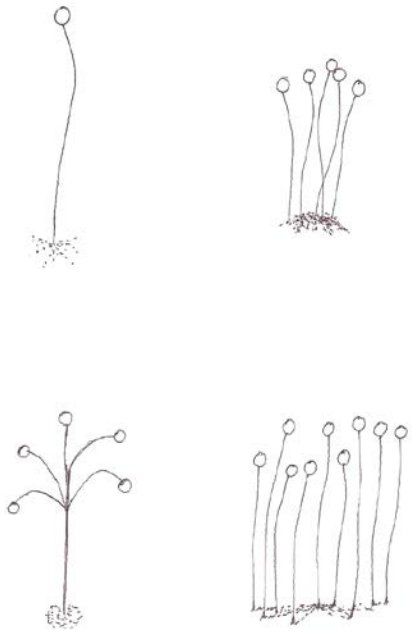
(after Anonymous, 2007; magnification: x 50).

No *Dictyostelium* species exhibits a regular branching, which is the defining character of *Polysphondylium*, though occasionally, irregular branching may be seen especially in unpigmented species and *P. violaceum*. Some *Dictyostelium* species e.g. *D. minutum* or *D. sphaerocephalum* may produce unbranched or irregularly branched sorocarps though the majority produce solitary sorocarps as opposed to sorocarps arranged in a unique characteristic fashion, e.g. clustered (*D. fasciculatum*) which occasionally produce solitary ones) or a coremium (*D. polycarpum*). The different growth habits are shown in figure 1.12

Depending on the size of the sorocarp, Dictyostelids can be broadly divided into three groups: based on sorocarp size; (1) those with small (≤ 2 mm) and delicate sorocarps e.g. *D. minutum*; (2) intermediate sized (3-9 mm) such as *P. pallidum* or *D. aureo-stipes*; and (3) large (≤ 10 mm) sorocarps exemplified by *P. violaceum* or *D. sphaerocephalum*.

Figure 1.12 Growth habits of dictyostelid sorocarps; a) left: solitary, right: clustered b) left: coremium, right: gregarious.

a)



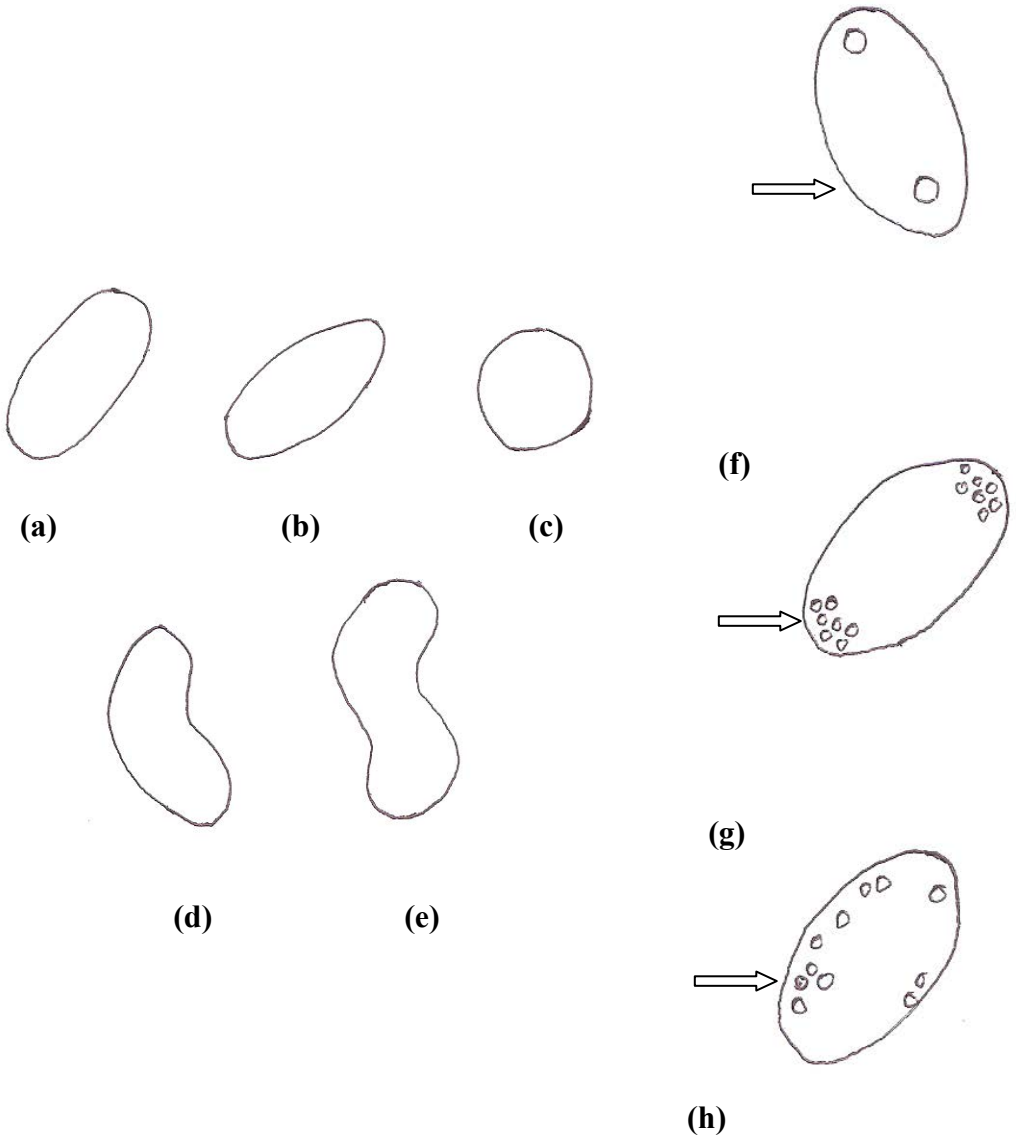
b)

Spore morphology, is according to Hagiwara (1989), another important diagnostic characteristic used in species identification. Dictyostelids spores show a considerable degree of variation in size and shape, even amongst single isolates and often overlap between the sizes of many species. Generally spores of all species are smooth and elliptical in shape, although shape can range from spherical to elongate. Spores also show differences in the arrangement of granular inclusions as shown below. Figure 1.13 shows different spore shapes and the arrangement of polar granules.

Dictyostelids also display an interesting behaviour in the soil. For instance, larger species, which normally feed in soil, are capable of migrating upwards to produce a fruiting body at the soil surface. The soil surface is a major invertebrate dispersal route and therefore the optimal spore dispersal location. The adhesive spores can be picked up by passing invertebrates and moved to new locations with potential bacterial food sources. As migration is affected by gravity, light, temperature or gas gradient the absence of any factor will cause slime moulds to migrate downwards instead of upwards, while the presence of ammonia will block all slime moulds movement. The migrating amoeba masses are phototactic and sensitive to temperature gradient which allows them to migrate upwards in darkness, e.g. at night. Gas gradients only initiate upward migration if light is absent. Some species, e.g. *D. rosarium* and *D. polycephalum* are not phototactic; and only so exhibit gas orientation (chemotaxis) (Bonner and Lamont, 2005).

While cellular slime moulds display a number of morphological differences, all their fruiting bodies consist of a slender stalk supporting a mass of spores called a sorus (fig 1.9), which is either very small ($\leq$ 1 mm) or very long (10 mm). Migration is determined by size, in that, generally smaller species do not migrate and therefore produce their fruiting body *in situ* where they feed, while larger species migrate to a new locations for spore dispersal and to exploit new food resources. These migrant species, e.g. *D. discoideum,* may migrate without the stalk formation (stalkless migration) and only form the stalk after they stop, while *D. mucoroides* or *D. purpureum* and others form the stalk as they move.

Figure 1.13 Spore shapes and the examples of arrangement of polar granules (arrows).



Legend; (a) – oblong; (b) – elliptical; (c) – spherical; (d) – reniform; (e) – sigmoid; (f) – consolidated; (g) unconsolidated; (h) – irregular; polar granules arrowed.

Spores placement on the stalk is variable; the most common form is a single sorus, found in many *Dictyostelium* species e.g. *D. discoideum,* while *Polysphondylium* species, have numerous sori placed on one stalk, which in regular sori, radiate outwards and held out laterally by a small and delicate mini-stalks (Bonner and Lamont, 2005; Raper, 1984). Although this type of fruiting body is most common in *Polysphondylium,* similar types occur in some *Dictyostelium* species, e.g. *D. rosarium* (figure 1.14) produces its fruiting body in which whorls are absent but sori are placed at regular intervals-like beads along the solitary stalk, reminiscent of a rosary, from which this species took its name.

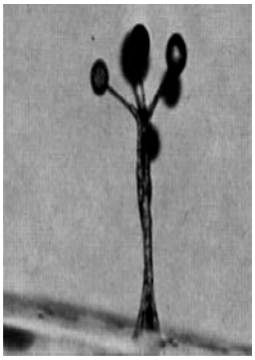
Figure 1.14 *Dictyostelium rosarium* sori.

(after Anonymous, 2009; magnification: x40).

D. polycephalum is capable of long distance migration after which it forms an interesting and unusual fruiting body. It initially produces between two and six papillae, each of which forms tiny fruiting bodies in which stalks adhered together for three quarters of their length, finally separating to form a whorl of sori (figure 1.15). The fruiting body formation is also determined by external growth conditions. For example, *D. polycephalum* easily produces masses of fruiting bodies in soil but is very resistant to fruiting on the agar medium; development of *D. rosarium* may be enhanced by the

presence of soil, while, by contrast, *D. discoideum* will thrive in both soil and on agar medium.

Figure 1.15 *D. polycephalum* fruiting body.



(after Waddell, 2008; magnification x40).

The reason why there is so much variation in dictyostelids morphology and physiology is not yet clear. It is well known that species from both genera have cosmopolitan distributions often co-occurring if temperature regimes are suitable. There are however, some exceptions e.g. *D. septentrionalis* from Alaska will only grow at 16º C (a rarely attained temperature in an Arctic/sub Arctic region). There is also no explanation why some species form multiple sori distributed in whorls while others produce a solitary stalk with single sorus, or why some species migrate and others cannot. The fact that both morphologically different species can cohabit would suggest that these differences may be the result of chance point mutation which has no advantage and is therefore selectively neutral (Bonner and Lamont, 2005).

1. 6 Early Dictyostelid Systematics

As described in section 1.5 the first taxonomy of dictyostelids was entirely based on morphological features and has been revised several times. The limited morphological studies have suggested similarities with both the Fungi and the Protozoa, though there

was a tendency to regard them as taxonomically closer to Fungi, as exemplified by a study on mitosis in *P. violaceum*. However, the mycologist de Bary (1887) placed them in the group Mycetozoa, erected by him in 1858, to harbour both slime moulds and plasmodial-forming Myxomycetes to show that they are distinct from Fungi. Olive (1902) subsequently decided on the basis of the dictyostelids' need for holozoic nutrients and their possession of tubular mitochondrial crystae, to transfer them from the kingdom Fungi to the kingdom Protista, further suggesting that the dictyostelids formed a monophyletic group along with the protostelids and the myxomycetes. Olive's suggested classification is as follows:

Kingdom:	**Protista**
Phylum	**Gymnomyxa**
Subphylum:	**Mycetozoa**
Class:	**Eumycetozoa**
Subclass:	**Dictyostelia** (dictyostelid cellular slime moulds) non-flagellate amoebae aggregating to form a multicellular pseudoplasmodium, that gives rise to a multi-spored fruiting body, sexual reproduction only in some species.
Order:	**Dictyostelida**

Cavalier-Smith (1993) on the other hand considered the cellular slime moulds to be polyphyletic placing them in the subphylum Dictyostelia and class Dictyostelea but within the phylum Mycetozoa. Protostelids and myxomycetes were placed as separate Protostelia and Myxogastrea classes in the subphylum Eumyxa. This classification scheme was derived from variety of morphological, biochemical, ecological and behavioural characters.

Olive's classification is still generally adhered to but now regarded as being less useful below the subphylum level and has been revised to introduce Raper's (1984) key to the classification of the cellular slime moulds:

a. Family - **Dictyosteliaceae** Rostafinski (1875). Fructifications (sorocarps) simple or branched, consisting of cellular stalks bearing globose to citriform sori (a spore producing structure).

Genus - ***Dictyostelium*** Brefeld (1869). Sorocarps simple or irregularly branched; sori globose to citriform, usually terminal but may be sessile.

Genus - ***Polysphondylium*** Brefeld (1884). Sorocarps with branches in whorls; sori globose and terminal on central axis and branches.

Genus - ***Coenonia*** van Tieghem (1884). Sorocarps simple or branched with prominent crampon-like bases, sori gelatinous, borne in terminal, cellular cupules.

b. Family - **Acytosteliaceae** Raper and Quinlan (1958). Sorocarps delicate, consisting of very thin, acellular stalks bearing terminal globose sori.

Genus - ***Acytostelium*** Raper (1956), also Raper and Quinlan (1958) diagnosis as per family.

1.7 Problems of Alpha-Systematics

The taxonomy of the family Dictyosteliaceae is based on morphological features with *Dictyostelium* and *Polysphondylium* grouped, on the basis of a common amoeba type, together in the family Dictyosteliaceae, yet the character that differentiates these two genera is principally branching type (see & 1.5). The term 'alpha' refers to alpha taxonomy being the first and the most basic in taxonomy. Other diagnostic features include e.g. spore patterns microcysts formation or variety of environmental pressures that can influence dictyostelids growth and development.

Since the first description of the genus *Dictyostelium* in 1869, the idea of recognising the genus *Polysphondylium* as being separated from *Dictyostelium* has prompted much discussion, yet even researchers including van Tieghem (1880) commented that the differences observed in relation to types of branching may not be sufficient to define the new genus. Indeed Potts (1902), Olive (1902), Rai and Tewari (1961) in Traub and Hohl, (1976) expressed similar doubts. Other researchers have argued that other significant structural and physiological differences between these two genera justify their separation (Bonner, 1967; Raper, 1973), while Traub and Hohl (1976) drew attention to the fact that many species already classified as *Dictyostelium* possessed a number of characters shared with *Polysphondylium* e.g. polar granules mentioned in table 1.1. The latter work showed that a certain group of strains assigned to the *D. mucoroides* complex exhibit some features which clearly related them to the genus *Polysphondylium* even though they do not possess the regular branches diagnostic of *Polysphondylium*. Several other *Dictyostelium* species, including *D. lacteum, D. minutum* and *D. polycephalum* exhibit similar features. Traub and Hohl's findings support the existence of at least two groups/ genera but also suggest the need for erecting a new genus as illustrated by the presence or absence of polar granules and therefore a revision to be needed (Traub and Hohl, 1976).

Traub and Hohl (1976) noted that spores of *Polysphondylium* spp. all contain polar granules (PG), transparent vesicles visible via phase contrast light microscope, based in the polar regions of the spores (see figure 1.8). Their presence is believed to be related to the pre-aggregative behaviour of cellular slime moulds. Significantly, Traub and Hohl (1976) also noted that some *Dictyostelium* species are PG-positive yet no *Polysphondylium* species were PG-negative. On the basis of the presence/absence of PG vesicles they proposed a new genus *Heterosphondylium*, intermediate to *Dictyostelium* and *Polysphondylium* to accommodate those *Dictyostelium* spp. which were PG-positive and yet shared characters with *Polysphondylium* but lacked whorled sorocarps (Traub, 1977 in Raper, 1984). This new genus has not however been universally adopted. It is generally argued that the principal morphological features of systematic value include colour of spores, sorocarp dimensions (Singh 1947), and sorocarp branching type. However, as noted by both Raper (1984), and Evans and Hughes (1988), all of these features vary to some extent with various aspects of culture conditions. This could imply that there are limitations to the valid classification of cellular slime moulds using only morphological features. The list of morphological features of *Dictyostelium* and *Polysphondylium* used in systematic studies is shown in table 1.1.

Dictyostelid systematics is obviously far from stable. When considering what characters should be used as the basis for determining taxa criteria, it becomes clear that the use of only morphological, physiological or biochemical characters have failed to produce an unequivocal classification of the cellular slime moulds. The failure to reach consensus has prompted a few recent attempts to apply molecular techniques to slime moulds classification.

Table 1.1 Dictyostelids morphological features used diagnostically in systematic studies: features characteristic for *Dictyostelium* and *Polysphondyliym*, respectively in the centre, features shared by species from both genera.

Morphological features	type of amoeba (numerous elongate pseudopodia)
	spore colour/shape
	fruiting body (sorophore: unbranched, branched)
	chemo-attractants (e.g. cAMP, glorin)
	formation of microcysts
	possession of phosphodiesterase inhibitor system
	possession of refractile polar granules

Dictyostelium	***Polysphondylium***
• cAMP sensitive	• not cAMP sensitive
• posseses an inhibitor system	• lack of inhibitor system
• do not form microcysts	• forms microcysts
• sorophores unbranched	• sorophores branched
• lack of polar granules	• polar granules

Common to both genera
• Types of amoeba
• Branched and unbranched sorophores
• Spores colour/shape
• Presence of polar granules

1.8 Molecular Systematics: The Solution?

Until the 1960s systematics was based mainly on analysis of morphological characters and behavioural variations (Hillis *et al.,* 1991). However, increased understanding of the mechanics of inheritance, particularly in terms of gene structure and genetic markers, has shifted the focus towards using molecular data as estimators of species relationships.

Molecular data show a clear inherited basis and can deliver a huge number of potential characters, which seem to be limited only by the size of the studied genome (Li and Sadler, 1992; Stephan and Langley, 1992). They also appear to be less influenced by environmental factors (Iqbal *et al.,* 1995), which allows the study of genes that lack morphological expression. In contrast some morphological characters may not be suitable for phylogenetic study because they are not heritable but result from environmental influences, while others, e.g. skeletal structures may contain convergent similarities which often confound systematics. Furthermore, evolutionary changes shown in molecular data e.g. at different nucleotide or amino acid sites, are easily comparable between taxa with the use of modern data analysis tools and are especially important when conflicting data are compared (Ridley, 2004). Molecular data also allow direct comparisons between essentially any groups of even distantly related organisms (Whellis *et al*., 1992). However, the use of molecular or morphological data is not without controversy. Both types of data were suggested to be inherently 'weak' and misleading (Kluge, 1983; Sibley and Ahlquist, 1987) but soon it has become clear that both types of data provide valid estimates of systematic relationships, although represent independent information sources that follow different rules and respond to different evolutionary pressures (Wilson *et al.,* 1974, 1977).

1.9 Problems of Molecular Systematics

Molecular data, however, show one major disadvantage; that of very limited possible character states (only four DNA or RNA bases) which lead to problems with distinguishing homoplasy (a shared character state derived from convergence and not common ancestry) from homology (a shared character state derived from common ancestry). Evolutionary classification considers only true homologies as a record of genealogical ancestry where 'homology' infers a common ancestry between species. If two DNA sequences share numerous similar base pair positions they are considered similar but not necessary homologous because similarity can result from independent processes such as gene duplication, gene transfer or speciation forces (Moritz and Hillis, 1991). This is especially important when comparing molecular sequences from different species from different sources; for instance, if the sequence resulting from the copy of a gene is wrongly compared with the sequence resulting from the original form of the same gene (Kimura, 1983; Gillespie, 1987; Moritz and Hillis, 1991; Freeman and Herron, 2001). Molecular homology is also confounded by "alignment" problems, where the same gene in different species is of different lengths as a result of extra deletions/ insertions accumulated from mutation. As presented, not all phylogenetic problems can be addressed from the analysis of molecular data nor can they be fully resolved by the morphological approach. In such cases many phylogenetic approaches may benefit from combining the molecular and morphological data**.** Furthermore, phylogenetic studies often require the use of different molecular markers or different analytical techniques in order to provide data that can be informative at different hierarchal levels and will contribute to understanding both the micro- and macro-evolutionary processes (Ridley, 2004; Watson, 2004; Avise, 2006). Thus, the level of variability shown by a particular marker as well as the ease of analysis will determine the choice of a particular type of a marker, at the outset of a research project (Hillis and Moritz, 1990; Avise, 1994). The list of commonly used molecular markers and their key characteristic is given in table 1.2

Table 1.2 Commonly used molecular markers, techniques and their key features.

Features	Markers		Molecular techniques				
	Allozymes	**Minisatellites**	**Microsatellites**	**RFLP**	**RAPD**	**AFLP**	**RAM**
Frequency	high	medium	high	high	high	high	med/ high
Level of polymorphism	med	high	high	Low/med	med	med	med
Codominance of alleles	no	no	yes	yes	no	no	no
Reliability	med	high	high	high	med	med	med
Steps involved	Extraction, separation, staining interpretation	RE Digestion-separat-ion, blotting,marker screening	PCR Electrophoresis Marker screening	RE Digestion Southern blott Probe labelling, Hybridization, Marker screening	PCR, Electrophoresis, Marker screening	RE digestion Ligation with adaptors, 2 step PCR, 2 selective primers	PCR Electrophoresis Marker screening
Applicability in systematics	med high	low high	med low	med high	low low	low med	med low
Technical demands, labour intensity	high	high	low	high	low	med	low
Amenability to automation	no	no	yes	no	yes	yes	yes

Allozymes - protein products of different alleles (Buth and Murthy, 1999); Minisatellites – stretches of monotonously repeated sequences with the basic repeat units of 7bp to100bp (Jeffreys *et al.,* 1992); Microsatellites - as above ,with the basic repeat units of <7bp (Jeffreys *et al.,* 1992); RFLP – Restriction Fragment Length Polymorphism (Evans *et al.,*1987); RAPD – Randomly Amplified Polymorphic DNA (Harris, 1999); AFLP – Amplified Fragment Length Polymorphism (Vos *et al.,* 1995); RAM – Randomly Amplified Microsatellites also known as ISSR-PCR – Inter Simple sequence Repeats (Zietkiewicz, 1990)

1.9.1 Protein Markers

Early applications of molecular systematics were largely based on proteins. Although, the first use of proteins (from over 500 different animal species) as taxonomic markers was done by Nuttall (1904) influenced by the earlier work of Landois (1875) and Landsteiner (1900) on blood agglutination (Hughes-Jones and Gardener, 2002), it was Reichert and Brown (1909) who demonstrated that it is possible to use proteins to differentiate individuals, species and genera. The analysis of protein variability as a means of establishing taxonomic relationship however did not become widespread until after some methodological developments. The first major breakthrough in assessing protein variability came with the development of paper electrophoresis and later on the introduction of starch gel as a supporting medium, which significantly improved that technique, followed by the discovery of an effective histochemical stain to visualize enzymes on a gel (Landsteiner *et al.,* 1939; Smithie, 1955). Since then protein electrophoresis has become a fully established method (May, 1998). Over the years there continued to be other developments in protein electrophoresis techniques, mainly in the nature of the supporting medium with a clear preference for agarose or polyacrylamide gel (Harris and Hopkins, 1976; Hillis and Moritz, 1996). However, it was during the 1950s and early 1960s, when increasingly efficient techniques for the extraction and analysis of individual proteins were developed, prompting further studies on proteins, which resulted in recognition that many proteins exist in multiple forms. For instance Markert and Moller (1959) in Buth and Murthy (1999), proposed the term ‘isozyme’ to describe different molecular forms of proteins with the same enzymatic specificity. Furthermore, allelic isozymes, or allozymes were recognized as the products of the different alleles of the same locus differing by one or more amino acids in their primary structure because of nucleotide variation in the encoding gene. Allozymes, being variants of a genetic locus, are inherited in a Mendelian fashion and can be considered as different allelic products of the same gene. Thus, it was soon realized, that both isozymes and allozymes represent potentially useful molecular markers for molecular systematics (Smith *et al*., 1982; Ayala, 1983; Kilias, 1987).

1.9.2 Allozyme Markers

During the 1970's and 1980's there was explosion of studies where allozyme marker systems were used to investigate phylogenetic relationships (Buth, 1984, 1999; Richardson *et al*., 1986). Allozymes were widely used in avian (Johnson *et al*., 1984; Matson, 1984 Smith *et al*., 1994), plant (Crawford, 1989, 1990) and fungal molecular systematics (Spieth, 1975) but in relatively few studies on prokaryotes (Selander *et al*., 1986). There were also a number of reports of the use of allozyme polymorphisms to study phylogenetic relationships within the slime moulds (Ramagopol and Ennis, 1984**;** Briscoe *et al*., 1987), which were followed by other work on proteins (Grant *et al*., 1985; Witke *et al*., 1986; Smith *et al*., 1989).

The first attempt at molecular systematics was that of Briscoe (1987), who employed allozyme electrophoresis together with the presence or absence of reaction to a monoclonal antibody against the spore coat glycoprotein gp 138 to assess the degree of genetic diversity of *D. discoideum* isolates. His findings revealed heterogeneity suggesting that there are at least four biological species represented in the studied *D. discoideum.* He also suggested that *D. purpureum* contained three species and *P. pallidum* two species. His findings also suggested that despite having a wide geographic range, there is relatively little genetic diversity among sexually developing *D.discoideum*. This caused surprisingly little concern among those working on dictyostelids systematics. Indeed it was suggested that the allozyme technique does not provide useful taxonomic information, since any technique that shows significant variation between all isolates would appear to be differentiating at the level of the clone rather than the species. Wheller and Hodgson (pers comm., Hodgson, 1999) repeated some of Briscoe's work without achieving consistency, again casting doubt on the efficacy of allozyme analysis as a taxonomic tool.

Another work using proteins, focused on amino-acid composition (Ramagopol and Ennis 1984). In an attempt to resolve the taxonomic relationship between the two major *Dictyostelium* and *Polysphondylium* genera, Ramagopol and Ennis (1984) used a high resolution two dimensional gel electrophoresis method to study the amino-acid composition of ribosomal proteins from a selection of species in both genera including four *Dictyostelium* and two *Polysphondylium* species. Their data confirmed that the morphological dissimilarity recorded between these two genera was reflected in variation among the ribosomal proteins. Also, more differences were observed between *Dictyostelium* and *Polysphondylium* than

between individual species within one genus. Although these data supported the existence of two genera, their results also demonstrated the strong conservation of amino-acid sequences of the ribosomal proteins. The advantage of allozyme data for phylogenetic studies is derived from the fact that they are easily obtained for a large number of individuals and that the data are less vulnerable to systematic anomalies resulting from mismatches between genetic and species trees when compared to other data (e.g. that delivered from mitochondrial DNA sequences). However, it became apparent that there are limitations to the use of proteins as informative markers for the effective characterization of individuals, mainly resulting from their low level of polymorphism, destructive sampling or the ongoing controversy over analysis methods (Mickevich and Johnson, 1976; Buth, 1984; Swofford and Berlocher, 1987; Pamilo and Nei, 1988; Murphy, 1993; Hillis and Moritz, 1996; Mable and Moritz, 1996; Dowling *et al*., 1996; Jane and Lagoda, 1996; Carvalho, 1998; Buth and Murthy, 1999; Wiens, 2000).

1.9.3 DNA Markers

Just as advances in protein technology in the 1960's enabled the development of allozyme marker systems, the development of various new post-1980 DNA technologies enabled the introduction of many new DNA based marker systems (Carvalho, 1998). Probably the most significant development was the invention of the Polymerase Chain Reaction (PCR) by Kary Mullis enabling the *in vitro* amplification of targeted DNA sequences to millions of copies (Mullis *et al*. 1986; Brown, 1995; Dowling *et al.,* 1996; Newton *et al*., 1999). PCR has also prompted the development of a range of new DNA marker systems which could be employed for different scientific approaches. Each marker system possesses recognized advantages and disadvantages in terms of expense and effort. For example, direct DNA sequencing allows high resolution of intra-specific and inter-specific variation but it is time consuming and expensive (Dowling *et al.,* 1996). By contrast, hybridization based techniques, although technically straightforward, do not identify underlying sequence variation, require large quantities of DNA and can also be time-consuming (Newton *et al.,* 1999).

1.9.4 Randomly Amplified Polymorphic DNA (RAPD)

RAPD was the first marker system based on PCR and commonly used in phylogenetic research (Harris, 1999). This marker system requires the presence of only a single primer, no longer than ten nucleotides, chosen without regard to the sequence of the genome to be amplified. Thus, and in contrast to the standard PCR, no prior sequence knowledge is necessary. This primer acts as both forward and reverse and while its base composition is fixed for a given experiment, it is chosen at random. In order to fulfil the criteria of a successful amplification the primer must anneal to sites on opposite strands of the DNA within an amplifiable distance, i.e. ≤ 3 kb achieved by the use of the low annealing temperatures (25 to 40°C).

RAPD primers have the potential of detecting genome variability, and so form the basis of a molecular marker system, because each primer is able to amplify a certain number of different sized fragments. Therefore, the polymorphism detected by the RAPD technique is the result of several different mutational processes e.g. base substitutions, insertions, deletion or inversions which all lead to the creation of new binding sites or the abolition of the old ones (Bowditch *et al*., 1993; Williams *et al*., 1993). If a primer-binding site is affected by a mutation, either the relevant DNA fragment will not be amplified from an individual DNA sequence, or the amplified fragment will be of variable length or a new fragment will be generated (Andersen, 2000). Depending upon genetic relationships, the resulting RAPD band pattern is population-, species- or individual specific (Welsh and McClelland, 1990). The simplicity of the RAPD marker system (with its use of universal primers), requirement of small amounts of DNA (≤10 ng per PCR reaction) and quick data generation held considerable appeal to many researchers investigating a wide range of systematic problems (Demeke *et al.,* 1992; Hadrys *et al,.* 1992; Adams and Demeke, 1993; Boehm *et al.,* 1993; Williams *et al.,* 1993; Weising *et al.,* 1995). However, its early promise as a reliable and quick technique appropriate for many systematic purposes has not been fulfilled mainly resulting from major problems with non-specific binding of RAPD primer as a result of its low annealing temperature, product reproducibility and band homology. Micheli (1994) and Bielawski (1995), both showed RAPD-PCR is highly sensitive to different reaction condition for example the quality or concentrations of PCR reagents, slight changes in which could affect reproducibility (Jones *et al.* 1997; Wolfe and Liston, 1998). These problems may be of

concern in cases where this system is used for the identification of taxon-specific markers in phylogenetic or systematic studies (Bremer, 1991; Lomboy, 1994a, 1994b). Despite attempts to employ RAPD markers for establishing phylogenetic relationships within the plant kingdom, e.g. *Mango*, *Iris, Plantago* (Plantaginaceae) or *Alkanna* (Boraginaceae) species as well as between different strains within the phytobacterium *Xanthomonas campestris*, this system has not been applied to dictyostelids phylogenetic relationships (Arnold *et al.,* 1991; Hadrys *et al.,* 1992; Smith *et al.,* 1994; Wolff and Morgan-Richards, 1999; Lowe *et al*., 2000).

1.9.5 Amplified Fragment Length polymorphism (AFLP)

Gradually, however in the late 1990's a new and more reliable technique caught the attention of phylogeneticists: AFLP developed by Vos *et al*., (1995). This marker system ensures better specificity during PCR amplification and generates more reproducible data while retaining the advantage of the RAPD technique of not needing any prior knowledge of the targeted DNA sequence (Mueller and Wolfenbarger, 1999). Polymorphism is detected in a similar way as differences in the banding patterns (Vos *et al.*, 1995; Karp *et al.,* 1996), and can be traced to base changes in the restriction sites or to insertions/ deletions in amplified DNA sequences. The AFLP technique comprises four main steps: (1) Extracted genomic DNA is first digested with two different restriction enzymes, one which is a frequent-cutter, e.g. EcoR1, and the other a rare-cutter e.g. Mse1. These two enzymes must recognize different four-six nucleotides sequences and produce well-defined restriction fragments with sticky ends. (2) The obtained fragments are next ligated to double stranded synthetic adaptors (ca. 20 nucleotides in length) with matching ′sticky ends′ to generate template DNA for PCR amplification. The addition of adaptors of known sequence serves both to provide binding sites for primers on these restriction fragments and also eliminates the need for specific sequence knowledge of the target DNA. (3) Binding is followed by a selective amplification of these ligated fragments. There are in fact two PCR reactions. The first, or pre-amplification reaction, uses primers with a sequence complementary to the adaptor but also contain one extra nucleotide at the 3' end. The obtained fragments serve as a template for the next step, a further PCR amplification using another primer containing an extra three selective nucleotides at the 3'end. Only restriction fragments in which the sequences flanking the restriction sites match the selective primer will be amplified. These two selective

amplification steps ensure that only a limited number of DNA fragments are finally amplified (typically fifty to one hundred) to ensure clear separation via polyacrylamide gel electrophoresis. Subsequent visualisation of the amplified bands is typically achieved via incorporation of a fluorescent or radioactive label in the second amplification step.

Much has been written about the AFLP technique and most of it is positive; results are highly reproducible when compared with RAPD technique (Matthes *et al.*, 1998), it is more sensitive, providing many more markers (Lu *et al.,* 1996; Sharma *et al.,* 1996), and with a high level of resolution (Powell *et al.,* 1996). It also requires only small amounts of DNA, is relatively fast and standard kits are available (Robinson and Harris, 1999). However, as AFLP produces complex fingerprints, high quality DNA is required and the technique is experimentally demanding (Caetano-Anolles, 1998). Because AFLP reaction is able to generate a large number of fragments it has the ability to differentiate individuals and therefore used in paternity analysis (Krauss, 1999), analysis of population genetic variations (Krauss and Peakall, 1998; Russell *et al.,* 1999), and genetic mapping (Van Eck *et al.,* 1995). AFLP analysis has also been effectively used in a wide range of phylogenetic studies e.g. to separate species and their different strains of bacteria from genera *Xantomonas, Aeromonas* and *Clostridium,* where it proved pivotal in epidemiological research (Janssen *et al.,* 1996; Huys *et al.,* 1996). The suitability of the AFLP technique for systematic study of fungi e.g. the potato cyst nematodes, *Globotera* spp. (Nematoda) was demonstrated by Folkertsma *et al.,* (1996), Mueller *et al.,* (1996), or other fungal species (Hill *et al.,* 1996; Sharma *et al.,* 1996; Kardolus *et al.* 1998; Aggarwal, 1999; Barker *et al.,* 1999; Muluvi *et al.,* 1999; Robinson & Harris, 1999; Russell *et al.,* 1999). Again, AFLP analysis has not been applied to the dictyostelids phylogenetic relationships. This is understandable, as that this system not only suffers from being unable to distinguish homology from homoplasy so important in many phylogenetic studies, but is extremely time-consuming, expensive and technically complex. Another molecular technique, which is based on commonly known molecular markers, microsatellites, will be discussed in section 1.12 as it was used in this project.

1.10 Post-Molecular Systematics

So where do the phylogenetic studies of dictyostelids stand in the light of modern molecular techniques? DNA sequencing has proved invaluable for comparative studies. During the 1960s protein sequencing became an automated process, rapid, and, once different protein sequences became available from sufficient species, triggered large-scale phylogenetic studies. In the 1960s and 1970s protein sequencing was the only routine procedure used to investigate molecular evolution. Then DNA sequencing followed, improved further by the development of PCR (Mullis, 1986), which in the late 1980s enabled Sanger's technique, as opposed to that of Maxam & Gilbert (1977), to become the standard for sequencing (Sanger, 1977; Ridley, 2004), the development of which contributed to the early studies on molecular relationships of dictyostelids.

Since the discovery of Mycetozoa in mid 1800s, opinions on their monophyly and phylogenetic affinity has varied greatly as a result of a striking contrast in the trophic stages of their life cycle. This often led to them being classified as entirely unrelated. Therefore, the early studies mainly focused on establishing the phylogenetic position of dictyostelids in relation to the Tree of Life. The early analysis of rRNA gene sequences showed myxogastrid and dictyostelid slime moulds as unrelated early branching lineages. More recent phylogenetic analysis of elongation factor-1α gene sequences showed strong support for a monophyletic Mycetozoa with the myxogastrid and dictyostelid slime moulds being the most closely related sister taxa (Baldauf and Doolittle, 1997). This work also placed the Mycetozoan assemblage as emerging among the multi-cellular eukaryotes, supporting their close relationship with animals and fungi rather than with green plants. A comparative study of ribosomal RNA sequences suggested that *Dictyostelium* represents an early branch in eukaryotic evolution (Olsen and Sogin, 1982; Mccarroll *et al*., 1983). Furthermore, it was also suggested that dictyostelids phylogenetic branches pre-date plant, animal and fungal clades but follow the divergence of *S. cerevisiae*; what is roughly concurrent with the branch that eventually led to the Protista (Loomis and Smith, 1995). Baldauf and Palmer (1993) and Baldauf and Doolittle (1997), both used Olive's higher-level taxonomy, in an attempt to clarify the relationship of the Mycetozoa to other eukaryotes and within the Metazoan

protostelid, myxogastrid and dictyostelid subclasses. They produced and analyzed elongation factor-1 alpha (EF alpha) amino-acid sequences as well as the DNA sequences of their encoding genes of these three Mycetozoan subclasses. The results supported the tenet of Mycetozoan monophyly (Baldauf and Doolittle 1997), which was subsequently supported by Spiegel *et al.,* (1995) and Eichinger *et al.,* (2005) - see figure 1.16. Moreover, the beta-tubulin and actin amino-acid sequences concluded that the Mycetozoa are late arising eukaryotes with a close affinity to the animal-fungal clade (Baldauf and Palmer, 1993). The last indication that dictyostelids are rather late-diverged eukaryotes than the early branch of eukaryotic evolution, suggested by the earlier studies, was also further supported by the discovery of signal transducers and activation of transcription (STAT) proteins, unique to higher organisms, in *Dictyostelium* (Kay, 1997).

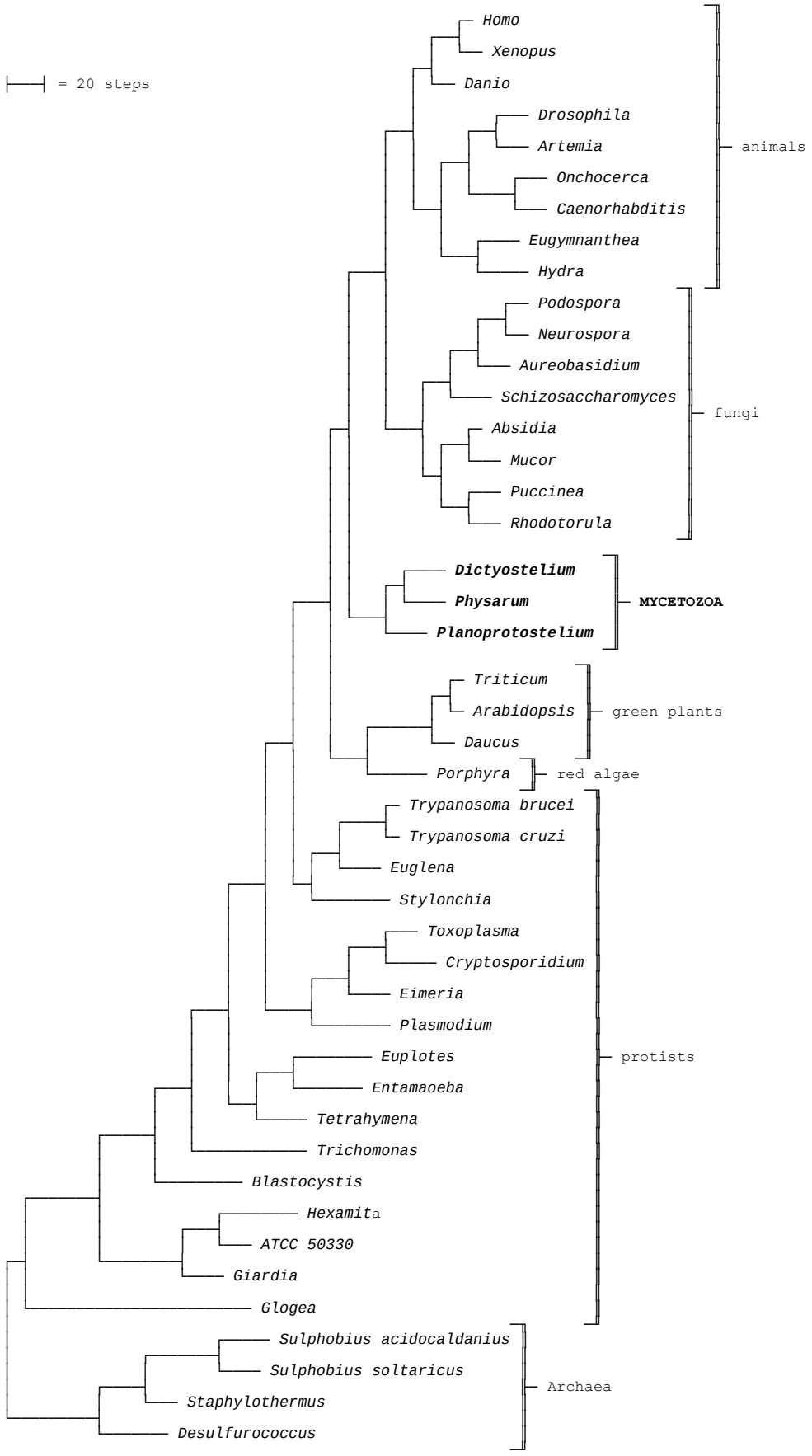


Figure 1.16 A phylogenetic 'Tree of Life' showing the Mycetozoa (bold) as a monophyletic assemblage (adapted from Baldauf & Doolittle, 1997).

1.11 How Many Mycetozoan Genera?

Whether an early or late origin of the Mycetozoan is established, there remains the key systematic question as to whether there are two main genera – *Dictyostelium* and *Polysphondylium*, or whether the species of those two genera should be regarded as one large single genus.

Shaap *et al.,* (2006) used a small 5.8 S subunit of rDNA and alpha-tubulin data from more than 100 Dictyostelia isolates (*Dictyostelium, Polysphondylium* and *Acytostelium*) to construct a molecular phylogeny identifying four major taxonomic subdivisions. Groups One and Three consisted of morphologically diverse *Dictyostelium* species, Group Two contained species from all three genera and finally Group Four contained mainly large, robust *Dictyostelium* species with some *Polysphondylium* species, e.g. *P. violaceum*. These groups were identified using the rRNA sequence data and confirmed (with some exceptions) by the sequence data of alpha-tubulin gene. Schaap *et al.* (2006) showed that none of the four 'molecular groups' correspond to traditional genera and none of these traditional genera appear to be monophyletic. This suggested that dictyostelids taxonomy requires revision and further indicated that morphology of the fruiting body used in the traditional dictyostelids classification is a variable (plastic) trait of little taxonomic value. Furthermore, Romeralo *et al.* (2007) used the 5.8 S unit and both ITS (internal transcribed spacer) regions of rDNA to study the molecular phylogeny of the dictyostelids. Parsimony analyses performed on 28 species and isolates from all three genera and consensus trees obtained in these analyses showed that isolates of the same species were consistently grouped together and therefore have proved the utility of these regions for studying phylogenetic relationships of dictyostelids. Only two studies (Schaap *et al.,* 2006 and Romeralo *et al.,* 2007), have hitherto used sequence data to address dictyostelids phylogeny.

Systematic study of the dictyostelids based on morphological features has failed to establish the number of dictyostelid genera. Although the allozyme studies have shed some light on the dictyostelids genetic diversity, they did not provide any useful taxonomic information and did

not focus on relationships within the dictyostelids (Briscoe *et al.*, 1987, Hodgson, 1999). The majority of other molecular studies focused on establishing the general phylogenetic position of dictyostelids or their position on the Tree of Life but not on differences between the two major dictyostelid genera. The most recent study on rDNA genes has highlighted the relationship between different species but did not clarify the validity of and interrelationships between genera (Romeralo and Baldauf, 2008).

The relationship between the two major genera of *Dictyostelium* and *Polysphondylium* remains unresolved and controversial. The ecological importance of the cellular slime moulds has highlighted the imperative to resolve these uncertainties; in particular to finally establish whether there are one or two dictyostelid genera. Molecular, especially DNA phylogenetics is fast establishing itself as a productive and informative discipline. Any molecular based attempt to resolve the phylogenetic relationship between *Dictyostelium* and *Polysphondylium* has to consider closely related species and so must focus on a molecular region of the genome that will show sufficient variability for comparisons.

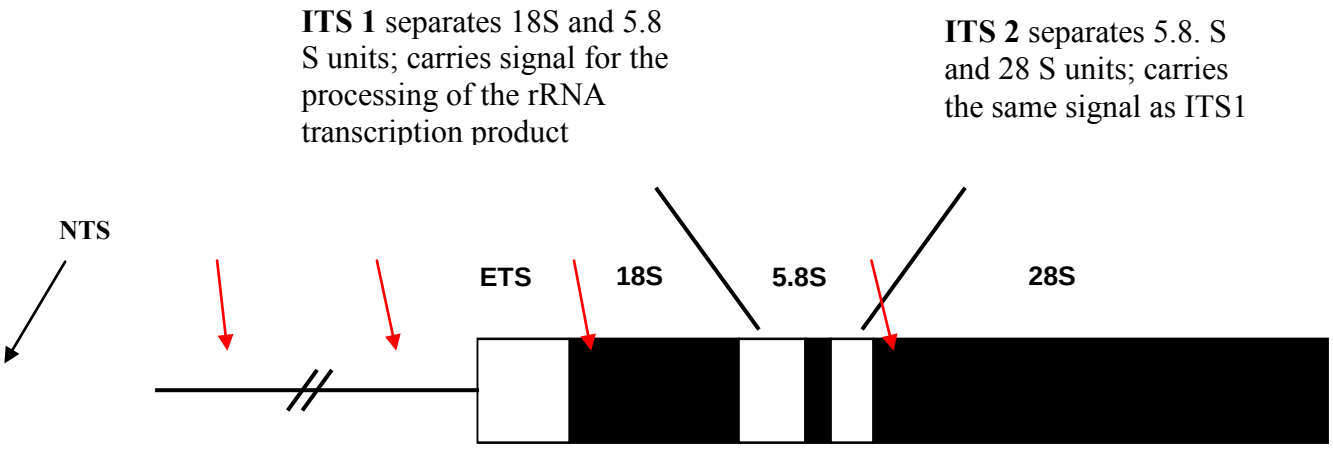
1.12 Markers and Techniques Used in This Study

1.12.1 Ribosomal DNA as a marker

The ribosomal RNA genes and their associated spacer regions are collectively called ribosomal DNA (Mindell and Honeycutt, 1990). Several different rRNAs combine with ribosomal proteins form ribosomes which consists of three subunits, often characterized in terms of sedimentation velocity unit of 23-28 S, 16-18 S, and 5.8 S units. In eukaryotes there are two internal transcribed spacers (ITS), carrying signals for the processing of the rRNA transcription product. Ribosomal genes can be integrated within a chromosome or exist extra-chromosomally. If integrated they are usually organised as a set of tandemly repeated copies of the transcription units and non-transcribed spacers. In eukaryotes the number of DNA copies of this transcribed unit varies from one to several thousand depending on species. The rDNA in *Dictyostelium discoideum* and other slime moulds is organised as an extra-chromosomal unit in the form of palindromic rDNA at 88 kb (Eichinger *et al.,* 2005). The structure of rDNA is shown in figure 1.17.

There has been a recent rapid growth of interest in the use of the internal transcribed spacers of rDNA for systematic studies, particularly the relationships between closely related species. Hillis and Dixon (1991) and Hillis *et al.,* (1991, 1996) in their work on different taxa from a number of different families such as fungi, plants or basal eukaryotes, assessing the suitability of different parts of the ribosomal gene complex for investigating phylogenetic relationships, suggested that the ITS 1 and 2 spacer regions show high level of inter-specific variation, relative to other regions of the ribosomal gene complex, evolve rapidly, and thus could be most valuable in inferring the phylogeny of closely related species. Also, other features of ITS 1 and ITS 2 sequences, beneficial for analysis, supported my choice of molecular markers for this project. Spacer regions are relatively small (600-700bp) and at high copy number (up to 30000 per cell) so are easily amplifiable (Dubouzet and Shinoda, 1999; Xu and Sun, 2001).

Figure 1.17 Structure of r DNA.



18S, 28S and **5.8 S** are conserved gene-encoding rDNA units of ribosomes; **ETS** (external transcribed spacer); **NTS** (non-transcribed spacer) separates the adjacent copies of the rDNA and serves as enhancer of transcription.

Spacer regions can also provide a great deal of resolution, however, their rapid rate of changes, and their relatively short lengths, may limit their effectiveness to infer phylogeny between species or even across the time scales. Moreover, ITS regions are non-coding regions and therefore prone to accumulate mutations fast and without any limitation as it is the case in functional genes, so they can show a relatively higher level of homoplasy (Alvarez and Wendel, 2003), which may influence the taxon alignment and therefore the final analysis. Despite this, Internal Transcribed Spacers are currently the most widely sequenced DNA regions, particularly with fungal species where they have proved useful in establishing phylogeny at and below the generic level (Bain and Jansen, 1995; James *et al* 1996; Scheiber *et al.,* 2000; Xu and Sun, 2001). James *et al.* (1996) for example analysed sequences from the small subunit 18 S and two internal transcribed spacers of two sibling yeast genera of *Zygosaccharomyces* and *Torulaspora,* and found out that analysis of their ITS sequences highlighted and evaluated many interspecies differences. Another study performed with ITS sequences from *Rhododendron* spp. (Scheiber *et al.,* 2000) contributed to a better understanding of the relationships between species within its subgenus *Anthodendron*, section *Pentanthera,* again supporting the usefulness of ITS for phylogenetic studies**.**

1.12.2. Polymerase Chain Reaction (PCR)

PCR is a useful molecular technique introduced in 1985 by K. Mullis, which has transformed the way DNA analysis is performed and has become an essential research and diagnostic tool (Mullis, 1990; Erlich *et al*., 1991). PCR relies on thermal cycling, i.e. cycles of repeated heating and cooling reactions, which cause DNA melting the physical separation of the double helix strands at high temperatures in the first stage, and then enzymatic replication of the DNA (the separated strand is used as a template for the replication performed at lower temperatures). The PCR reaction is based on key components, the annealing and extension of two synthetic sequences of single stranded DNA (of ≤ 30 nucleotides), called primers that flank the region of interest in the target DNA and enable selective and repeated amplification. Nearly all PCR applications employ a heat-stable polymerase e.g. *Taq* polymerase, which enzymatically assembles a new strand of DNA on the basis of single-stranded template DNA using DNA oligonucleotides as building blocks. Standard PCR techniques can successfully amplify products up to 10kb and once the reaction progresses, newly generated DNA is itself used as a template for replication, setting in motion a chain reaction (Brown, 1995). Although PCR is primarily used for producing copies of a sequence of interest, it can be a powerful means of modifying a PCR product to perform a wide array of genetic manipulations, for example, creating new restriction enzyme sites (DuBose and Hartl, 1990; Erlich *et al.,* 1991; Kain *et al.,* 1991; Newton and Graham, 1994).

PCR is simple, sensitive and rapid technique. It has the ability to amplify DNA from as little as 1 pg of DNA (ca. 980 Mbp) obtained from a wide range of different sources, e.g. blood, tissue, single cell, individual hairs as well as from unpurified (e.g. containing traces of proteins) or degraded DNA (Higuchi *et al.,* 1988). Such fine sensitivity allows new methods of studying molecular pathogenesis and has found a number of applications in for example forensic science or genetic linkage studies (Higuchi *et al.,* 1988; Erlich *et al.,* 1991). Its attributes make it a very popular technique which can be performed in relatively short time of only few hours as it consists of generally 30 cycles each lasting 3-5 minutes, compared with cell-based cloning which may take several weeks. However, PCR shows a number of limitations; its extreme sensitivity can be counterproductive as the great care has to be taken to avoid sample contamination from external DNA sources and in order to construct PCR

primers, which permit selective amplification of the target DNA, prior sequence information is required (Gibbs, 1990; Arnheim and Erlich, 1992).

1.12.2.1 Brief introduction to the PCR requirements

It is well known that when developing a new technique incorporating the use of PCR the most important step is to optimize the PCR reaction conditions in order to maximize its sensitivity and specificity. The key to optimization lies in determining what changes to the standard conditions should be made to fully accomplish this. For instance, incompletely denatured template will be unavailable for primers in the subsequent stages of a PCR cycle (Kidd and Ruano, 1991; Bangham, 1991; Hoelzel and Green, 1998).

The annealing temperature is the critical parameter to be optimised for successful PCR. It is generally predicted to be 5°C below the calculated melting temperature for the primers, estimated by the commonly used formula, T m (˚C) =2× (A+T) +4× (G + C) and to some extent depends, on the base composition and length of the primers. An annealing temperature in the range of 50°C to 60°C and annealing time between 30 sec and 2 min is regarded as standard, as long as primers are either not very short (for example 10 bp) or AT rich. The extension time, at 72°C for 1 min is an optimal working temperature for most PCR polymerases and a target sequence of 1 kb and usually depends on the length and the concentration of the target sequence. Estimations of the rate of DNA polymerase synthesis are between 2 and 4 kb per minute equivalent to 35-70 bases per second (Kidd and Ruano, 1991). This rate, however, is influenced by the enzyme used, the buffer composition and the nature of the template (Innis *et al.,* 1988). Prior to cycling, an initial denaturation at 94°C for 5 minutes is needed when amplifying from complex genomic DNA and the final extension at 72°C for 10 min is used to ensure that the annealed primer in the final cycle is fully extended (Newton & Graham, 1994). In order to obtain sufficient PCR product, the reaction normally needs between 25 and 40 repeated cycles, depending on various factors. For example, samples with less than 1000 template molecules may need 40 or more cycles. Normally the number of cycles should be kept to the minimum required for reasonable DNA yield as this protects against non-specific amplification (usually evidenced as a PCR product of a different weight than expected), which is likely to occur after the plateau phase is reached at 20-25 cycles.

If the standard temperatures or times do not work properly for a certain experiment, optimization may be needed. For example, where there is a need to increase the amount of the initial single stranded template for amplifying genomic DNA, increasing the denaturation time may be useful as well as increasing the time for the extension step of the PCR process, to ensure that all annealed primer is fully extended (Hoelzel and Green, 1998; Kidd and Ruano, 1991). While optimizing the annealing temperature it should be also remembered that GC regions in the sequence are usually more stable than AT regions (Hoelzer, 1998; Miesfeld, 1999; Newton and Graham, 1994)**.**

In this project PCR is used to amplify ITS 1 and 2 regions prior to sequencing and forms the basis of the second molecular technique used: ISSR-PCR. Furthermore, there is widespread knowledge of Sanger's chain termination sequencing method in opposite to ISSR-PCR technique which is not so commonly used. Thus, the next section will discuss this technique in more detail.

1.12.3 Inter Simple Sequence Repeats- PCR (ISSR-PCR)

ISSR-PCR also known as Randomly Amplified Microsatellites - PCR (RAM-PCR), or Microsatellite-Primed PCR (MP-PCR) was developed in the early 1990's by Zietkiewicz (1994). Initial ISSR-based studies focused on cultivated species and as a result demonstrated the hyper-variable nature of ISSR markers. A later test on the general applicability of this technique to natural populations used a hybrid complex of four species of the plant genus *Penstemon* and compared the ISSR-PCR data with three available molecular data sets. The results clearly demonstrated the utility of ISSR markers for addressing questions of hybridisation as well as diploid hybrid speciation. Several other studies also confirmed the utility of ISSR markers in a wide range of applications, including molecular ecology, conservation biology and systematics (Gupta *et al.*, 1994; Wolfe *et al.,* 1998). It was not however, until the early 21st Century, that this technique began to find favour in a wide variety of different applications, including taxonomy and phylogenetic relationships. The ISSR-PCR technique is virtually identical to the aforementioned RAPD, differing only in the sequence of the designed primer and a higher annealing temperature. The novelty of this technique lies in the fact that while it is based on microsatellites it has the universality of RAPD analysis.

Inter-simple sequence repeats (ISSR) are DNA fragments found between adjacent, oppositely oriented, microsatellites. These microsatellites generally contain common SSR motifs since the technique requires two SSRs with the same motif to be situated on opposite strands of the DNA within an amplifiable distance of one another (generally ca. 2kb). The typical PCR amplification of a targeted region requires two distinct flanking primers designed on the sequence of interest. However, in ISSR-PCR technique, sufficiently short primers designed on the basis of an 'arbitrary' sequence, commonly found in the genome, amplify sequences from a large number of random loci, going throughout the whole genome. Such a 'microsatellite' primer could anneal at the 5′ or 3′ end of a repeat region and then extend outside into the flanking region. To ensure that the "anchoring amplification" will always start at the 5′ or 3′ end of the microsatellite, an extra two or three 5′ or 3′ nucleotides are present at either 5′ or 3′ end of the particular repeat. This permits the successful amplification of the "inter-simple sequence repeat" fragment, flanked by the microsatellites, on the basis of complementarity to the anchor nucleotides of the designed primers. Such a primer construction also protects from priming from within a repeat and reduces the number of targeted loci to those that can be clearly separated by the subsequent agarose gel electrophoresis. The addition of anchoring nucleotides also allows the design of different specificity primers by for example, extending a primer's repeat-anchoring part or by exchanging its non-repeated part with other unique or degenerate nucleotides. This ISSR primer will target sites at different genomic locations (Zietkiewicz *et al*., 1994). The ISSR designed primer must be highly specific where microsatellite density is high and the genome highly complex.

A suitably designed ISSR primer would generally generate a high degree of polymorphic bands in a single-primer amplification with fragments ranging from 100-2000 nucleotides that could be next easily resolved via agarose gel electrophoresis and finally scored as a given size fragment's presence or absence (Wolfe and Liston, 1998). The number of bands depends on the frequency of the particular repeat and whether or how many anchoring nucleotides were added to the primer. For example $(CA)_n$ repeats have been shown to be less abundant in plant genomes (Lagercrantz *et al*., 1993). Fewer bands are generally obtained from species with small or low complexity genome, for example, *Arabidopsis thaliana*. Such band patterns, visualized via gel electrophoresis, and which come from different species, are never identical and therefore treated as unique profiles of the analysed DNA. However, band patterns obtained from sibling or congeneric (belonging to the same genus) species often

show clear similarities. This means that some bands will be shared between related species but other bands in the profile will be characteristic of particular individuals. The clear similarities and differences between obtained profiles would also suggest that repeats used to design primers for ISSR amplification, although largely species-variable in relation to the number of repeats per unit, in terms of their genomic localisation are relatively stable (Zietkiewich *et al*., 1994). All these factors could indicate that ISSR-PCR is a molecular marker system that could be successfully applied to study genomic profiling at inter- and intra-species level as well as to study evolutionary boundaries between species. Moreover, as this technique allows a good examination of different individuals of the same species, it could also be considered a successful multiple-locus DNA marker system.

The confirmation of the suitability of ISSR-PCR technique for evolutionary biology comes from studies on fungi, e.g. on the differentiation of yeast isolates or on the identification of mycorrhizal fungi e.g. *Gremmeniella abietina* (Lieckfeldt *et al*., 1993; Meyer *et al*., 1993; Longato and Bonfarte, 1997). These proved not only the utility of this technique in generating many fungal species-specific patterns, but also its utility to identify fungi belonging to different species and/or different inter-sterility groups (of e.g. *Heterobasidion annosum*), different races (e.g. North American and European). ISSR-PCR has also proved successful in estimating the genetic diversity between individuals in many different species of fungi and was considered reliable and efficient at genomic-wide estimation of genetic diversity between individuals (Meng and Chen, 2001). Additionally, ISSR-PCR shows the advantage over the earlier mentioned RAPD marker system in finding more markers for any given primer, e.g. 6 to 7 markers instead of only one or two per RAPD primer (Hamelin *et al*., 1993; Hantula *et al*., 1996). There have also been reports on the applicability of ISSR-PCR to study genetic diversity or taxonomical relationships as well as its use as a diagnostic tool to separate morphologically similar species (Hantula *et al*., 2000; Grunig *et al*., 2001; Meng and Chen, 2001; Muthumeenakshi *et al*., 2001; Zhou *et al*., 2001; Luque *et al.,* 2002; Hao *et al*., 2002; Rao *et al*., 2005).

This molecular analysis of dictyostelid species and isolates focused, for justifiable reasons, on the distinction between the genera *Dictyostelium* and *Polysphondylium* previously established on the basis of morphology. As this technique was quick and highly reproducible, it was assumed that it was capable of screening of a large number of isolates/ species in a short time and therefore also capable of discriminating genetic variability at both the intra- and

interspecies levels. This capability would aid the identification of closely related dictyostelid species and therefore a reassessment of their classification which was the key area of this project investigation.

1.13 Phylogenetic Analysis

Molecular phylogenetics, first investigated by Zuckerkandl and Pauling (1962), compares specific characters between related taxa under the assumption that species with more similar molecular and/ or alpha-systematic features are more closely genetically related. The relationships between organisms under scrutiny is therefore based on studies of gene sequences where DNA nucleotide or protein sites are considered as characters representing units of information encoded in organisms and deduced from blocks of conserved sequences constructed in the process of sequence alignment. Molecular and morphological data (the latter using phenotype as the base of phylogeny) are related because the genome contributes to phenotype (Wiley, 1981; Swofford, 1991; Page and Holmes, 1998; Avise, 2006).

The accuracy of phylogenetic analysis is highly dependent upon initial homology inferences at the nucleotide or amino acid level and these inferences are particularly important in non-protein coding genes, e.g. rDNA, in relation to their higher rate of mutation and therefore possible high level of homoplasy as a result. This means that all characters and their character states, as well as inferences of shared-derived states, should be defined by alignment prior to any phylogenetic analysis (Miyamoto, 1991; Page and Holmes, 1998; Avise, 2006). Where non-coding sequences are involved, constructing the alignment can prove problematic, as they contain no phylogenetic 'signals' to aid alignment. Therefore, the ambiguity in the constructed alignment of e.g. rDNA sequences may have profound effects on phylogenetic inference (Morrison and Ellis, 1997). This problem can however, be solved by a character weighting procedure, where tenuous positional homology regions are zero weighted and therefore can be effectively removed prior to further phylogenetic analysis. Moreover, computational problems with multiple alignments could be improved by constructing a set of alignments by the use of different parameters such as gap penalty costs (Gatsey *et al.,* 1993; Thomson *et al.,* 1997). Gaps result from the alignment of sequences of unequal length during the primary homology assessment and contain historical information suitable for

phylogenetic analysis. In order to decide which alignment is preferred among competing hypothesis a common denominator, a gap penalty is introduced. The system of assigning a gap penalty is based on the idea how frequently gaps (deletions/insertions) occur relative to point substitutions (bases mismatches). For instance, exploring a range of different ratios of gap opening to gap extension costs (GO: GE), a penalty for the initiation of the gap to extension of the gap, will lead to the construction of different set of alignments and in this way assess which regions of the given alignment are "unambiguous" or rather identical between alignments (Giribet and Wheeler, 1999). The alignment can be then used for the construction of a cladogram, being the basis of cladistic analysis, and representing hypothesized sister-group hierarchies, obtained by morphological, physiological or other characters analysis and presented in the form of branching. The simplest measure for assessing the data fit to a cladogram is cladogram length which indicates the total number of character state changes necessary to support the taxon position in a tree and therefore the shortest (or most parsimonious) tree fits the data best and is assumed to have least homoplasy. Three widely used indices have been proposed to enquire how well the distribution of character states is explained by the tree. The Consistency Index (CI) measuring the amount of homoplasy in the given character or data set (Kluge and Farris, 1969), the Retention Index (RI) measuring the amount of similarity interpreted as synapomorphy (shared derived character state), expected from the data and retained as synapomorphy on cladogram with RI being better fit between data set of different sizes or tree length. The third, the Homoplasy Index explains the level of homoplasy of a character and is defined as hi = 1 – ci (Archie, 1989; Farris, 1989 a, b).

Cladistics as a method of classification, first explicitly used by Hennig (1966), classifies taxa hierarchically, so that taxa sharing derived characters are grouped more closely together than taxa which do not share any. Hennig argued that only shared derived characters deliver phylogenetic information about taxon relationships. Therefore, the principal step in cladistic analysis is to determine which of the analyzed character states are ‘primitive’ or plesiomorphic and these which are ‘derived’ or apomorphic. The cladogram is supposed to reflect true genealogical clades from homoplastic ones, where two sister analyzed taxa are more closely related to each other than either of them is to a third taxon. In order to ‘polarise’ ‘primitive’ and ‘derived’ characters the outgroup comparison was used. It suggests that character state is considered plesiomorphic if it is shared by a taxon not considered a member

of the group of classified organisms and by all members within the group (ingroup) (Kitching *et al.,* 1998).

Two principal methods are distinguished in construction of phylogenetic trees (Kitching *et al.,* 1998), phenetics, e.g. distance matrices, and cladistics e.g. maximum parsimony, the latter being used in this study The most parsimonious tree (MPT) is the tree which requires the fewest numbers of steps and therefore is considered the optimal tree while other trees require more than the minimum number of steps in order to explain character distributions. The parsimony method may derive several equally parsimonious trees for a given character set. In that case, preference will be given to one particular solution (cladogram) chosen on the basis of other criteria, e.g. character weighting, where more 'weight' is given to one type of character change in relation to another. This method, therefore, compares different parsimonious trees and chooses the tree having the least number of evolutionary steps, e.g. nucleotide substitutions, to explain the phylogeny of the species under scrutiny. Moreover, parsimony tests all possible combinations of all character changes at all nodes of all trees. This means that individual characters are seen as phylogenetic homologies in relation to other independent characters. There are a number of methods used to search for MPTs, with the heuristic method usually employed for larger data sets. It selects most parsimonious trees by selecting an initial tree and then swapping branches to find a better one. The number of possible trees which can be obtained increases with the number of taxa studied. The heuristic approach does not always guarantee to find all or even any of the MPTs, as it suffers from one major problem - entrapment in local optimum, particularly where analysis yields multiple "islands of trees'' with their own local optimum (minimum-length cladograms). In cases like that the shortest tree (global optimum) can only be found by running many analyses, each starting with a topologically distinct cladogram (Kitching *et al.,* 1998).

Despite the disadvantages, maximum parsimony seems to be one of the most commonly used methods for inferring phylogenetic trees. Additionally, it displays a number of properties such as being informative, predictable, repeatable and natural, which are significant when the reconstruction of evolution is considered. Maximum parsimony is considered a simple and popular approach, as it does not seem to depend on any explicit model of evolution. It delivers reliable results, if data are well constructed, with homoplasy being rare or randomly distributed on the tree. Furthermore, the parsimony is based on an auxiliary principle proposed by Hennig, that "simpler hypotheses are preferable to more complicated ones," thus

tree construction requires the smallest number of evolutionary changes in order to explain phylogeny. Moreover, it classifies organisms on the basis of synapomorphies, which are considered the best evolutionary evidence (Hennig, 1966). Parsimony also follows the principle of hypothetico-deductive testing of evolution, which discovers the evolutionary trends from the tree while by contrast, likelihood methods assume these trends and then force the data to fit them. Furthermore, parsimony employs a more precise analytical procedure for handling the data and therefore is more scientific and repeatable, fulfilling the properties of predictability and repeatability. Parsimony is also informative and natural as it delivers classification of organisms with more information content than e.g. phenetic methods. Information content is generally indicated by counting the number of deviations of the character state distribution from the tree. However, the lower the number of deviations, the higher the information content (Schuh and Polhemus, 1981). As parsimony groups the organisms on the basis of synapomorphy (shared derived character state) and not by similarity, as it is in case of phenetic methods, therefore, parsimony will always give more informative classification (Farris, 1983). Finally, if classification of organisms is performed with the highest content of information used, such classification will always show the maximum congruence of characters and therefore will be the most natural (Lipscomb, 1998).

1.14 Objectives

The aim of my study was to explore the phylogenetic relationship between species, which on the basis of morphological features, mainly their fruiting bodies, were assigned to two major genera of *Dictyostelium* and *Polysphondylium,* using genetic marker techniques, in order to clarify their position in the dictyostelid family tree with the main objective of establishing the exact number of genera.

As discussed in previous sections dictyostelids comprise closely related species (species which diverged less than 50 million years ago) grouped in two main genera *Dictyostelium* and *Polysphondylium*. Their main taxonomy is mainly based on morphological features e.g. microcyst formation, spore pattern, presence or absence of polar granules or the sensitivity to chemotactic cAMP. Although both *Dictyostelium* and *Polysphondylium* were grouped together in the family Dictyosteliaceae, on the basis of amoeba type and the presence of the

predatory phase in the asexual life cycle, the main morphological character used to distinguish between these two genera was the type of branching being either regularly branched or unbranched sorophores (see section 1.5). Quite aside from such differences in form between these two genera, the pattern and dimensions of sorocarps may also vary within individual species, depending largely upon the relative abundance of the vegetative amoebae and upon the cultural environment. This can profoundly influence the manner in which little amoebae coordinate their movement during aggregation and then subsequently differentiate to form their fruiting bodies (sorocarps). Indeed, ideally the classification of species/genera should have been based upon all aspects of dictyostelids growth and development, but in practice, it was defined primarily upon the physical characteristics of their fructifications. Moreover, the genus *Dictyostelium* containing many more species than *Polysphondylium* shows that *Dictyostelium* species substantially differ between each other. In fact many species share certain characteristics with genus *Polysphondylium*, for example, the presence of polar granules or absence of chemotactic response to cAMP. Therefore, the idea of recognising the genus *Polysphondylium* as a separate entity has prompted ongoing discussion since 1869 (Olive 1902; Rai and Tewari 1961; Bonner, 1967; Raper, 1973 in Traub and Hohl, (1976)**.** Despite the fact that certain differences between both genera were recognised and acknowledged, the discussion led to the conclusion that significant structural and physiological differences do not justify the separation of these two genera. Moreover, it was also suggested that those differences may not be sufficient to define a new 'intermediate genus', even though Traub and Hohl (1976) drew attention to the fact that many species already classified as *Dictyostelium* possessed a number of characters shared with *Polysphondylium*.

The work of Traub and Hohl (1976) not only supported the existence of at least two groups/ genera but it also suggested the need for erecting a new genus. Indeed, in 1977 a new genus, *Heterosphondylium,* was introduced, although not formally described, by Troub in order to accommodate species, which were richly but irregularly branched and which showed the absence of branches arranged in definite whorls. That new genus also accommodated species favouring substrates with a reduced nutrient content. Such species were too different to be included in either Brefeld's genera (Raper, 1984), but this newly introduced genus was not universally adopted. The fact that some aspects of culture conditions may exert significant influence over the variability of morphological features would suggest the limitations to a valid classification of dictyostelids based entirely on morphological features. In that aspect

dictyostelids taxonomy seems to be far from valid as it has failed to produce an unequivocal species classification. Thus, lack of validity in dictyostelids classification prompted a new focus on molecular markers.

The few published molecular studies discussed in more detail in section 1.10 have focused on establishing the phylogenetic position of dictyostelids on the Tree of Life e.g. Baldauf and Palmer, (1993) or Baldauf and Doolittle, (1997) as well as the isolation and description of new species rather than on seeking a resolution of the number of genera, a constant source of confusion. One of the attempts, discussed in more detail further on in this section, was the use of allozyme electrophoresis to assess the genetic diversity of the number of *Dictyostelium* species (Briscoe *et al.,* 1987). It suggested very little genetic variability within this group of species. This idea was further pursued by Hodgson (1999) but raised some doubt in relation to the efficacy of this technique for taxonomic purposes. Moreover, Ramagopol and Ennis (1984) made some attempts in relation to resolve the taxonomic relationship between the two genera *Dictyostelium* and *Polysphondylium* on the basis of amino acid composition of ribosomal proteins. Although their study seemed to support the existence of two genera with more differences in amino acid composition between genera then between congeners this demonstrated strong conservation of ribosomal amino acids and therefore a low level of variability. This cast some doubts on suitability of amino acids as genetic markers to study closely related species and therefore phylogenetic relationships of dictyostelids. It seemed that such task would need a special approach in relation to the choice of suitable genes, which would be variable enough to be able to distinguish the existing differences between closely related species.

When resolving relationship between closely related species, target genes, or gene sequences, should fulfil a number of requirements; in particular they should be sufficiently variable to distinguish between different species but not so variable as to cause alignment problems and therefore difficulties in comparing sequences. The Hillis and Dixon (1991) review of the suitability of different parts of ribosomal DNA to infer phylogenetic relationships provided a wealth of information about phylogenetic relationships across a wide spectrum of organisms from basal lineages of life to study closely related species. This work also showed that although rDNA sequences are a rich mine of evolutionary information which can solve many problems previously considered intractable by morphology, different regions of this gene are useful when addressing different phylogenetic problems. The reason for such versatility of

the rDNA gene comes from different rates of evolution among different regions of this gene, the fact that this gene exists in many copies as well as the pattern of concerted evolution that occurs between different copies of this gene. Originally the alignment of sequences was performed by Clustal X and then subsequently by eye. Preliminary alignment performed on sequences of the small, 18 S subunit of rDNA, performed with exemplar species of *Dictyostelium discoideum*, *Dictyostelium mucoroides* and *Polysphondylium violaceum* showed very little phylogenetic signal appropriate to distinguish between closely related species. There were also some brief attempts to sequence and align calmodulin and then beta-tubulin gene sequences (data not shown), also with exemplar species from both dictyostelid genera. These alignments also showed too little phylogenetic signal to be suitable to distinguish between closely related species. Indeed these results should not come as surprise, especially as it is a well known fact that the process of choosing a suitable region that is likely to be appropriate for a particular systematic question is the most critical step in any analysis. As Hillis and Dixon (1991) showed, if the chosen region is evolutionarily too conserved i.e. compared sequences in the alignment will be nearly the same for all studied taxa, collecting such invariant data would be a waste of time in relation to the addressed phylogenetic question. Their work also showed that the small subunit of rDNA is among the slowest evolving sequences and therefore most suitable to infer ancient evolutionary events and not recent ones. This finding also supports my other preliminary results performed much earlier in my research (data not shown) with small 18 S subunit rDNA, proving that coding regions are too conserved to be suitable to study closely related species.

Finally, the decision was made to use non-coding regions of rDNA (see figure 1.17), principally ITS1 and 2 which show generally high level of inter-specific variation in comparison with other rDNA regions (Hillis and Moritz, 1996). My choice of this molecular marker was supported by the fact that there has been a recent rapid growth of interest in the use of the internal transcribed spacers of rDNA for systematic studies, particularly the relationships between closely related species, suggesting at the same time that the level of variation in the spacer regions is enough to make them suitable to identify species and even different strains (Hillis and Dixon, 1991; Hillis and Moritz, 1996). When, as here, species-level comparisons are considered, the most appropriate markers to use are those that vary significantly between the species under consideration but not within them. The inter-species differences should not be too large otherwise the true phylogeny could be affected by, for example, convergence. My choice of gene was also supported by the fact that multiple-copy

gene families are easier to analyse. Since they are so uniform and abundant it gives them the advantage that regions of this gene can be amplified with the use of PCR and also can directly be sequenced. An additional advantage is the fact that PCR amplification of both internal spacers is facilitated by three conserved flanking regions of 18S, 5.8S and 28S subunits of rDNA (see figure 1.17) which allow the design of universal primers for use in PCR amplification.

Moreover, there have been three previous attempts to study rDNA sequences of the slime moulds. Evans *et al.* (1987) used DNA probes especially designed for rDNA coding regions, the spacer regions and two genes, actin and discoidin from chromosomal gene families in order to examine 27 wild isolates assigned to *Dictyostelium discoideum* morphotypes, (isolates assigned to the same species on the basis of morphological criteria). Their results showed that phylogenetically important differences in sequence have been accumulated in the spacer regions of these examined sequences, whereas the coding regions of these examined rDNA sequences proved much more conserved. Additionally, their findings also showed that four probes designed for rDNA spacer regions, either did not hybridize or had decreased levels of hybridization to the DNA of four isolates of *Dictyostelium discoideum*. As it was shown hybridization failed in these four isolates because they were morphologically distinct from other isolates of *D. discoideum* and displayed unusual mating behaviour. These differences were interpreted by Evans *et al.* (1987) who indicated that these four atypical isolates, although previously assigned to *D. discoideum*, now should be considered species other than *D. discoideum*. Furthermore, Evans *et al.* (1987) also showed that the changes accumulated in spacer regions enabled the examination of isolates and therefore the identification of new species, showing at the same time the power of ITS analysis for systematic purposes. Also Schaap *et al.* (2006) used the small subunit of ribosomal DNA, in parallel with the alpha-tubulin gene sequences, obtained from around 100 dictyostelid species and isolates to infer dictyostelids phylogenetic relationships. Their findings based on both genes suggested that dictyostelids consist of more than one group and indeed identified four groups. However, on the basis of morphological features none of these groups were traditionally recognised nor they were monophyletic as they contained species from both genera. Therefore, they concluded that dictyostelids taxonomy required complete revision and therefore remained unresolved. The findings of Schaap *et al.* (2006) correspond to another recent study by Romeralo *et al.* (2007) on the molecular systematic of dictyostelids. They also used rDNA sequences with the focus on ITS 1 and on the 5.8 S region of rDNA to

examine their efficacy in identifying dictyostelids phylogenetic relationships. Their results confirmed that small differences in ITS regions, between isolates and congeners, make these regions very informative for studying phylogenetic relationships. Moreover, the ITS sequences were able to differentiate closely related species, while the 5.8 S region of rDNA was considered too conservative for that purpose. In conclusion, analysis of molecular data showed that (a) *Dictyostelium* and *Polysphondylium* consist of more than one major group; (b) none of these groups correspond to their traditional genera, and, (c) none of the traditional genera are monophyletic. They also indicated that some morphological features, e.g. fruiting bodies upon which their traditional classification was based appear to be of little taxonomic value.

In order to achieve the objectives of my study I intend to sequence the ITS 1 and 2 spacer regions of the chosen species and isolates. The sequences were to be amplified in the process of Polymerase Chain Reaction (PCR) and then directly sequenced. The obtained sequences were to undergo parsimony analysis via PAUP (Swofford, 2002). In addition to direct DNA sequencing of spacer regions of rDNA, I also decided to use another molecular marker, ISSR, also amplified by PCR and analysed by PAUP, in order to determine whether this second technique could aid the reassessment of dictyostelids classification together with forming an effective and rapid tool to identify newly isolated, but closely related species. I am interested whether results obtained by this second technique would support the direct sequencing results. Clearly new isolates and species cannot be defined in terms of morphology alone. The identification of new soil isolates is better achieved via analysis of DNA rather than morphology or a combination of both. There is a need for a rapid and highly reproducible molecular technique, able to screen a large number of newly isolated samples and which can identify new isolates in a short time. The ISSR-PCR profiling technique (see 1.12.3) is rapid and simple to perform, capable of discriminating genetic polymorphism at both the intra- and inter-species levels, and at the same time able to yield a large amount of data in a short time (Zietkiewicz, 1994; Wolfe *et al.,* 1998). Therefore, it was considered to be suitable for use in this study**.**

Furthermore, I assume that any designed ISSR-PCR primer is likely to amplify a different target site and these amplified sites would vary between species and isolates, producing different electrophoretic profiles, thus could be used to differentiate both species and isolates. I am also interested to further test this technique for its suitability as a multi-locus marker

system in relation to the changes made in the anchor sequence of the used ISSR-PCR primers. These anchor sequence changes would produce a number of different primers, annealing to the different parts of the sequence, and as a result produce different sequence profiling, which thus should enable clear identification of different dictyostelid species and isolates. I also want to investigate whether the variable profiles, generated from individual species and isolates could be further subjected to full phylogenetic analysis, and yield additional information on phylogenetic relationships within the dictyostelids.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Origins of cellular slime moulds

To date ca. 70 dictyostelid species are recognised world-wide, of which 60 < represent the genus *Dictyostelium* with few (ca. seven) in the genus *Polysphondylium.* My work will examine a total of 49 species and isolates including sixteen different *Dictyostelium* species, some of unknown designation, and three *Polysphondylium* species. Some species from the two genera e.g. *P. violaceum* and *D. discoideum* are common, widely available, and will be represented by a number of isolates taken from different sites. The single species status of *D. mucoroides* remains uncertain, therefore seven isolates of this species were included in my analysis. The genus *Acytostylium* (Protostelida) and *Amoeba proteus* (Proctista) were both used as phylogenic outgroups. The 51 species and morphotypes (including outgroups) used in this thesis came from the stock held by Denis Wheller in the Department of Life Sciences at Anglia Ruskin University (ARU) and from a stock held in the USA. Eight were American species donated by Dr Landorf from USA. Most of the ARU species are of U.K. origin though a few were collected in the United States. The list of all species (including American species) with their place of origin is shown in table 2.1.

All species were preserved as desiccated spores on crystals of silica gel and stored in a refrigerator at 4°C. Sterile desiccated grade 40 mesh silica gel granules by Sigma Aldrich Scientific, (Epsom, U.K.) were used for preserving dictyostelids spores.

Table 2.1 Dictyostelid species and isolates used for ITS and ISSR-PCR analysis.

Species/isolates shortenname/code	Species and isolates	Isolate code	Culture no.	Origin
Acytostelium	*Acytostelium spp.*		45	USA
D.brefTNC11	*D.brefeldianum*	TNC 115	11	U.K.
D.citCu2A4	*D.citrinum*	2A4C	4	Cuba
D.citD.dCR	*D.citrinum/D.discoideum*	CH14A	3	Costa Rica
D.d ATN119	*D.discoideum*	ATN 1,19	13	USA
D.d ATN122	*D.discoideum*	ATN 1/22/6	12	USA
D.dDcit Mex	*D.discoideum/D. citrinum*	CF3B	6	Mexico
D.discNC4	*D.discoideum*	NC4	37	U.K.
D.discV12	*D.discoideum*	V12	10	U.K.
D.impEPN1 or EPN11	*D.implicatum*	EPN 1,1	20	Epping Forest, Essex, U.K.
D.minNWM12	*Dictyostelium minutum*	NWM 1,2/2	2	USA
D.muc BPR31	*D.mucoroides*	BPR/3,1/21	16	Bedford Northants, U.K.
D.muc FLM or FLMN28	*D.mucoroides*	FLM/1,1/1	28	Felshamhall Wood, Suffolk, U.K.
D.muc HTL or HTLN29	*D.mucoroides*	HTL/2,1/1	29	Hatfield Forest, Essex, U.K.
D.muc PYR31 or PYR	*D.mucoroides*	PYR/3,1/20	30	U.K.
D.mucBPR11	*D.mucoroides*	BPR/1,1/1	21	Bedford Purlieus, Northants, U.K.
D.mucDDS orDDS21	*D.mucoroides*	DDS/2,1	32	U.K.
D.mucEW5	*D.mucoroides*	EW5,2,7	35	U.K.
D.ros EDF3	*D.rosarium*	EDF 3,1	22	Norfolk, U.K.
D.ros IL	*D.rosarium*	IL	18	USA
D.rosAlabF or Alab	*D.rosarium*	F2	7	Alabama
D.sp HLM 411	*D. species*	HLM/4,1/1	27	U.K.
D.sphaGMN3	*D.sphaerocephalum*	GMN 3,3,1	17	Gamlingay Wood, Cambridge, U.K.
D.sphaSXN2	*D.sphaerocephalum*	SXN/2.1/1	1	U.K.
D.spWLD1 or WLDN12	*D. species*	WLD/1	12	U.K.
DaursBDW4 or BDW	*D.aureo-stipes*	BDW 4,1/3	23	U.K.
Dimp12	*D.implicatum*	EPN 1,2	16	Epping Forest, U.K.
DimpBCN21	*D.implicatum*	BCN 2,1	21	Norfolk, U.K.
Dmin ATN16	*D.minutum*	ATN 1,6/21	8	USA
DminGMN3	*D.minutum*	GMN 3,1	11	USA
Dpurp1K or AT1K	*D.purpureum*	1K	19	USA
DpurpAT17 orATN13	*D.purpureum*	ATN 1,7/1	13	USA
DpurpATN1	*D.purpureum*	ATN 1	20	USA

DpurpCRLC	*D.purpureum*	LC2A	9	Costa Rica
DspBDW112	*D.species*	BDW/1,1/2	19	U.K.
Dspha HYY1	*D.sphaerocephalum*	HYY 1,1/1	15	Hayley Wood, Essex, U.K.
DsphaGMN31	*D.sphaerocephalum*	GMN/3,1,1	25	Gamlingay Wood, Cambridge, U.K.
DsphaSib 6	*D.sphaerocephalum*	Sib2/5	6	Siberia, Republic of Russia
DspHLM42	*D.species*	HLM/4,2	17	U.K.
DtenueCu2G3	*D.tenue*	2G3A	5	Cuba
P.aur 36 or N36	*P.aureum*	SLR/1,1/1	36	U.K.
P.pallidum or Ppall ATN18	*Polysphondylium. pallidum*	ATN1,8/10	1	USA
P.violATN18	*P.violaceum*	ATN 1.8	14	USA
PviolATN	*P.violaceum*	ATN	38	U.K.
PviolATN1	*P.violaceum*	ATN 1	10	USA
PviolATN1112	*P.violaceum*	ATN/1,11/2	3	USA
PviolDNC1	*P.violaceum*	DNC 1,1/1	43	U.K.
PviolDNC3 or D3	*P.violaceum*	DNC 3,1/1	44	U.K.
PviolFCM	*P.violaceum*	FCM	40	U.K.
PviolRTN	*P.violaceum*	RTN	42	U.K.
PviolSTR	*P.violaceum*	STR	39	U.K.
PviolTDN	*P.violaceum*	TDN	41	U.K,

2.1.1 Growth of cellular slime moulds.

Cellular slime moulds from the genera *Dictyostelium* and *Polysphondylium* were grown using two media, Lactose-peptone agar medium (LP) and Hay Infusion agar medium (HIA) developed in our laboratory by Helen Hodgson. The experience of Cavender & Raper (1965), who tried out many different solid media for the growth of dictyostelids, resulted in the suggestion that *Poa* species hay infusion agar buffered to ca. pH 6.0 with a dense inoculum of bacteria was the best medium on which to grow dictyostelids as it supported the growth of all dictyostelids, whilst limiting microbial contamination (Hodgson, 1999). Lactose-peptose agar medium is a simple medium used as the first growth medium to revive species from the silica gel. Hay Infusion agar medium was used to maintain cultures of cellular slime moulds. Both media were prepared according to the protocols available in the Department of Life Sciences, ARU as described below.

2.1.2 Culture media

Lactose-Peptose agar medium (LP)

Agar (tech N°3, without nutrients)	90.0 g
Lactose	5.0 g
Peptose (bacteriological)	5.0 g
Water	5.0 litres

The technical agar N°3 (Sigma Aldrich Scientific, Epsom, U.K.) was first dissolved separately in 4 l of distilled water then the remaining ingredients were added and dissolved. The whole mixture was made up to a final volume of 5 l and bottled in 400 ml aliquots. All bottles were autoclaved at 110°C at full pressure for 20 minutes.

Hay Infusion Agar medium (HIA)

Meadow hay	40.0 g
Agar (technical N°3)	75.0 g
Potassium dihydrogen orthophosphate	7.5 g
Disodium hydrogen orthophosphate heptohydrate	3.1 g
Water	5.0 litres

First 40 g of meadow hay was dissolved in 3 l of distilled water, autoclaved at 120°C for 20 minutes and filtered through cotton cheese cloth with a 0.5 µm mesh. Next 75 g of technical N°3 Agar (Sigma Aldrich Scientific, Epsom, U.K.) was added, and made up to 4.5 l. The whole mixture was autoclaved until the agar was dissolved. Then, 7.5 g of potassium dihydrogen orthophosphate and 3.1 g of di-sodium hydrogen orthophosphate heptohydrate

were dissolved separately in 500 ml of water and added to the 4.5 l hay-agar mixture which was divided into 400 ml bottled aliquots and autoclaved at 121° C for 15 minutes.

2.1.2.1 Preparation of an inoculum of *Klebsiella aerogenes*

The bacterium *Klebsiella aerogenes* was used as the food source for cellular slime moulds, as recommended by Turner (1978) and Hodgson (1999), as an alternative to *E.coli*, to achieve better dictyostelids isolation. Both bacteria, however, would adequately support the vigorous growth and development of dictyostelids.

Firstly the *K. aerogenes* was streaked on the nutrient agar (NA) in a 90 mm plastic Petri dish and left in an incubator at 37°C for 24 h. Next day the bacterial culture was checked for any contamination and then, if contaminant-free, a few colonies from the plate were transferred to 10 ml of freshly made broth, prepared according to the protocol, used in Life Sciences ARU (see below, section 2.1.2.3), in a sterile glass bottle and incubated at 37°C overnight, to a thick bacterial suspension.

Nutrient Agar (NA)

Agar	15.0 g
Lab-Lemco powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Distilled water	1.0 litre

All 28.0 g of the ingredients were suspended in 1 l of distilled water, and brought to the boil to dissolve completely. The pH was adjusted to 7.4. The solution was sterilised at 121°C for 15 minutes. Nutrient agar (NA) was supplied by Sigma Aldrich Scientific, (Epsom, U.K.).

Nutrient broth (NB)

Lab-Lemco powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Distilled water	1.0 litre

All ingredients were dissolved in 1 l of water and adjusted to a pH of 7.4, then autoclaved at 121°C for 15 minutes. Nutrient broth (NB) was supplied by Sigma Aldrich Scientific (Epsom, U.K.).

2.1.2.2 Culturing amoebae

The life cycle of cellular slime moulds (see Chapter 1) comprises two main stages, tiny amoebae feeding on bacteria and secondly when they start to produce the fruiting body and take up the appearance of fungi. This second stages starts when the food resources are limited (the plated *K. aerogenes* has been consumed) and amoebae become starved.

The easiest way to extract DNA from cellular slime moulds is via their amoeboid cells. Amoebae were obtained as follows. Spore containing crystals were placed on the Lactose-Peptose agar medium (LP) to prompt the slime moulds to grow by firstly allowing spores to give rise to amoebae and then to produce the spore-containing fruiting bodies. This step revived slime moulds from the preserved form. The spores in the form of sori were then placed on fresh LP medium, with streaks of bacteria to provide food. New amoebae and then fruiting bodies were produced. This stage enabled recognition of the desired species and

elimination of any contamination. The sori of the clean and correct species were mixed with a solution of bacteria and placed on the new medium to obtain amoebae. This step allowed the amoebae to multiply to the amount sufficient for DNA extraction. The amoebae were harvested and used for extraction.

2.1.2.3 Growing amoebae

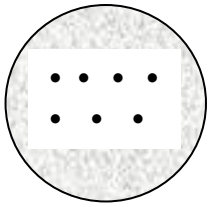
The Petri dishes with LP medium were poured a day in advance of inoculation, then on the next day 0.2 ml of *Klebsiella areogenes* broth suspension were spread over the surface of the medium with a disposable spreader, so that the surface was completely covered, and left to dry for a few minutes. Then a few crystals of every dictyostelid species were placed on top of the medium with the bacteria, with a separate plate used for each species. All plates were incubated at 22°C for a few days and examined for the presence of dictyostelid colonies starting at second day of incubation. The examination was repeated daily over a 1-2 week post-inoculation observation period due to the fact that species growth and development from inactive silica crystals occurred to be highly variable. *Dictyostelium* species generally showed sufficient growth for further study within 3-7 days, while *Polysphondylium* species required up to 13 days. Indeed the first colonies of *Polysphondylium pallidum* were not observed until 12 days post-inoculation. Each colony was assumed to arise from one spore, amoeba or microcyst (Hodgson, 1999).

Soon after translucent areas in the bacterial lawn were visible around the crystals, dictyostelids fruiting bodies started to appear. Plates with HIA medium were prepared in advance and streaks of bacteria *K. aerogenes* applied to them. Next, three to five spore containing sori of each species were carefully picked using a sterile hypodermic needle and placed onto these streaks. One plate with HIA medium and two streaks of bacteria were prepared for each species and secondary cultures were then incubated at 23°C for 7-13 days. Primary cultures were also kept in the incubator in case growth of these secondary cultures failed. Then after three days of incubation the plates were checked using an Olympus SZ4045TR zoom stereo microscope at magnifications ranging from 6.4 to 40 times for the presence of dictyostelid fruiting bodies. Daily examination was carried out for the next 10 days.

When the fruiting bodies of each species of dictyostelid were well established in secondary cultures, new plates with HIA medium were poured. Then the 5-10 sori containing spores were carefully transferred using a sterile hypodermic needle (a new needle for each new species) into the disposable plastic bottle containing 0.2 μl of freshly incubated broth suspension of *K. aerogenes* bacteria. After mixing by tilting, the mixture of bacteria and spores was spread over the top of the plate with HIA medium and left to dry (two new plates per each species) and dry plates incubated at 22°C. Plates were then examined daily for the presence of small amoebae. It was important to get all species at the right growth stage to achieve successful DNA extraction. To achieve the amoeba stage, optimal for DNA extraction, some 2-4 days of post-plating incubation of the sori and bacterial mixture was generally required. The culturing stages of *Dictyostelium,* from silica gel crystals to extraction of DNA from amoebae, are shown in figure 2.1.

Figure 2.1 The culturing stages of dictyostelid: from silica gel crystals to DNA extraction.

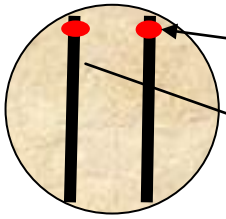
1) Clean Petri dishes are filled with LA medium mixed with bacteria *K. aerogenes.*

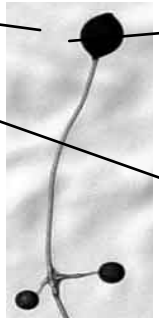


2) The silica crystals containing spores are spread on the medium and cultured for 3-7 days.

3) Through amoeba stage to fruiting body and spores stage.

4)

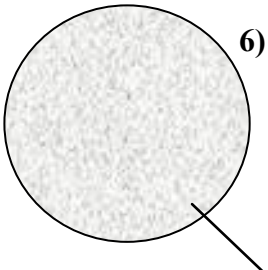




Sori with spores are placed on HIA medium with streaks of *K. aerogenes* and cultured until amoeba and then fruiting bodies appear (3-7 days).

Streaks of bacteria *K. aerogenes*

Sori with spores are placed again on HIA medium with streaks of *K.aerogenes* and cultured until fruiting bodies appear (3-7 days). 5)



6) Spores (sori) mixed with medium and bacteria are spread on the dish.

7) The plates are observed daily for amoeba. Once they occur, they are harvested for extraction of DNA (full description & 2.1.2.2 to 2.1.2.4)

2.1.2.4 Amoeba harvesting

Two to four days after plating the sori, translucent areas were generally visible in the bacterial lawn previously inoculated and where dictyostelids appeared in the form of bacteria eating amoebae-like cells. These cells were harvested at a time when nearly all bacteria were consumed which was important to reduce the chances of extracting significant bacterial DNA contamination along with the target dictyostelid DNA, even though PCR conditions were optimized to be specific for dictyostelid DNA. To harvest the amoeba, the lawns of amoeba were scraped off the medium surface with a plastic disposable loop and washed with 5-10 ml of sterilised NaCl (Ringers 1/4 strength tablets solution). The suspension of amoeba of each species from the two plates was then pipetted into sterile 10 ml plastic disposable bottle and centrifuged at 3500 rpm for 10 minutes. The pellets were next resuspended in phosphate buffered saline (PBS tablets) for DNA extraction.

Ringers solution pH 7 (1/4 strength tablets) by Oxoid

Sodium chloride	2.25 g (per tablet)
Potassium chloride	0.105 g
Calcium chloride ($CaCL_2$ 6 H_2O)	0.12 g
Sodium bicarbonate	0.05 g
SDS water (sterile distilled water)	1.0 litre

Phosphate buffered saline (PBS tablets) by Oxoid

Sodium chloride	8.0 g (per tablet)
Potassium chloride	0.2 g
Disodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
Distilled water	1.0 litre

2.2 DNA Extraction

There is a well established procedure (see 2.2.1) for extracting DNA from dictyostelid amoebae developed by Robert Kay in 1995 working in the Laboratory of Molecular Biology at the University of Cambridge (see 2.2.1.1). This technique was however, time consuming and prompted me to search for a more rapid and simple procedure (see 2.2.4) for the present study.

2.2.1 DNA extraction method I - according to the protocol by Kay, (1995)

2.2.1.1 Solutions for extraction (Kay, 1995)

Lysis buffer

Tris in distilled water	0.6 g/ 250 ml
0.1 M HCl	40.3 ml
distilled water & buffer to pH 7.5	500 ml
sucrose	54.75 g
$MgCl_2$,	0.51 g
sodium azide	0.10 g
triton X-100	5.0 ml

Digestion Buffer

Tris	0.3 g
distilled water	250 ml
0.1 M HCl	20.15 ml
distilled water	500 ml
sodium EDTA	0.63 g
SDS	3.5 g
	Store at 4°C

5 M Ammonium Acetate, pH 7.4

ammonium acetate	7.7 g
distilled water	6 ml

Mixture phenol: chloroform

phenol	5.0 g. in -
TE buffer (19mM TrisHCL, 1mM EDTA pH 7.4)	9.0 ml
chloroform	9.0 ml (cooled)
8-hydroxiquimoline	0.08 g

2.2.1.2 The procedure

This extraction protocol was based on the use of approximately 100 million dictyostelid cells.

1. After harvesting, the cells were washed to free them of bacteria, then spun in KK2 centrifuge at 2000-4000 rpm for 4 h.
2. The cells were resuspended in 1ml of Ringers 1/4 strength solution per plate.
3. After resuspending the cells 0.5 ml of ice cold cell lysis buffer (see recipe above) was added and mixed.
4. Nuclei were spun down at 3600 rpm in a bench centrifuge for 10 min at 4°C and then the supernatant carefully tipped off from the very loose pellet.
5. The pellet was resuspended again in 0.5 ml of the lysis buffer, mixed well and pelleting repeated.
6. The pellet was further resuspended in 0.3 ml lysis buffer, taking care to resuspend the pellet.
7. After resuspention 2.7 mg of Proteinase K was added to the resuspended pellet so that it gave 100 μg/ml after the digestion buffer was added.
8. Then 1 ml of digestion buffer was gently added, mixed carefully and incubated for 1h at 60°C for the Proteinase K to work.
9. The mixture was extracted with 1 ml of phenol by gentle inversions (not vortexing) and next the aqueous phase was taken off and leaving the interphase.
10. The extraction was repeated with 1 ml phenol: chloroform (equal ratio) two to three times until there was no interphase then the extraction repeated once more with chloroform.

11. 0.6 ml volume of 5 M ammonium acetate pH 7.4 was added and mixed cautiously. The DNA was spooled out by layering 1-2 ml of ice-cold ethanol on top. In case it failed, mix the ethanol gently and pick out the tangle of DNA with a hooked Pasteur pipette.
12. Finally it was rinsed in 70% of ethanol and dissolved in TE without ever letting it dry. This might take some time as the DNA might not dissolve immediately.

2.2.2 DNA extraction method 2 - by boiling

Harvested amoebae were suspended in 200 µl of NaCl (suspension contained ca. 10,000 amoebae) and placed in a bath of boiling water for 2 minutes. The cooled suspension was used directly for PCR.

2.2.3 DNA extraction method 3 - by microwaving

The microwave procedure was outlined by Goodwin and Lee (1993), as follows. A pellet of ca. 10,000 amoebae was placed in 50 µl of lysis buffer (see protocol below) and microwaved at 650 W for a total of 20 s in three separate segments of 10 s, 5 s and 5 s. A further 300-350 µl of lysis buffer was added and the mixture incubated at 80°C for 10 minutes. After incubation 400 µl of centrifuge mixture 1 (see protocol below) was added and centrifuged at 10,000 g for 15 minutes. After that the aqueous phase (top layer) was removed and placed in a fresh sterile plastic tube together with 0.54 ml of isopropanol and 10 µl of 5 M sodium acetate (mixture 2-see protocol below). The mixture was centrifuged again at 10,000 g for 2 minutes. After the supernatant was discarded the pellet was washed with 80 % ethanol and resuspended in 50-150 µl of the resuspension mixture (see protocol below) from which DNA was obtained for PCR.

2.2.3.1 Reagents for DNA extraction method 3

Centrifuge mixture 1

1:1 chloroform: phenol solution	12 ml

Centrifuge mixture 2 (100 ml)

41.0 g of 5M sodium acetate dissolve in -	41.0 g
distilled water, then add -	100 ml
isopropanol	0.54 ml

Lysis buffer (100 ml) and centrifuge mixtures

50mM of Tris HCl (pH 7.2)	0.424 g. in -
distilled water	70 ml
0.480 g of 50mM EDTA	0.480 g. in -
distilled water	26 ml, then add -
sodium dodecyl sulphate	3.0 g
2-mercaptoethanol	1.0 ml

Resuspension mixture pH 8 (100 ml)

10 mM Tris HCL	0.121 g
0.1 mM EDTA	0.0372 g, dissolve in -
distilled water	100 ml
adjust pH to 8	
80% of ethanol	final rinse

2.2.4 DNA extraction method 4 - the use of QIA Amp® DNA Mini Kit

The DNA extraction Qiagen Kit was performed on spin columns, which fit into standard microcentrifuge tubes and are designed to prevent cross-contamination of extracted species. All components needed for extraction together with Proteinase K were supplied by Qiagen, (Crawley, Sussex, U.K.).

2.2.4.1 Qiagen protocol for culturing cells

The protocol applies to a maximum of 5 million cells.

1. A pellet of cells was obtained and resuspended in PBS to a final volume of 200 µl.
2. Then 20 µl of Proteinase K was added.
3. After that 200 µl of AL lysis buffer was added and mixed by vortexing for 15 s.
4. The mixture was incubated at 56°C for 10 min in water bath.
5. Then it was centrifuged briefly to remove drops from the inside the lid.
6. After that 200 µl of 100% ethanol was added and mixed by vortexing.
7. The mixture was applied to the QIAamp spin column in a 2 ml collection tube and centrifuged at 8000 rpm for 1 min.
8. Then the spin column was placed in a new collection tube.
9. 500 µl of washing buffer AW1 was added to the spin column and centrifuged at 8000 rpm for 1 minute.
10. The supernatant was discarded and 500 µl of washing buffer AW2 was added to the spin column and centrifuged at 14000 rpm for 3 minutes.
11. The supernatant was discarded and spun again at 14000 rpm for 1 min to eliminate any chance of buffer AW2 being carried over into the next stage.
12. Finally the spin column was placed in the fresh collection tube and 50-200 µl of elution buffer AE was added to the spin column.
13. It was incubated at room temperature for 5 minutes and centrifuged at 8000 rpm for 1 minute. The obtained DNA was kept in a refrigerator at 4˚C.

2.3 PCR procedure

2.3.1 Designing primers for application of ITS 1 and ITS 2

The sequence for the ribosomal RNA gene (for the structure of rDNA gene see chapter 1, figure 1.14) for *Dictyostelium discoideum* was downloaded from ′GenBank′, accession number X00601. According to the rules (Hoelzel and Green, 1998) of designing primers they should be ca. 18-26 bp long, of similar G&C content and with no complementarity to each other. The homology at the 3` end with the template DNA should be 100 %. The parts of the sequence that fulfilled those rules were chosen and two pair of primers was designed for them, one pair for ITS1 and one for ITS2 (table 2.2).

ITS 1 Forward primer:

The ITS 1 region lies between nucleotides 3665 and 3994 of the rDNA gene complex. However the sequences chosen to design primers lie within part of the 17S unit of the rDNA sequence for the forward primer and a major part of the 5.8 S unit of the rDNA for the reverse primer. The forward primer was designed to be complementary to nucleotides 3506 to 3524 (5′ GCCCGTCGCTCCTACCGAT 3′). These nineteen bases were chosen as the forward primer because the sequence is G&C rich, in contrast to the majority of the *Dictyostelium* sequence being A&T rich. The melting temperature was established as 64ºC on the basis of the formula:

T_m (ºC) = 2×(A+T) + 4×(G+C)

A, C, G, T representing nucleotide proportions

ITS 1 Reverse primer:

The ITS 1 region ends at 3994 bases. A suitable 22 base template sequence was identified between nucleotides 4135 and 4156 (5′ TTATACTTGGGTGAGAGTGGTC 3′). Therefore, the sequence for the reverse primer had to be complementary to that template sequence, (3′

AATATGAACCCACTCTCACCAG 5′). The melting temperature for this reverse primer was also calculated to be 64°C.

ITS 2 Forward and reverse primers:

The ITS 2 region lies between nucleotides 4157 and 4731. Similar criteria used in the design of ITS 1 forward and reverse primers were applied to the design of the pair of ITS 2 primers. The forward primer was 20 bases long (5′ CGATGAAGACCGTAGCAAAC 3′) complementary to nucleotides 4029 to 4049, with a melting temperature of 60°C. The reverse primer was 20 bases long and constructed on the basis of 4769 to 4788 template sequence (5′ GCATATCAGTAAGCGGAGGA 3′). The final sequence for the reverse primer is 5′ TCCTCCGCTTACTGATATGC 3′, with a melting temperature of 60°C. Table 2.2 shows sequences of both primers for ITS1 and ITS2.

Figure 2. 2 The DNA sequence of small subunit of rDNA with highlighted exemplar pair of primers for ITS 2.

3665 to 3994 ITS 1 (internal transcribed spacer); 3995 to 4156 5.8S ribosomal RNA; 4157 to 4731 ITS 2; 4732 to .>7972 26S ribosomal RNA

```
3421 gtaattattg atcataaacg aggaattcct tgtaagcgta agtcattacc ttatgctgaa
3481 tatgtccctg ccctttgtac acaccgcccg tcgctcctac cgatcgaatg atacggtaaa
3541 gttaacggat cgttttatct gtggcaacac tgatataaat taaaagttat ttaaatctca
3601 ttgtttagag gaaggagaag tcgtaacaag gtatccgtag gtgaacctgc ggatggatca
3661 ttttttatct taatatcata aactttatgt ttttttggat aatggaactg cttgcgtata
3721 aagaaattag gctttttaat taaggtttaa gagtaaagtt ggcttgtcag atttattgtc
3781 tcgatgctat taatatgttt tcttaagaca aattgtttat agtattttga taaaaaaagt
3841 caattgggta acaccaattg aatccatcta gcaatatgtt acacatgtta cgacataaaa
3901 tcccgactca aaaatttaca tcacaggctt gccagtgata tttttctagt acgtcaaaca
3961 aaacatttaa aatatgtctt tagattttaa atctttaagc ataaacggtg aatacctcga
4021 ctcctaaatc gatgaagacc gtagcaaact gcgataattc acttgaattg cagcctactg
4081 ggatagttga aatgttgaac gcacatgatg acatcggtcc tttcggatta ggtgttatac
4141 ttgggtgaga gtggtcctga tagaatccct tttgggtgtg tctattgaac ttgattagat
4201 ggtggtaaaa agtgcgcgag tgcatagcag ctttcgtcct aataaactat gcgaggaccg
4261 cccgaaatga gccaaatgtt aatcaaggga gatagataac gcctgattgg acgtatgcct
4321 agggtatctt tccgtctgtt gccccgcaag ggggggctta ctgggtgact taggctacta
4381 tgttgtaatt ctctcgattt gatcacctgc cattcggcta gcattcggca gcatttagag
4441 gttaacgtaa tcaatataca ttggtcacaa aaaacttttt aatggtggaa ggtaaattta
4501 atgtcactaa taatcggtcc gtcaaattgg ttagttagag ggcattaagc gtgctgaaag
4561 ctattagaaa ggatcaattg tgttgattcg acctatctct aatatgttag ttgggtatgt
4621 ccggcagttt agcggcaggg aaaacggtgt acgcgctggg tttctgacca ccgttgcagt
4681 agcaagtaag atctgcgtaa tactagtact tgtccgaaag attattctaa ttccgcctca
4741 cctttgtaag attacccgct gaacttaagc atatcagtaa gcggaggaaa agaaaccaac
```

Forward ITS2 primer (in yellow) and reverse ITS2 primer (in green).

Table 2.2 Sequences for ITS 1 and ITS 2 primers.

Locus	Primer	Primer sequence	T_m (melting temperature)	Length (bases)
ITS 1	Forward	5′ GCCCGTCGCTCCTACCGAT 3′	64ºC	19
	Reverse	5′GACCACTCTCACCCAAGTATAA3′	64ºC	22
ITS2	Forward	5′ CGATGAAGACCGTAGCAAAC 3′	60ºC	20
	Reverse	5′ TCCTCCGCTTACTGATATGC 3′	60ºC	20

T_m calculated according to formula 2×(A+T) + 4×(G+C)

2.3.2 Preparation of primer working solutions

All primers were received from the manufacturer, Sigma Aldrich Scientific, (Epsom, U.K.); formerly Sigma Genosis, as a freeze dried powder, which was rehydrated and diluted as described below to produce a final working concentration of 0.01 nm/µl. The primer was first rehydrated to produce a stock solution of 0.5 nm/µl, then diluted 1 in 50 to give a working solution of 0.01 nm/µl. Both stock and working solutions of primers were stored in a freezer at a temperature of minus 20°C.

Preparation of PCR Master Mix

The quantity of each PCR reagent is prepared per 20 µl of PCR reaction, and is as follows:

750 mM Tris HCl (pH 8.8); 200 mM $(NH_4)_2$ SO_4; 0.1% (v/v) Tween reaction buffer	2.0 µl
0.25 mM dNTPs	2.0 µl
25 mM $MgCl_2$	1.25 µl
Taq polymerase	0.20 µl
Sterile distilled water (SDW)	9.55 µl

Forward primer (0.01nm/μl)	2.0 μl
Reverse primer (0.01nm/μl)	2.0 μl
DNA	1.0 μl

The working mixture was always prepared for multiple PCR reactions. Sufficient volumes of each ingredient were added to a master mix to ensure sufficient reagents for the number of PCR reactions being undertaken.

Sterile plastic tubes and sterile disposable tips for calibrated pipettes were always used. A 19 μl sample of the working mixture was pipetted into each sterilised PCR plastic tube to which 1 μl of relevant DNA was added. The negative control had an extra 1 μl of SDW water substituted for the DNA and was used to check for the presence of any unwanted DNA contaminations of the PCR samples. To prevent any cross-contamination a new sterile tip was used for each new addition of template DNA and the whole procedure was carried out in a laminar flow cabinet. The tubes were then placed into a Perkin Elmer Geno-Amp PCR machine and the appropriate PCR program was run.

2.3.3 The PCR programme

The standard cellular slime mould program used for PCR is as follows:

Initial denaturation at	94°C for 3 minutes
Denaturation at	94°C for 1 minute
Annealing at	55°C for 2 minutes
Extension at	65°C for 5 minutes

This protocol was run for a total of 35 cycles.

During the later stage of the project the standard *Taq* PCR Kit available from Qiagen, (Crawley, Sussex, U.K.) was used on a daily basis. The decision to use the standard *Taq* PCR

Kit was made because of occasional inexplicable failure to produce PCR products. Apart from the standard components for making a PCR working mixture, Qiagen recommends the use of its own special component-solution Q. In case when Q solution is used, 4.0μl of solution Q (5× concentrated) per 20 μl of PCR reaction is recommended.

2.3.4 Gel electrophoresis

The 20 μl of PCR products was divided as follows. 10 μl was pipetted into a new sterile plastic Eppendorf tube and retained for sequencing. Some 1μl of loading dye was added to the remaining 10 μl of PCR product. A 10 μl aliquot of a DNA ladder, ϕX174/Hae III, for size comparing purposes, was prepared according to the protocol.

TE buffer	7.0 μl
DNA ladder ϕX174/Hae III	2.0 μl
Bromophrnol blue (loading dye)	1.0 μl

Both bromophenol blue and the DNA ladder were supplied by Fisher Scientific, (Loughborough, U.K.).

2.3.4.1 Agarose gel electrophoresis preparation

A 2% agarose gel was made by dissolving 2 g agarose in 100 ml of 1×TBE buffer (see protocol below) and melting the agarose in a microwave oven at 650 W for 3-4 min until well dissolved. The melted agarose was left to cool until its temperature reached at c. 50˚C, then 50 ml of melted agarose was poured into the clean gel platform and left for approximately 30

min to set. Once the gel was set**,** the tray was placed in electrophoresis apparatus filled with approximately 800 ml of 1×TBE buffer and 12.5 µl of ethidium bromide (10 mg/ml).

10×TBE buffer

Tris	105g
Boric Acid	55g
0.5 EDTA at pH 8	40 ml

This buffer was then used to prepare 1×TBE buffer. To run the gel electrophoresis 10 µl of each sample containing 1 µl of loading dye were loaded into the wells. Then 10 µl of the prepared ladder was added to the first and the last of the rows. The apparatus was connected to a power pack and run at 70 V for ca. 2 h. The TBE ingredients were supplied by Fisher Scientific, (Loughborough, U.K.).

2.3.5 Sequencing procedure

The sequencing procedure consists of the three stages of DNA purification, Cycle sequencing and DNA Precipitation. After the PCR amplification of the target DNA electrophoresis was performed using 3% standard agarose gel in TAE (Tris Acetate EDTA) buffer at 90V for 2 hours to give optimum band resolution (Hillis and Moritz, 1996). In the first step of DNA purification, gel separated amplicons were viewed under UV light, and bands of interest excised from the gel and placed in separate GFX columns. Then the samples were purified with the GFX ™ DNA and Gel bands Purification Kit and GFX spin columns supplied by Amersham Biosciences, (Buckinghamshire, U.K.). During that process the gel slice containing the target band was mixed with capture buffer supplied by the GFX kit, vortexed and incubated at 60º C until the agarose completely dissolved. This allowed target bands to be freed from the gel. Then the samples were washed with wash buffer and finally the target

DNA was eluted on the GFX columns with the use of water or elution buffer and purified. The purified target DNA was then ready for the next stage, cycle sequencing.

Cycle sequencing, like standard PCR, also utilizes thermostable polymerase as well as a temperature cycling format of denaturation, annealing and DNA extension. However, in contrast to a standard PCR reaction it employs a single primer, either forward or reverse, so that the amount of PCR product increases linearly and not exponentially as in standard PCR (Surdhar, 2002). Purified DNA was then set up for the cycle sequencing procedure.

The cycle sequencing mixture was prepared in the way shown below with two sets of clean tubes.

The cycle sequencing mixture per tube contained:

water	4.8 μl
primer (separate forward or reverse) [3.5 picomoles]	1.0 μl
Ready Mix	2.2 μl
purified DNA sample	2.0 μl

The reaction was prepared by adding the cycle sequencing mixture to each tube, and then to the first group of tubes only forward primer and to the second group only backward primer was added. The mixtures were then amplified in 35 PCR cycles with the following program: denaturation at 96°C for 30 sec; annealing at 50°C for 15 sec; extension time at 60°C for 4 minutes. Ready Mix was supplied by Amersham Biosciences, (Buckinghamshire, U.K.). The cycle sequencing procedure was then followed by the final step, the precipitation of the amplified DNA. For this purpose the following mixture was prepared:

Precipitation mixture per each tube;

100% ethanol	64 µl
water	26 µl

Some 90 µl of this precipitation mixture was added to each clean tube with 10 µl of DNA obtained in the cycle sequencing and finally either forward or reverse primer was added. The samples were first spun for 30 minutes at 15.000 rpm on a benchtop centrifuge, the ethanol was removed then 1 ml of 70% of alcohol was added and centrifuged for 5 minutes. After removing the entire alcohol with a pipette, the samples were left to dry and then sent for sequence reading to Imperial College, Ascot.

2.4 Materials used for ISSR-PCR

2.4.1 Strains and growth conditions

The same species and isolates from both *Dictyostelium* and *Polysphondylium* genera, used to generate the sequencing data analysed in the previous chapter were also used for ISSR-PCR. These species and isolates were taken from the collections of the Department of Life Sciences at Anglia Ruskin University and from the USA (see 2.1 and table 2.1). All were cultured using the same protocols as outlined in the sequencing experiments, and used in the ISSR-PCR study (listed in table 2.1).

2.4.2 DNA isolation

DNA was extracted using the DNA extraction kit QI Aamp Tissue QIAGEN, (Creawley, Sussex, U.K.) though two isolates of *P. violaceum* (DNC1, 1/1 and DNC 3, 1/1) failed to deliver good quality DNA as a result of recurring culture contamination.

2.4.3 Primer design and PCR amplification

Eight ISSR-PCR primers were tested for their ability to produce clear reproducible PCR products. Four out of eight had been designed and previously used for amplification of fungal DNA (Hantula *et al*., 1996), while the remaining four were newly designed for this study. Dictyostelid DNA sequences were obtained from EMBL/GenBank and a search conducted for the most common nucleotide repeats, which were found to be AT, AAT, CCA and GAA. These repeat sequences were used to design primers, which would amplify inter-microsatellite regions. Any short repeat sequence chosen for use as a primer was extended by the addition of a few bases at the 5'- end. Such a specifically designed primer contains a region with high sequence homology to a specific DNA region, and therefore ensures the primers anneal exactly at the beginning of the desired short repeat sequence. This ensured amplification of the primers together with the region flanked by them. Following initial screening, (data not shown) only four primers were chosen for further work. The other four were excluded from the further work (see 4.4.1). The original eight anchored ISSR-PCR

primers sequences are listed in table 2. 3, all primers were synthesized by Sigma Aldrich Scientific, (Epsom, U.K.).

PCR amplification was carried out using a Perkin Elmer Geno-Amp PCR machine in 20 µl reactions containing 20 mM Tris-HCl (pH 8.4), 50 KCl, 1.5 mM $MgCl_2$, 4µl of Q solution, 0.25 mM dNTPs, 10 ng of each primer, 1U of *Taq* polymerase (ABgene, Poole, U.K.) and 1µl of DNA extract. All amplification used four-step cycle, of an initial 3 minutes at 94°C, followed by 30 cycles of 1 minute at 94°C (denaturation time), then 2 minutes annealing time, with the different annealing temperatures established for each primer (see table 2. 2) and the last step, 5 minutes at 65°C (extension time). After the final extension step, samples were held at 4°C until further processing. The reproducibility of each primer was confirmed by three times repeating the PCR followed by the gel electrophoresis.

Table 2.3 Sequences of tested primers. (primers rejected are in bold)

Primer number	Primers sequences. **H** = A,T or C; **B** =G,T or C; **Y** =G,A or C; **D**= G,A or T		Annealing temp. (°C)
1	$DD(CAA)_5$		55
2	**$BDB(AAT)_7$**	**excluded**	43
3	DD(GAA)5		45
4	**$BDB(ACA)_5$**	**excluded**	45
5	$DD(CCA)_5$		60
6	$DHB(CGA)_5$		60
7	**$YHY(GT)_5G$**	**excluded**	45
8	**$DHD(AT)_7$**	**excluded**	25

Note; For instance, the primer one with formula DD $(CAA)_5$ is a mixture of 8 primers (there are 2^3 permutations). The reason for this flexibility is to maximize chance of picking up binding sites.

2.4.4 Electrophoresis conditions

The amplified products were separated using gel electrophoresis. Some 10 µl of amplification products and 1 µl of run/stop solution bromophenol blue 50 mM EDTA were mixed and separated on an agarose gel. Initially 1.5% New Sieve agarose, supplied by Invitrogen, U.K. was used as it was hoped that this agarose will give superior resolution. However, problems with agarose preparation and obtaining reproducible results led to New Sieve agarose being abandoned in favour of ordinary agarose. Three different percentages of gel agarose (1%, 1.5% and 2%) were tested. The 2% agarose produced the best band resolution and so was used for all electrophoresis of ISSR-PCR amplification products. A 20 µl aliquot of a 100 bp ladder was used as a standard, a 10 µl sample of the ladder was added to the first and the last lane of each gel and used in final scoring. Then 10 µl of ethidium bromide (concentration 10 mg/ml) was added to the TBE buffer. Electrophoresis was carried out in 0.5×TBE buffer. After an initial 30 minutes at 35 V, gels were run at 70 V for 3-4 hrs. Electrophoresis was stopped when the bromophenol blue dye marker had migrated 10 cm. The amplification products were visualised using a UV transilluminator and photographed using a Polaroid camera. Negative controls with no template DNA were included to ensure that reagents used were not contaminated with extraneous template DNA. An initial problem of curved bands was solved by performing electrophoresis at 4°C.

CHAPTER THREE – ESTABLISHING CULTURING, DNA EXTRACTION AND PCR METHODOLOGY

3.0 Optimisation of culturing conditions and an extraction technique

3.1 Optimisation of amoeba culture conditions

Amoeba from a total of 51 species and isolates (table 2.1) were cultured and harvested. The bacterial suspension of *Klebsiella aerogenes* proved a successful food source for growing cellular slime moulds. Approximately 80% (40 out of 51) species, particularly *Dictyostelium* rather than *Polysphondylium* species, grew well and when the selected growth conditions were sustained, few problems were encountered in their culturing. Slight changes to the growth conditions (see section 2.1.2.3) such as decrease of the temperature or humidity did not produce any significant growth differences. However, species and isolates from the genus *Polysphondylium* were more specific in their growth requirements, as were a few *Dictyostelium* species, particularly *D. minutum* and *D. sphaerocephalum.* There were also continual problems with first reviving then culturing and maintaining the viability of most species, particularly those from the USA. All were however eventually successfully cultured and amoeba from all the troublesome species and isolates finally harvested.

Whilst culturing the dictyostelids it was noticed that some suggested growth conditions for primary and secondary cultures needed modification to be optimal. Raper (1984) suggested that humidity is a key factor. Yet, while humid conditions helped to sustain dictyostelids growth, it also led to rapid culture fungal contamination and once contaminated, there was considerable difficulty in re-culturing pure colonies. Even though transferred sorocarps were apparently not infected by fungi, some fungal spores were inevitably transferred. The solution was to decrease humidity. Initially cultures were placed inside plastic boxes covered with damp tissue, but when this was abandoned in favour of culturing outside the plastic box, the contamination was significantly reduced. As the adjusted conditions did not seem to affect growth these were adopted for all subsequent cultures.

The time needed to get fully developed sorocarps varied. Raper (1987) suggested that the time needed for sufficient *Dictyostelium* species growth was generally three to seven days, but up to 13 days for *Polysphondylium*. In this study fully developed sorocarps often appeared after only two or three days in *Dictyostelium* spp. and at seven to ten days in *Polysphondylium* spp. The more rapid growth of dictyostelid species resulted in an earlier appearance of the amoeba, thus the cultures were observed twice each day to ensure that the critical amoeboid stage important for DNA extraction was not overlooked.

3. 2 Development of optimal DNA extraction technique

The efficiency of three different DNA extraction procedures was tested with a high quality and quantity DNA being the desired criteria. The first and simplest extraction method was by boiling for ten minutes. The second employed a two-hour microwave treatment and the third a Qiagen QIAamp® DNA Mini Extraction Kit which took 2-3 hours depending on the number of extracted samples.

Boiling the cells, if successful, would be relatively simple and rapid. As dictyostelids in the amoeboid state are a suitable form for the DNA extraction, it was hoped that it would be sufficient to merely break open the cells. Although the boiling extraction of all harvested species and isolates was repeated 3 times it did not prove successful. No amplifiable DNA was ever obtained via this method.

The second technique of DNA extraction using microwaves is a rapid procedure used for the extraction of genomic DNA from a variety of plant, fungi, protist and animal tissue (Goodwin and Lee, 1993). This method uses microwaves to break down the cell walls and membranes. To test its success the harvested amoeba from all 51 cultured species and isolates were subjected to a total of 20 s microwave radiation, which was delivered as (10 s, 5 s and then 5 s) pulses with a short 15 s refractory period between successive pulses. The extracted material was then tested for the presence of DNA via PCR and gel electrophoresis and the DNA extraction of each species and isolates were repeated three times. This procedure was straightforward and rapid when compared with the established but time consuming DNA extraction technique (section 2.2.1.2) used by Kay (1995), but yielded many problems. While the conditions suggested by Goodwin and Lee (1993) were followed, the cells harvested from

the process were often burnt. Cells were irradiated in two different microwave machines yet the change did not improve the extraction of viable cells. Shorter time intervals were tested in case the suggested irradiation time was too long, thus a total of 15 s was divided in three intervals of 5 s, then further decreased to a total of 10 s at three time intervals of 6 s, 2 s and 2 s. This yielded only minor improvement even though further stages of the extracting procedure were strictly followed. Sometimes the cells were unduly damaged while other times, under identical treatments and for no apparent reasons, they were destroyed. Such an unpredicted final product did not fulfil successful extraction criteria. Furthermore, amplifiable DNA was only obtained in 30% of species, mainly *Dictyostelium* spp. suggesting low yield and /or poor quality DNA. Thus microwave DNA extraction was also regarded as unsuccessful for the purpose of this project.

The third extraction technique tested in this project was the DNA extraction Kit available from the Qiagen company to deliver a single and reliable method for DNA extraction from many different sources such as blood and other body fluids, tissue, dry materials, buccal swabs and cultured cells. The standard enzyme Proteinase K and lysis buffer were used to break down the cells, a procedure performed in easy to use spin columns. DNA was extracted from all 51 harvested species and isolates, being performed twice on each species or isolate. The amplifiable DNA obtained and extracted from all 51 samples appeared to be of good quality producing a sharp, reproducible PCR product. As the results obtained from two independent extractions showed the same results, this procedure proved to be not only successful but also rapid, straightforward and cost - effective.

3.3 Optimisation of PCR conditions

3.3.1 Optimization of PCR parameters for amplification of dictyostelid DNA

Optimisation of PCR parameters was performed to establish the best possible PCR conditions for the work described in this thesis, as well as to check that the right products were being amplified. Optimisation was performed with only three samples. Two different *Dictyostelium* species (*D. discoideum* and *D. mucoroides*) together with one *Polysphondylium* (*P. violacum*) were selected as test examples. To avoid the occasional culturing problems, mainly in relation to contamination, slow growth or the dying off of cultures, it was decided to use DNA from species which caused relatively few culturing problems and would always yield

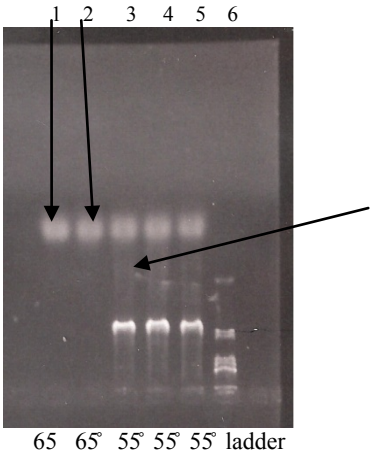
sufficient DNA for analysis. Moreover, it was believed that these species were representative of their parent genera and so if the established PCR conditions worked for these three species then they would most likely work for other dictyostelid species and isolates under study.

Initially attempts using the standard PCR protocol for the amplification of a 1 kb stretch of DNA (see & Materials and Methods) yielded no products and so considerable time and resources were needed to optimise PCR conditions for amplification of dictyostelid DNA. Optimization of the PCR process usually initially involves adjustment of the annealing temperature, which relates to the melting temperature of the primers used. Therefore, the first attempt to optimize the PCR conditions focused on establishing the optimal ITS 1 and ITS 2 primer annealing temperatures. All designed primers for ITS 1 had a melting temperature of 64°C and for ITS 2 a melting temperature of 60°C. Therefore, to establish the optimal working temperature for these primers, it was decided to test four different initial temperatures with a 5°C serial increments. As the melting temperatures were in the range 60°C to 64°C so it was decided to start at 50°C with a 5°C serial increments up to 65°C. Denaturation temperature and time as well as extension temperature and time were kept constant as in the standard PCR procedure (section 3.3.1). The annealing temperature of 55°C produced multiple bands or a weak band in *D. discoideum* but the results were not repeatable. It was therefore decided to try a tighter set of annealing temperatures in the 50°C to 65°C range but with 1°C increment. All 15 annealing temperatures tested failed to produce a strong band, but temperatures close to 55°C produced a weak band or multiple bands. Results are summarised in table 3.1 and figure 3.1. The same results were obtained for both ITS 1 and ITS 2 primers. This suggested that a reaction was occurring but that other PCR conditions needed optimising.

Table 3.1 PCR products obtained with a range of annealing temperatures using primers designed to amplify both ITS 1 and ITS 2 regions.

Annealing temperatures	***D.discoideum***	***D.mucoroides***	***P.violaceum***
65°C	No band	No band	No band
60°C	No band	No band	No band
55°C	Weak band	Multiple bands	Weak multiple bands
50°C	Smear	Smear	No band

Figure 3.1 Visualised PCR products obtained after the amplification performed at 55˚C and at 65˚C. Lane 1, *D. discoideum*; lane 2, *P. violaceum*; lane 3, *D. discoideum*; lane 4, *D. mucoroides*; lane 5, *P. violaceum*; lane 6, ϕX174/Hae III DNA ladder.



Lanes 1 and 2 show the lack of PCR product after the amplification at 65˚C, lanes 3-5 PCR products after the amplification at 55˚C.

When optimizing the PCR conditions consideration is given not only to adjusting reaction conditions, e.g. cycle temperatures, but also to concentrations of reaction components. As the previous experiments with adjusting the temperatures proved unsuccessful, it seemed reasonable to take a closer look at any other factors which could help to eliminate multiple

banding and smear, both of which may result from over-amplification, too short or degenerative primers, too high concentration of the dNTPs used in the PCR reaction mixture or too many cycles. Too high a concentration of dNTPs, short or degenerative primers as well as over-amplification due to too high number of cycles were all considered unlikely as the cause of multiple banding.

This led to considering other possible factors which could improve the results. It was decided to test a different polymerase, the Ampli *Taq* Gold polymerase (ABgene, Poole, U.K.) to see if it could improve amplification, reduce the presence of non-specific products and increase the chance of obtaining specific products via increased enzyme function in the later cycles. The new polymerase was tested, with the three exemplar species and the use of ITS 1 primers. Four separate PCR reactions were performed each consisting of 35 cycles and an annealing temperature set of 50°C, 60°C, 65°C. A particular focus was given at 55°C which previously showed some signs of activity. Unfortunately, no significant differences were noticed in the action of this polymerase and so I reverted to the ordinary and less expensive *Taq* polymerase. As the change of polymerase enzyme failed to improve amplification, it appeared reasonable to re-consider the denaturation and extension temperatures.

It is well established that full denaturation of the DNA strands is needed to anneal primers (Hoelzel and Green, 1998). Ordinarily, denaturation is performed at 94°C for 1 minute but extra time is added for fragments longer than 1kb. As the dictyostelids are particularly A&T rich, comprising < 85% of total genome nucleotide composition in introns and the regions over-flanking the genes, it was concluded that denaturation time was likely to be optimal. Thus attention was focused on extension temperature and time.

Although an extension temperature of 72°C for 1 minute is optimal for most PCR reactions, the rate of DNA synthesis may in some cases be affected by the nature of the template, assuming that the buffer composition is appropriate (Innis and Myambe, 1988). It was considered that if sequences were extremely rich in A&T bases the standard extension temperature of 72°C may not be optimal and that the polymerase may have difficulties in adding dNTPs when encountering A&T rich pattern of bases. It was therefore decided to (1) decrease the extension temperature which would slow the rate of synthesis and thus enable the polymerase to synthesise new DNA fragments in A&T rich regions and, (2) increase

synthesis time to possible completion. Table 3.2 and figure 3.2 present the results of varying extension temperatures and time regimes.

The decreased extension temperature of 65°C and increased extension time of 5 minutes proved to be successful in eliminating the multiple banding (figure 3.2), and produced reproducible results. Thus, the following adjusted PCR conditions were used in this study.

- 3 min initial denaturation at 94°C

- 1 min denaturation at 94°C
- 2 min annealing at 55°C
- 5 min extension at 65°C

} 30 cycles

- holding at 4°C

Table 3.2 Amplification results from varying extension temperatures and time regimes.

Extension time (minutes)	**Extension temperature of 65°C**	**Extension temperature of 68°C**
10	No product	No product
5	**Band obtained. No multiple bands/ smear**	No product
1	No product	No product

Figure 3.2 ITS 1 PCR product obtained with the extension time of 65° C for 5 minutes. The PCR was performed with the exemplar species. Lane 1, *D. discoideum*; lane 2, *D. mucoroides*; lane 3, *P.violacum;* lane 4, ϕX174/Hae III DNA ladder.

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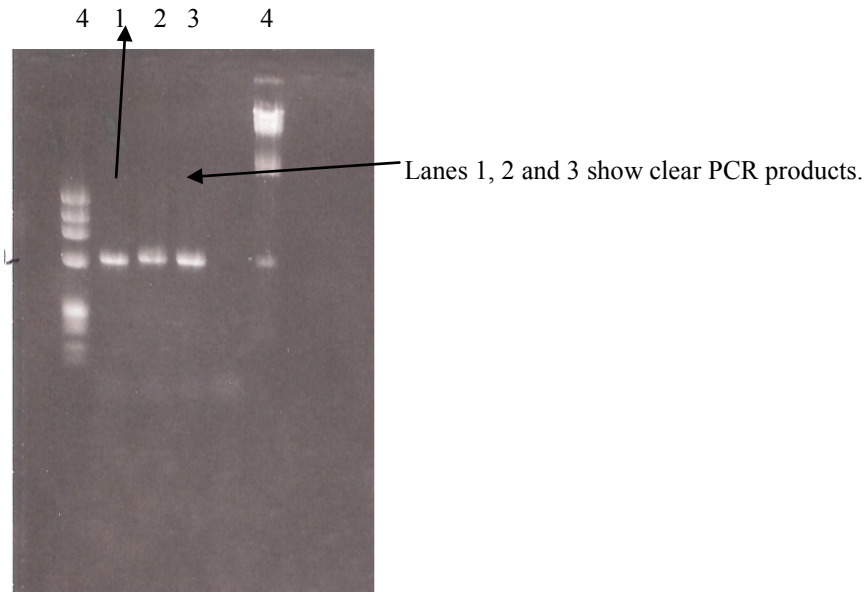


To ensure the complete elimination of problems associated with obtaining the desired PCR products, the standard *Taq* PCR Kit from Qiagen was, as mentioned above, routinely used. Qiagen also supplied "solution Q" to be used on 'difficult' templates because it facilitates amplification by modifying the melting behaviour of nucleic acids and will enable or improve suboptimal PCR systems caused by problematic templates which have for example a high degree of secondary structure or are G&C rich (Qiagen PCR handbook). Although care was given to design primers without secondary structure or G&C rich regions, the use of solution Q for the working PCR mixture nevertheless proved successful in obtaining repeatable PCR products in situations which had previously proved impossible. Thus Q solution was routinely included in the PCR reaction mix.

Electrophoresis conditions were also adjusted as from time to time a problem with curved bands appeared. Running the agarose gel in a cold room at < 18°C sufficiently improved

results while decreasing the initial voltage to 35V for the first 30 to 45 minutes of the gel run which was then raised to a standard 70V for a further 2.5 to 3 hours, produced superior results of strong, repeatable bands.

Once the PCR and gel electrophoresis conditions were optimised, the work on all 51 species and isolates could proceed. The products obtained for the ITS 1 part of the rDNA gene were checked against the ϕX174 Hoe III molecular marker size ' ladder' and were confirmed to be at the expected length of 680 bp. Products from all species and isolates except for *Acytostelium* spp. (used for rooting the trees) and *D. minutum* (ATN 1,6/21) were successfully amplified. *D. tenui* and *D. purpureum* ATN 1(20) both produced only a very weak band. All 49 obtained PCR products from the ITS 1 region were sequenced.

Once the PCR conditions were established for amplification of the ITS 1 region, they were tested for amplifying the ITS 2 region. The annealing temperature of 55°C was found to be suitable also for the ITS 2 primers. All samples were then subjected to PCR and the resulting products separated via agarose gel electrophoresis and checked against the same ladder for the anticipated product of 780 bp. However, not all species and isolates yielded successfully amplified ITS 2 DNA. Amplification failed in four species and produced very weak bands that also failed to be sequenced in four more. A total of 43 products were successfully obtained for ITS 2 and sent for sequencing.

The following chapter will introduce the results obtained from sequencing technique and their parsimony analysis performed by PAUP. It will also focus on the results obtained with the use of the second typing technique, the ISSR-PCR, employed in order to see if it would confirm the results obtained by sequencing. Moreover, it will discuss this ISSR-PCR technique in relation to its power to screen a large number of newly isolated samples and their further identification in a short time.

CHAPTER FOUR - RESULTS

4.0 DEFINING DICTYOSTELID GENERA: PHYLOGENETIC ANALYSIS OF ITS 1 AND ITS 2 SEQUENCES

As discussed in chapter one, dictyostelids phylogeny has been largely established on the basis of morphological features. Yet some *Dictyostelium* species show a number of features in common with *Polysphondylium* species, suggesting that dictyostelids taxonomy based exclusively on morphology is flawed. A new, molecular approach is vital to explore dictyostelids phylogenetic relationships, especially generic validity. The new molecular approach of sequencing of ITS 1 and ITS 2 spacer regions of rDNA gene employed in this project required some time to optimize the culturing condition of the dictyostelid species and PCR conditions for sequencing. This was a result of the absence of published PCR based studies on dictyostelid species. Once the optimization was achieved, the sequencing and phylogenetic analysis could follow. This section presents and discusses the results obtained by sequencing.

Forty three of the 51 *Dictyostelium* and *Polysphondylium* species/ isolates were successfully sequenced for the ITS 1 part of the rDNA gene. Despite many attempts six species and isolates produced no sequence or sequences that did not match the rest of ITS 1 region sequences and therefore could not be aligned with them. A further three sequences were incomplete, or of poor quality and were excluded from subsequent analysis. *Dictyostelium discoideum NC4* was used for rooting. Thirty six of the 43 species and isolates that amplified for ITS 2 were sufficiently complete and readable for phylogenetical analysis, including *Acytostelium* spp. which was used for rooting.

All ITS 1 and ITS 2 sequences were aligned using CLUSTAL X (Thompson *et al.,* 1997) with each of the following gap opening (GO) : Gap extension (GE) combinations 1:1, 2:1, 2:2, 4:2, 4:3, 4:4, 8:1, 8:2, 8:4, 8:6, and 8:8 and with transition : transversion costs set to 1. Each alignment was then subjected to maximum parsimony analysis using PAUP 4.0 b 10 (Swofford, 1987), where ITS 1 and ITS 2 data were analysed separately.

To establish primary homology (de Pinna, 1991) of aligned DNA sequences and thus allow a comparison of corresponding regions in the genomes of different species any insertion and/ or deletion 'gaps' must be reconciled. Gaps may be 'useful' if resulting from DNA polymerase slippage or mutation, or 'artefact' if merely inserted to align homologous DNA sequences of different lengths producing sequence mismatches (Li, 1997; Giribet and Wheeler, 1999). Alignment is further complicated, even prevented, by attempting to match sequences containing multiple gaps and mismatches at different parts along each sequence. The solution is a 'Gap penalty', i.e. assume how often gap events occur (Li, 1997) in terms of 'gap extension' (GE) after the initial 'gap opening' (GO) (see also 1.13). Lower GE costs favour longer, more 'contiguous' gaps as opposed to many small individual gaps (Giribet and Wheeler, 1999).

All ITS 1 and ITS 2 sequences are shown in full in Appendix 5.

4.1 PAUP ANALYSIS OF SEPARATE ITS 1 AND ITS 2 DATA

The initial phylogenetic data for the ITS 1 part of the rDNA gene, using *D. discoideum* NC4 as an outgroup were obtained with 100 random additions, holding only one tree at a time. Further PAUP analysis was performed with 10,000 random additions, holding only 10 trees at a time. Gaps were treated as missing. The initial PAUP analysis for the sequence alignments for the ITS 2 part of the rDNA gene were also performed with 100 random additions, holding only one tree at a time, in the first instance and then with 10,000 random additions holding 10 trees. Branch support was assessed by bootstrapping using the 50% majority rule consensus option. A total of 1,000 initial analyses performed subsequently for separate ITS 1 and ITS 2 segments as well as combined ITS 1 and ITS 2 segments, where the 1,000 additions were made over a 100 series of ten steps, holding only one tree at each step with option step-wise addition. Only clades with bootstrap frequencies of $\geq 50\%$ were retained.

The heuristic search of ITS 1 yielded 23 (in the initial 100 random additions) and 3543 most parsimonious trees (MPTs), in 10,000 random additions, of the length 687 and 688 for the repetitive GO1:GE1 gap penalties. The heuristic search performed with 100 and 10,000 random additions for the GO2:GE1 gap penalties yielded 28 and 3088 trees of the same (728) length. The results in terms of MPTs, tree length and statistics for all eleven GO:GE combinations for ITS 1 are summarised in table 4.1. These statistics, consistency index (CI), retention index (RI) and homoplasy index (HI), listed in the following tables, are discussed in chapter 1 & 1.13 (Quicke, 1993; Swofford & Begle, 1993; Kitching *et al.,* 1998).

Note that MPT length increases with gap penalty, reaching 1027 for GO8:GE8. Exemplar trees, one of 23 most parsimonious trees for the gap penalty GO1:GE1 and one of 28 trees for the gap penalty GO2:GE1, are showed in figures 4.1 and 4.3. The strict consensus tree constructed from 23 and 28 trees subsequently for the gap penalty GO1:GE1 and next for GO2:GE1 is shown in figures 4.2 and figure 4.4. Strict consensus trees for each of the further nine analyses (from GO2:GE2 to GO8:GE1) are shown in figures 6.1 to 6.5 (on the accompanying CD).

All strict consensus trees were well resolved if rooted with the *D. discoideum* NC4. The first consensus trees showed a characteristic grouping where nearly all *D. mucoroides* isolates (except *D. mucoroides* EW5) formed a crown clade. The same was true of all *P. violaceum* isolates with the exception of *P. violaceum* DNC 1,1/1 and DNC 3,1/1. Moreover all *D.discoideum* isolates as well as *D. citrinum* and *D. discoideum/ D.citrinum* (a species not fully identified) were also recovered together on the tree. The ITS 1 consensus trees placed *D. purpureum* AT17 and *D. purpureum* 1K in a clade, and as a sister clade to *D. purpureum* CRLC. Furthermore, *D. implicatum* BCN 21 and *D.* sp. BDW112 were always recovered as sister groups though appearing at different positions for different gap penalty settings. In the majority of cases *D. mucoroides* isolates formed a consistent clade, *D .mucoroides* EW5 formed a sister group with the *D. aureostipes* BDW4 and *D. implicatum* EPN1.

Table 4.1 Description of MPTs obtained for ITS 1.

GO/ GE ratios	MPTs (n)	Length	CI	RI	HI
1:1	23	687	0.7307	0.9130	0.2693
2:1	28	728	0.7390	0.9070	0.2610
2:2	34	752	0.7194	0.9029	0.2806
4:2	97	817	0.6903	0.8941	0.3097
4:3	45	823	0.6950	0.8968	0.3050
4:4	100	849	0.6938	0.9002	0.3062
8:1	100	955	0.6817	0.8896	0.3183
8:2	100	970	0.6753	0.8881	0.3247
8:4	97	989	0.6785	0.8900	0.3215
8:6	100	1024	0.6748	0.8879	0.3252
8:8	100	1027	0.6796	0.8930	0.3204

Notes: CI – consistency index. RT – retension index, HI – homoplasy index. For 100 random additions there is a cut off at100 tree.

The heuristic search for ITS 2 subsequently yielded 99 and 8441 most parsimonious trees of length 1706 for the alignment constructed with the gap penalty GO1:GE1. PAUP analysis of the alignment constructed with the gap penalty GO2:GE1 and 100 and 10,000 random additions yielded 96 and 5474 trees respectively each of length 1832. A summary of the results for all eleven GO:GE combinations is shown in table 4.2. Here again the larger gap penalty resulted in trees of longer length e.g. 2493 for GO8:GE6, however, with the gap penalty GO8:GE8 tree length dropped to 2429. Exemplar trees from the PAUP analysis for the alignment performed for gap penalties GO1:GE1 and GO2:GE1 are presented in figures 4.5 and 4.7. The strict consensus trees performed for both sets of shortest trees (GO1:GE1; GO2:GE1) are shown in figures 4.6 and 4.8 and for the further eight analyses are shown in figures 6.6 to 6.14(CD).

Table 4.2 Description of MPTs obtained for ITS 2.

GO/ GE ratios	MPTs (n)	Length	CI	RI	HI
1:1	99	1706	0.7485	0.8490	0.2515
2:1	96	1832	0.7216	0.8340	0.2784
2:2	98	1949	0.7101	0.8407	0.2899
4:2	98	2140	0.6944	0.8310	0.3056
4:3	75	2231	0.6957	0.8277	0.3043
4:4	99	2290	0.6843	0.8272	0.3157
8:1	99	2291	0.6888	0.8326	0.3112
8:2	85	2390	0.6770	0.8273	0.3230
8:4	93	2436	0.6691	0.8284	0.3309
8:6	88	2493	0.6611	0.8114	0.3389
8:8	68	2429	0.6513	0.8107	0.3487

Notes: CI – consistency index. RT – retension index, HI – homoplasy index. For 100 random additions there is a cut off at100 tree

The results obtained from both ITS 2 sequence presented shortest trees (figure 4.5; 4.7) which are comparable to those obtained from the previous ITS 1 sequence shortest trees, in that both show similar congeneric isolate clades of e.g. *D. discoideum*, *D, citrinum / D. discoideum* and *D. mucoroides* isolates. Although none of the obtained strict consensus trees for the ITS 2 sequences are fully resolved they show some distinct similarities. The same groups of species were recovered in all strict consensus trees obtained for ITS 2, for example the *D. purpureum* AT1K; *D. purpureum* ATN13 and *D.purpureum* CRLC2 were constant although in different clades, according to the different gap penalty settings. For example, for the gap penalty GO1:GE1 this group formed a crown clade but with respect to the GO2:GE1 they appeared in the middle of the tree. The four isolates of *D.discoideum* ATN122, NC4, V12 and ATN119 were always recovered together and despite different gap penalty settings always occupied the basal position. Furthermore,

another *Dictyostelium* species, U.K., *D. minutum* NWM 12, American *D. minutum* ATN16 as well as *D. minutum* GMN3 and *Polysphondylium violaceum* ATN 11 were, with the sole exception for GO1:GE1 the gap penalty where both isolates were left unresolved, recovered as a consistent clades. In many cases, for instance for the GO2:GE2 gap penalty, both monophyletic groups were recovered as a sister group. Interestingly, in all ITS 2 consensus trees *Polysphondylium pallidum* falls close to the base of the tree as an outgroup while other *Polysphondylium* species were recovered together with all *Dictyostelium* species in a single clade. Although ITS 1 and ITS 2 consensus trees differ in recovered groups, the same isolates AT17, 1K and CRLC of *D. purpureum* (see table 2.1) were recovered in both consensus trees. Despite the fact that none of the consensus trees are fully resolved, note that ITS 2 shows neither strict generic distinction between species and isolates nor an American (New World) and English (Old World) species distinction. In all cases the species and isolates of one genus are mixed with species from the second genus. Also, and again in all cases, an outgroup was consistent in its basal position which is explained by a high consistency index.

4.1.1 PAUP analysis of combined ITS 1 and ITS 2 data

The ITS 1 and ITS 2 alignment data obtained using Clustal X with the 11 gap opening (GO) : gap extension (GE) combinations 1:1; 2:1; 2:2; 4:2; 4:3; 4:4; 8:1; 8:2; 8:4; 8:6 8:8 and with transition : transversion costs set to 1 were combined together with the use of interleave command. The alignment data set was then subjected to maximum parsimony analysis using PAUP. The initial phylogenetic data were obtained with 100 random additions holding only 1 tree at a time (at the end of every random addition program retains one or more cladograms of the lower score, according to settings). Further PAUP analysis was performed with 10,000 additions holding 10 trees at a time. Gaps were treated as missing data (Swofford & Begle, 1993) and the *D. discoideum* NC4 isolate was used as the outgroup.

The heuristic search performed with 100 and 10,000 random additions for the combined ITS 1 and ITS 2 data yielded 42 and 4013 respective most parsimonious trees (MPTs) of the same 2380 length for the GO1:GE1 gap penalty. While, the different gap penalties yielded different numbers of trees and the different tree lengths, the same tree lengths were always recovered for the same GO:GE gap penalty, irrespective of whether the search was performed with 100 or 10,000 random additions. It was also noticed that, as in the previous case, where ITS 1 and ITS 2 data were analysed separately, the length of the most parsimonious trees increased with the bigger gap penalty from 2380 for (GO1:GE1) to 3746 (GO8:GE8). The phylogenetic results including number of trees, tree lengths as well as consistency and retention indices are summarised in table 4.3.

The combined analysis revealed well-resolved consensus trees, comparable to those obtained for separate analyses of ITS 1 and ITS 2, while the results confirmed the principal clades and sister groups identified in both ITS 1 and ITS 2 described above. The combined analysis did not reveal any new relationships between species and isolates although the positions of some species or isolates were better resolved e.g. *P. pallidum* and *D. sphaerocephalum* Sib 6. The *Acytostelium* was selected as the outgroup, however, the lack of any signal in one of the two partitions, caused PAUP to automatically select the alternative and more robust outgroup of *D. discoideum* NC4.

Table 4.3 Description of MPTs for combined ITS 1 & ITS 2

GO/ GE ratios	MPTs (n)	Length	CI	RI	HI
1:1	42	2380	0.7055	0.8587	0.2945
2:1	72	2562	0.6889	0.8440	0.3111
2:2	77	2699	0.6747	0.8462	0.3253
4:2	46	2955	0.6579	0.8382	0.3253
4:3	43	3054	0.6604	0.8382	0.3421
4:4	76	3140	0.6541	0.8397	0.3485
8:1	65	3268	0.6515	0.8373	0.3485
8:2	12	3353	0.6466	0.8371	0.3534
8:4	71	3402	0.6443	0.8404	0.3557
8:6	27	3490	0.6390	0.8308	0.3610
8:8	49	3746	0.6372	0.8203	

Notes: CI – consistency index, RI – retension index, HI – homoplasy index.

Strict consensus trees for each of the 11 gap penalty settings are shown in figures 6.15 to 6.25 (on CD).

4.1.2 Bootstrap analysis

Bootstrapping is one of the approaches that use randomisation procedures in order to assess statistical support for individual clades. This method randomly samples characters in an alignment with replacement and create a pseudo- replicate data set of the same size as an original one. The result of it is to randomly delete some characters and reweight others, with the sum of the weights being the same as the number of characters in the data matrix. The process is repeated multiple times e.g. 1000 and the results are compiled to allow an estimate of the reliability of particular grouping. The most parsimonious tree for each pseudo-replicate is then found and the degree of conflict between them estimated by 50% majority rule consensus tree (retain all those grouping which are supported by more than 50% of pseudo-replicates). The percentage given for a particular grouping might be interpreted as a confidence level associated with this group e.g. if the group is supported at 90 % it means that this group appeared in 90 % or more of pseudo cladograms (Kitching *et al.,* 1998). Bootstrap supports are statistically significant (Chernick, 1999), thus the recovered taxon relationships are valid.

In this study the majority of recovered bootstrap trees for ITS 1, ITS 2 and combined ITS 1 & 2 data showed very high bootstrap contingency values of 80-100% with marginally better-support for ITS 1. As expected the data showed considerable homology, with most conspecific (belonging to the same species) isolates forming coherent clades supported by high bootstrap values. ITS 1 showed 100% support for the majority of clades. A comparison of the shortest and the longest MP trees showed, with few exceptions, identical branch order with *P. pallidum* a sister group to *D. minutum* (D.min NWM12) supported by 96% in the case of the shortest MP tree (GO1:GE1) but recovered as a sister group with *D. citrinum/D. discoideum* (D.cit.D.dCR) with 70% support in the longest tree (GO8:GE8). The positions of the *P. violaceum* (Pviol STP; FCM; ATN1; ATN) and the *Dictyostelium* (D.aur BDW4; D.imp EPN1; D.muc EW5) isolates on both bootstrapped trees were different (in shortest and the longest MP trees) though the positions of isolates and species within the groups were consistent.

For instance, the GO 8:GE 8 bootstrap tree showed very high supports for the majority of clades. The clades (D. sp BWD 112; D.ros Alab; D.ros EDF3; D.ros 1L), then (D. aurst BDW; D.imp EPN 11) and clade (D.ten CU 2G3; P.pal ATN18), all showed 90-99% support. Moreover, clades e.g. clade including *D.purpureum* isolates (D.purp AT1K; D.purp ATN13 and D.purp CRLC2) or *D.discoideum* isolates (D.d ATN122; D.discoNC4; D.dATN119; D.discoV12) showed even higher support of 100%. Additionally, two sister clades, one comprising both *Polyshondylium* and *Dictyostelium* species (D.min GMN3; P.viol ATN11) and the other comprising two *Dictyostelium minutum* isolates (D. min NWM12; D.min ATN16) separately were given 100% support and then 90% for both sister clades, giving confidence to those clades. Interestingly, another clade (mentioned earlier) comprising species from both genera (D.ten CU 2G3 and P.pal ATN18) showed 96 % support and again high confidence. Although some clades on this tree showed lower bootstrap support, however, all of them were in the range of 69-72% with only one showing 55%, which again indicial of statistical support, the results were comparable with other bootstrap trees. Although, the shortest and the longest bootstrap trees could not adequately resolve the position of either Siberian *D. sphaerocephalum* (D.spha Sib 6) or *P. aureum* (P.aureum N36) species, they were given 50% support.

Bootstrap analysis of the combined ITS 1 and ITS 2 data showed similar values but significant differences in branch order. Despite the differences, many clades identified in either ITS 1 or ITS 2 were also observed in the combined analysis and with high (significant) bootstrap values. For example, sister groupings of *D minutum* (D.min NWM12 and D.min ATN16) were recovered in both, shortest and longest MP trees with 100% and 94% respective support.

To summarise, all bootstrapped trees for ITS 1, ITS 2 and combined ITS 1 & 2 data shared numerous similarities and in many cases recovered the same species/ isolate/ clade branch order (see figure 4.19). Most branches were supported by high bootstrap values suggesting robust relationships with few not fully resolved by a <50% bootstrap.

Examples of trees are shown below in figures 4.13 to 4.18. The bootstrap trees for other gap penalty settings are shown in figures 6.26 to 6.54 (on accompanying CD).

Figure 4.1 ITS1 tree of the length of 687 (GO1:GE1);CI 0.73;RI 0.91;HI 0.27;(23MPTs)

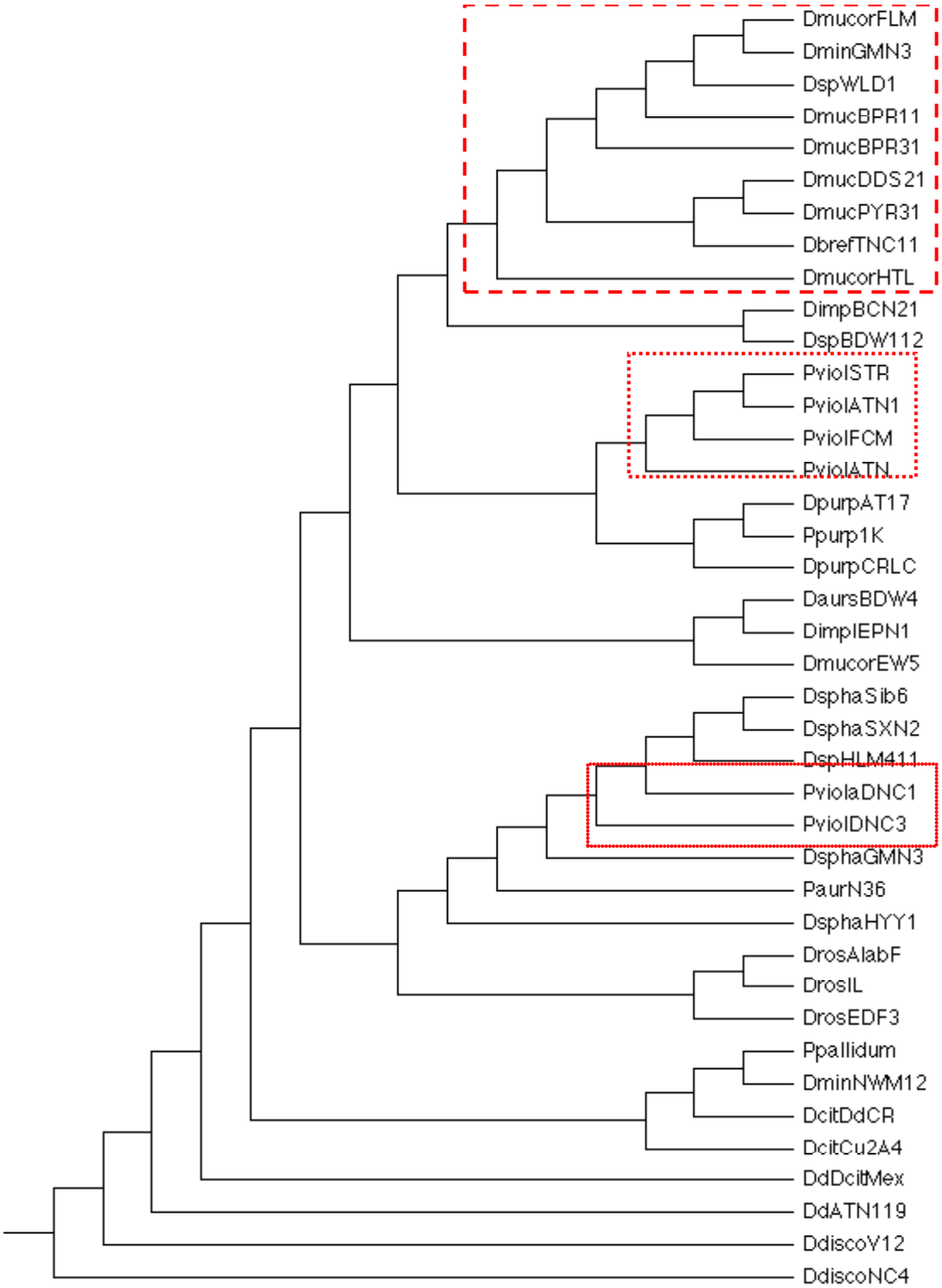


Figure 4.2 ITS 1Strict Consensus tree of 23 MPTs (length 687; GO1:GE1)

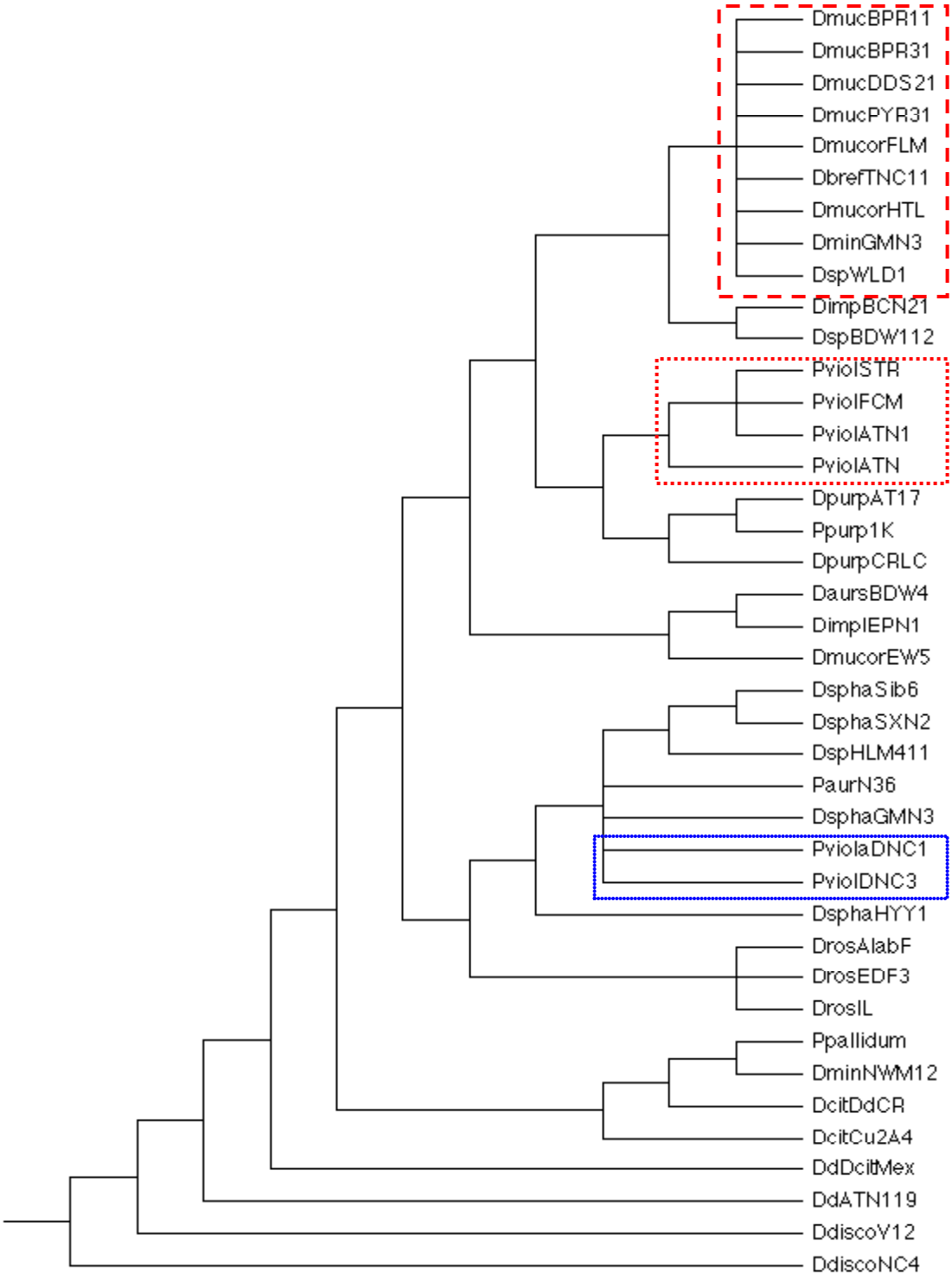


Figure 4.3 ITS 1 tree of the length of 728
(GO2:GE1), CI 0.74; RI 0.90; HI 0.26; (28 MPTs)

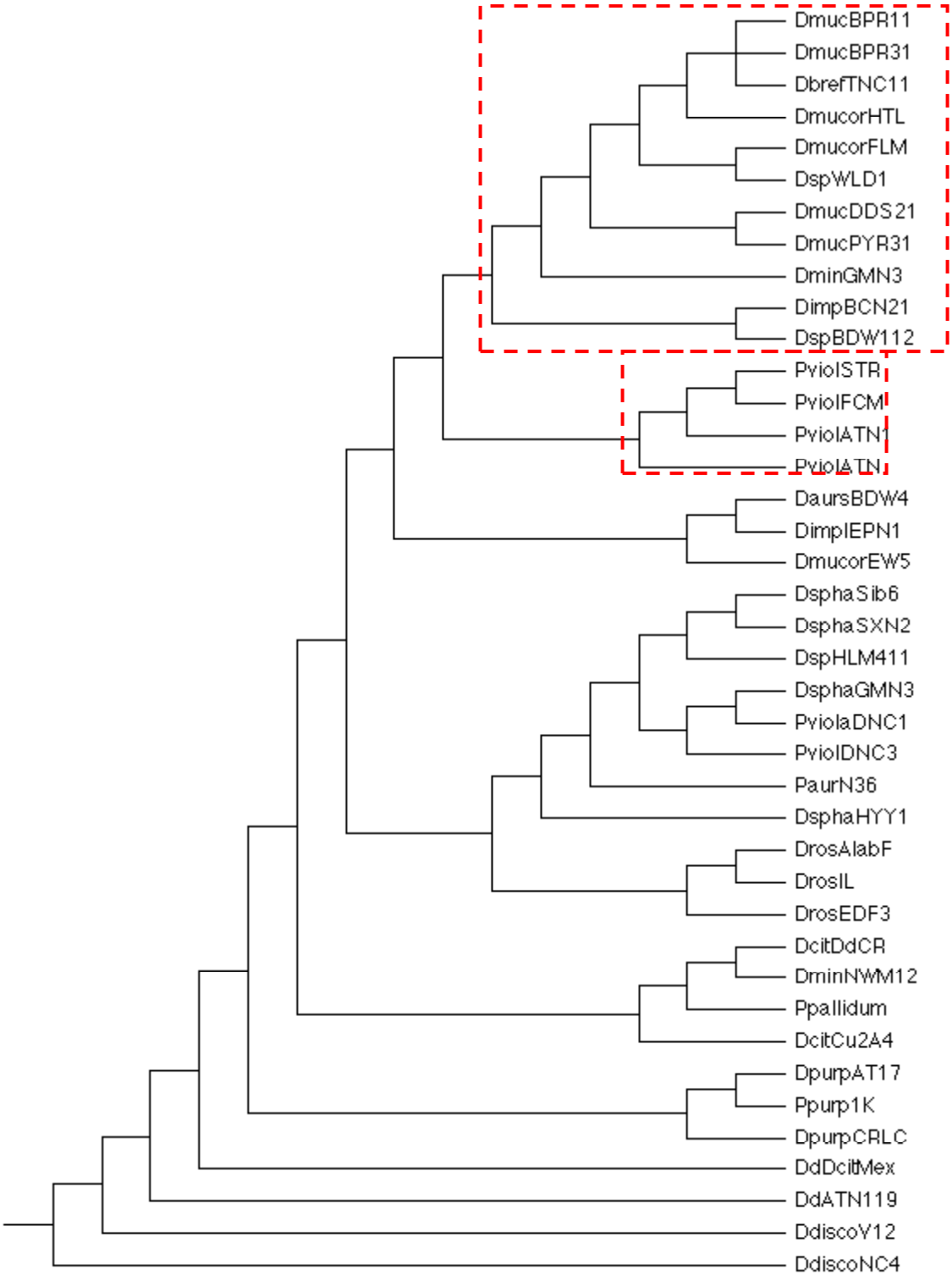


Figure 4.4 ITS 1 Strict Consensus tree of 28 MPTs (length 728 GO2:GE1)

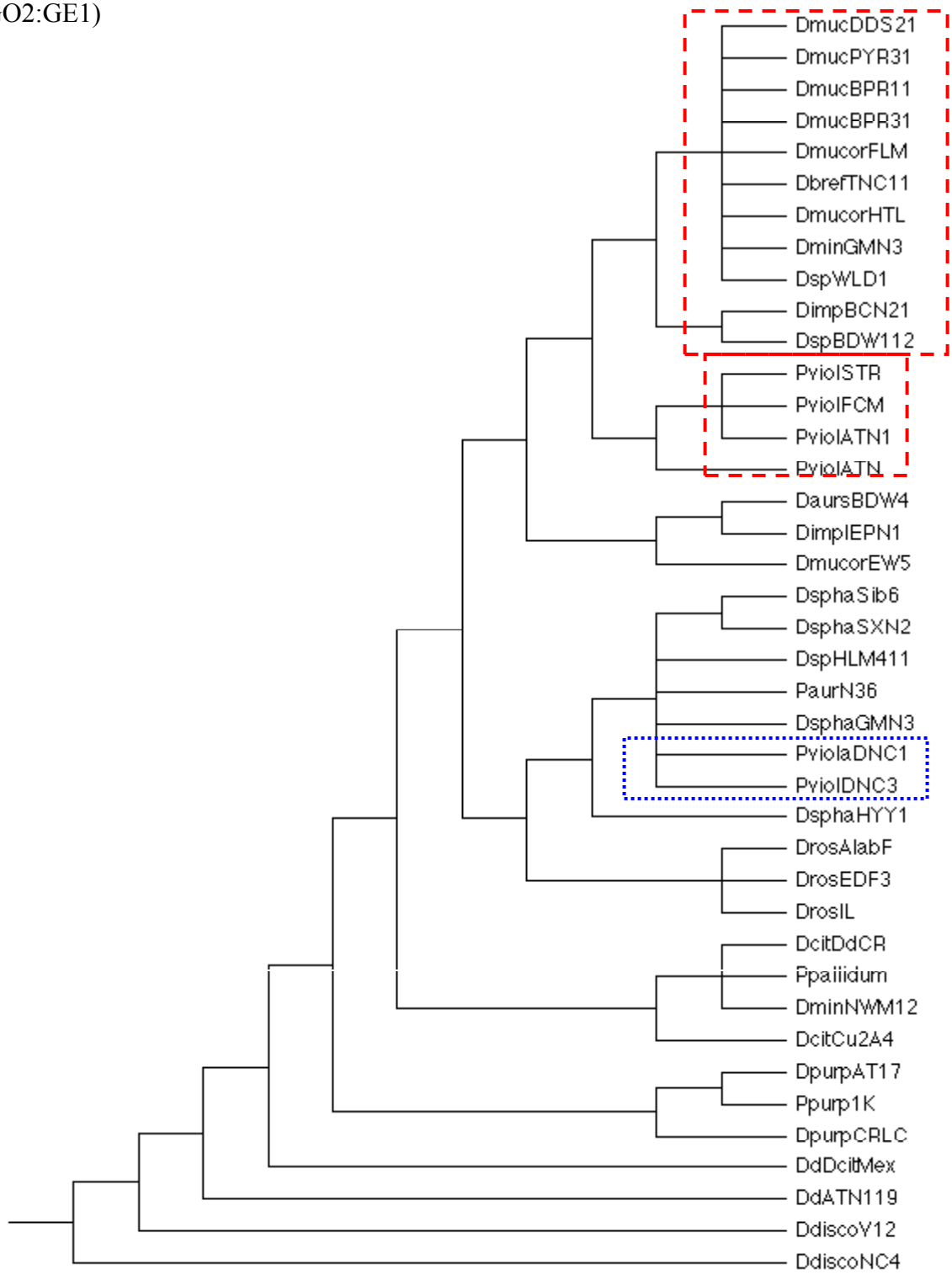


Figure 4.5 ITS 2 tree of the length of 1706 (GO1:GE1) CI 0.75; RI 0.85; HI 0.25; (99 MPTs)

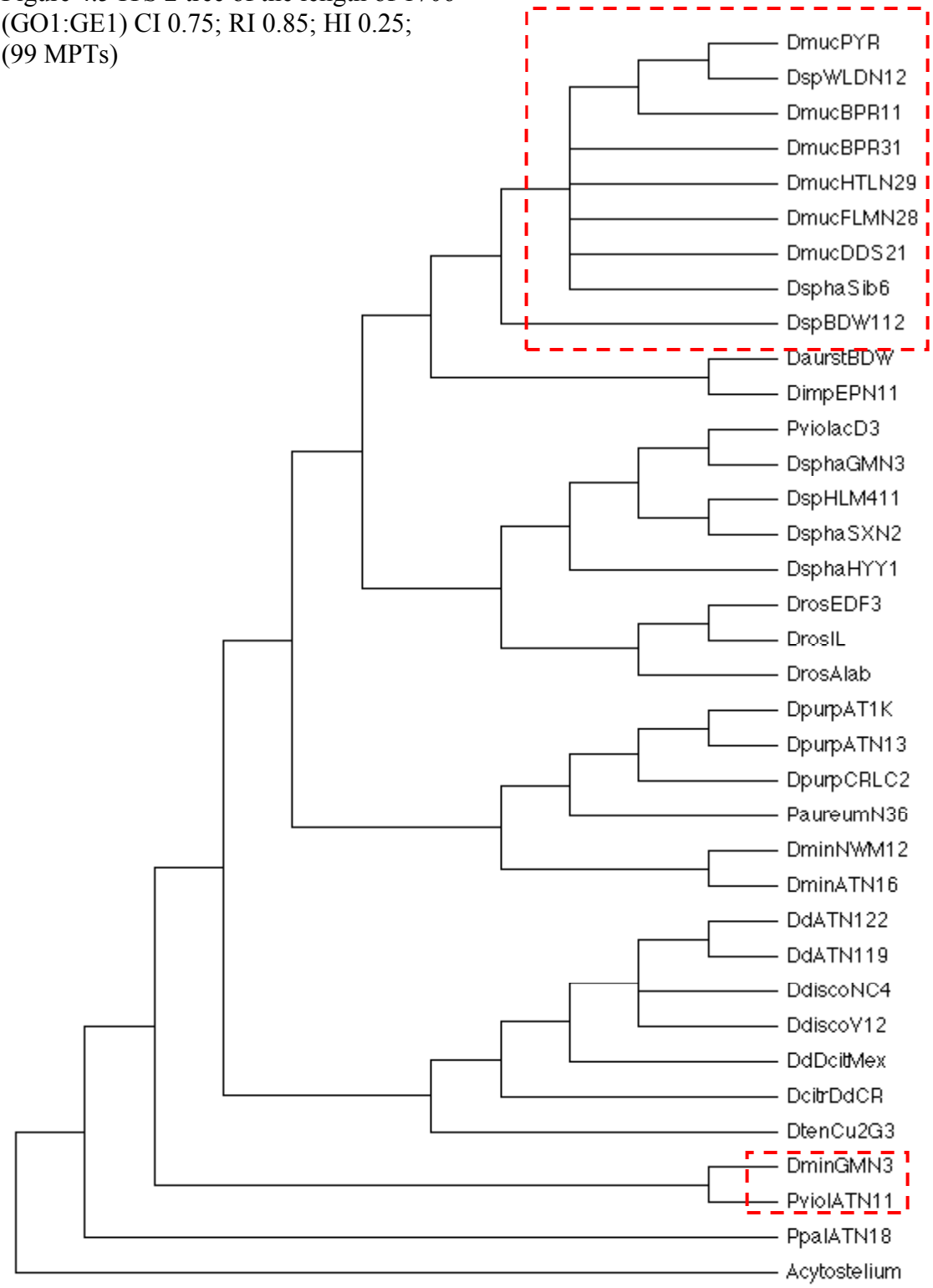


Figure 4.6 ITS 2 Strict Consensus tree of 99 MPTs
(length 1706 GO1:GE1)

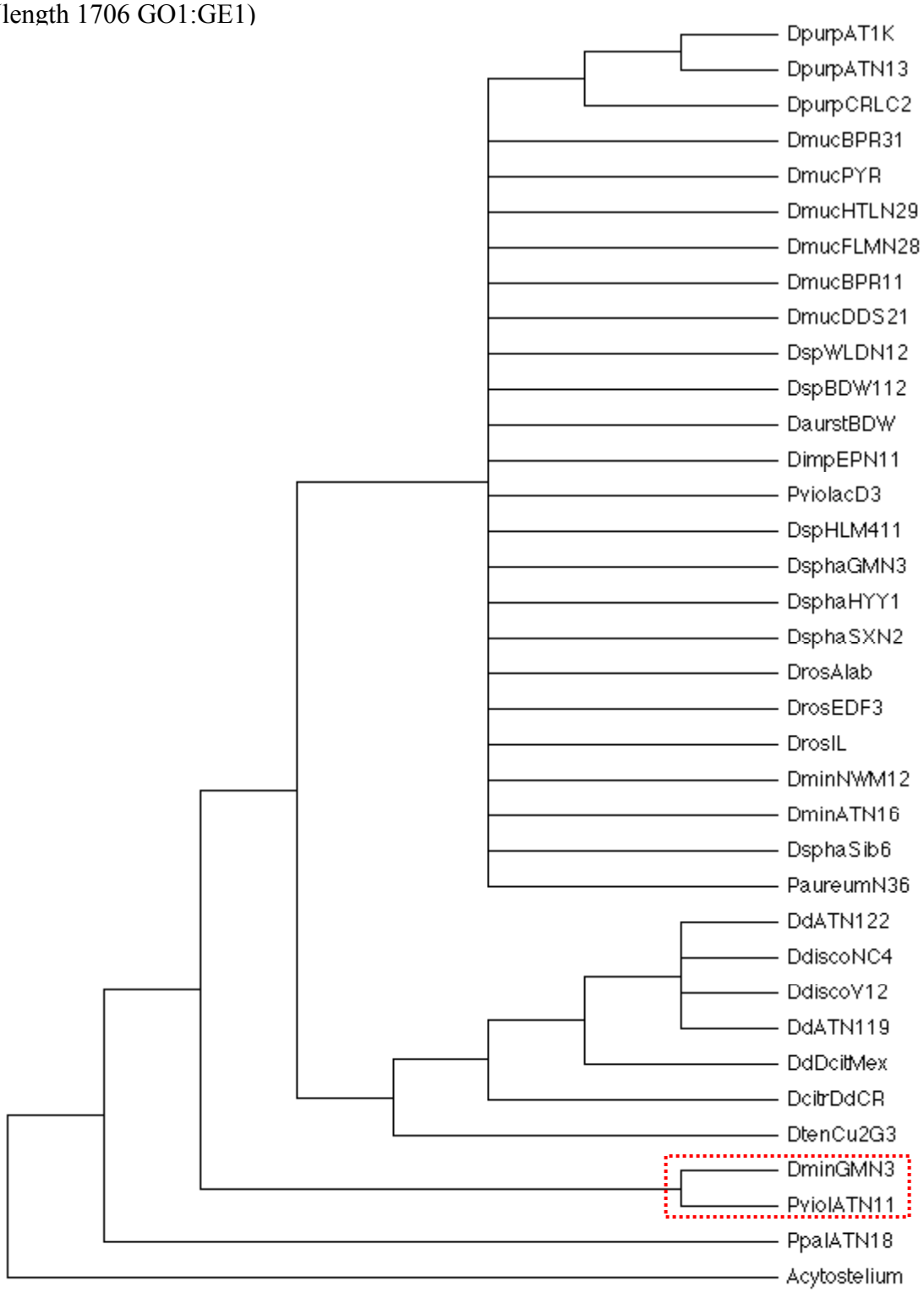


Figure 4.7 ITS 2 tree of the length of 1832 (GO2:GE1) CI 0.72; RI 0.83; HI 0.28; (96 MPTs)

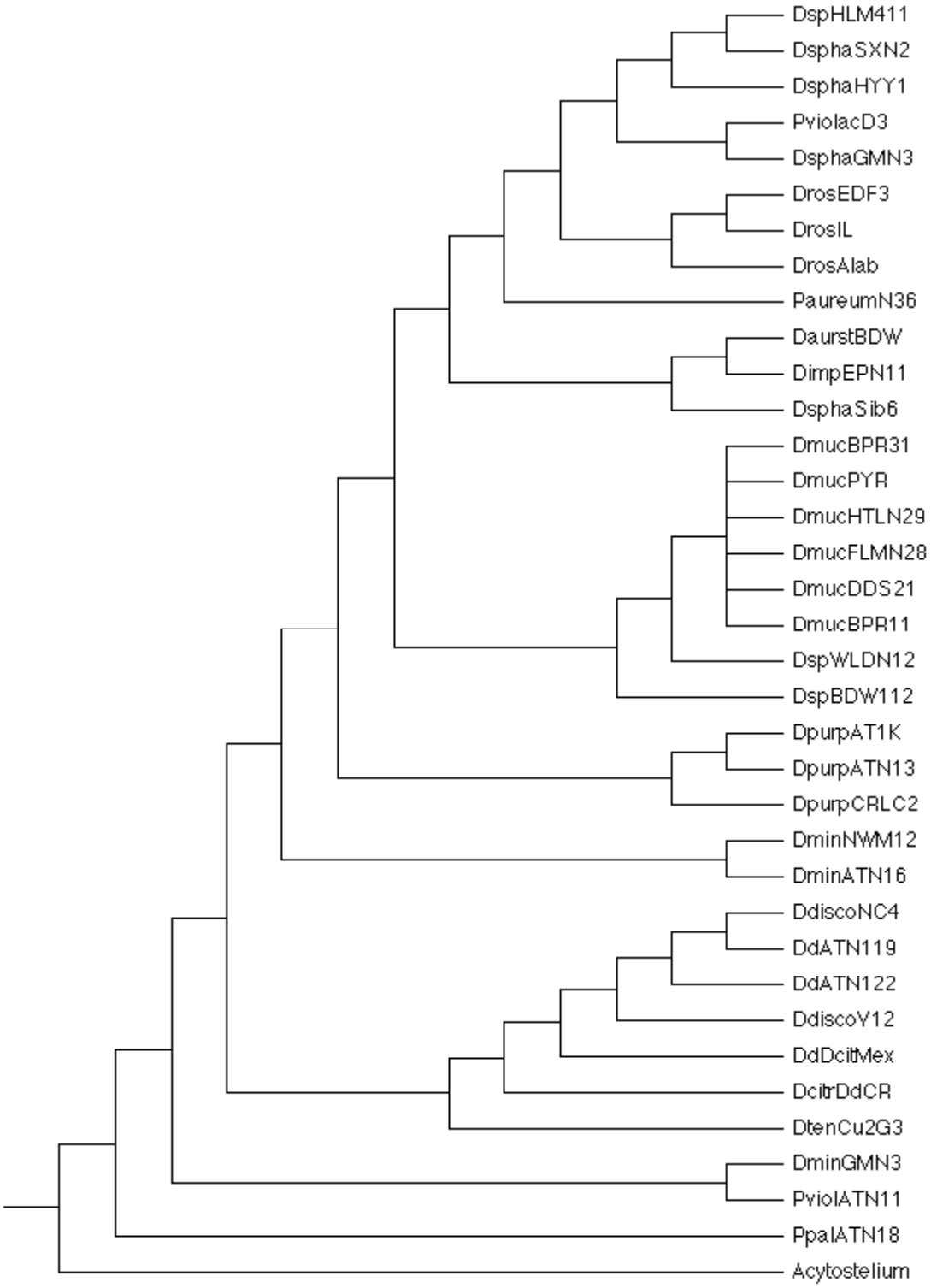


Figure 4.8 ITS 2 Strict Consensus tree of 96 MPTs
(length 1832 GO2:GE1)

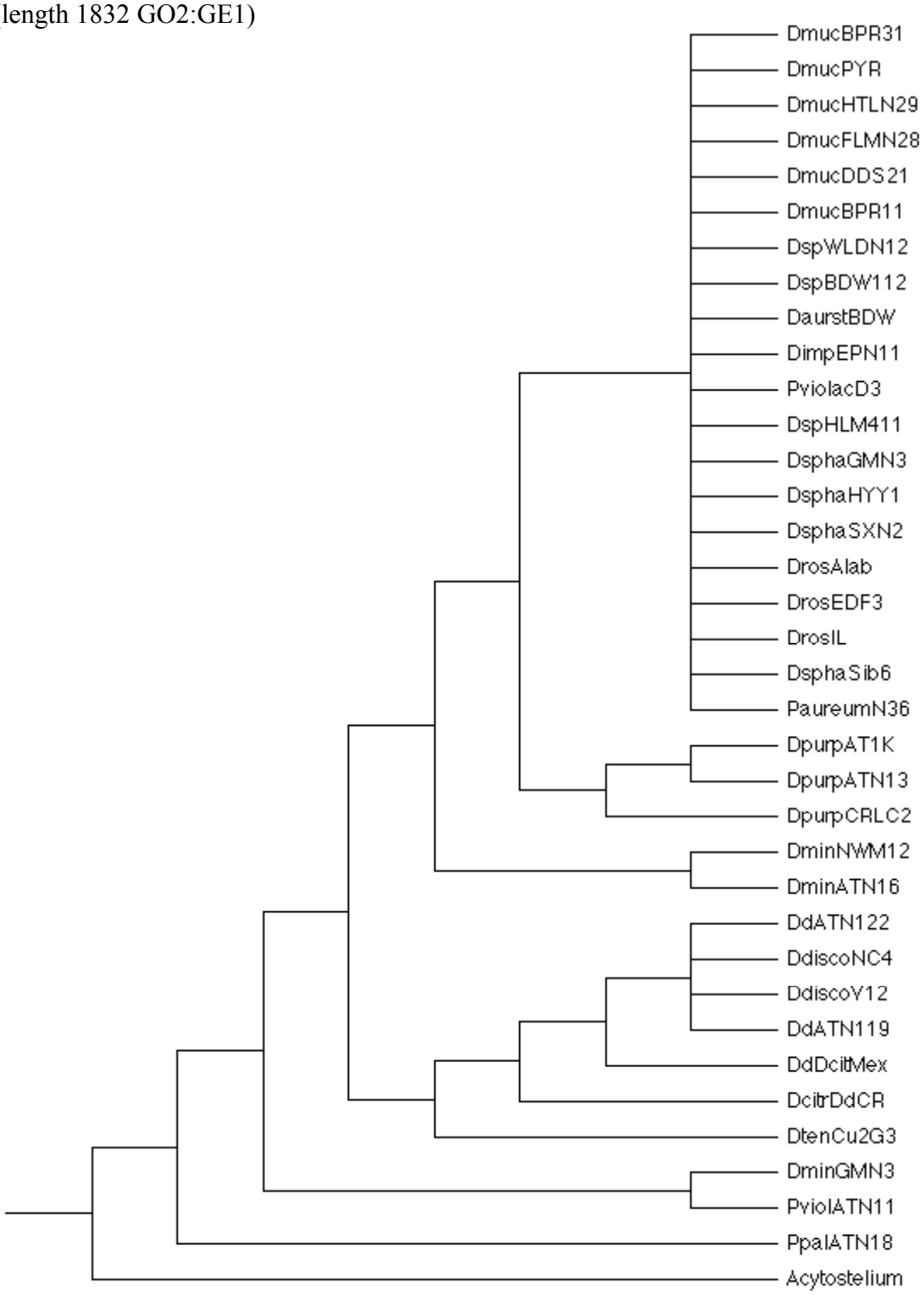


Figure 4.9 ITS 1 Strict Consensus tree of 100 MPTs
(length 970 GO8:GE2)

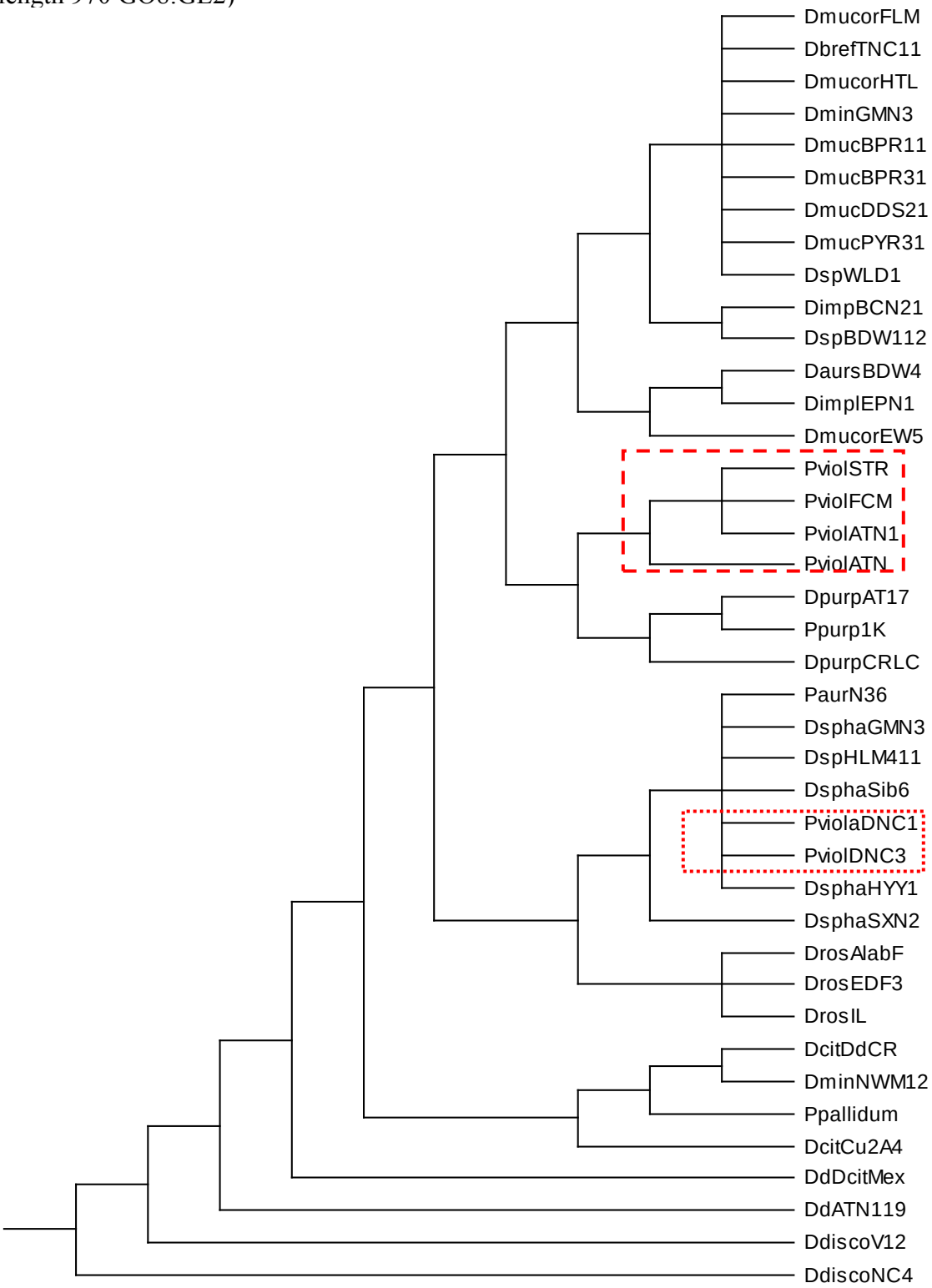


Figure 4.10 ITS 1 Strict Consensus tree of 97 MPTs (length 989 GO8:GE4)

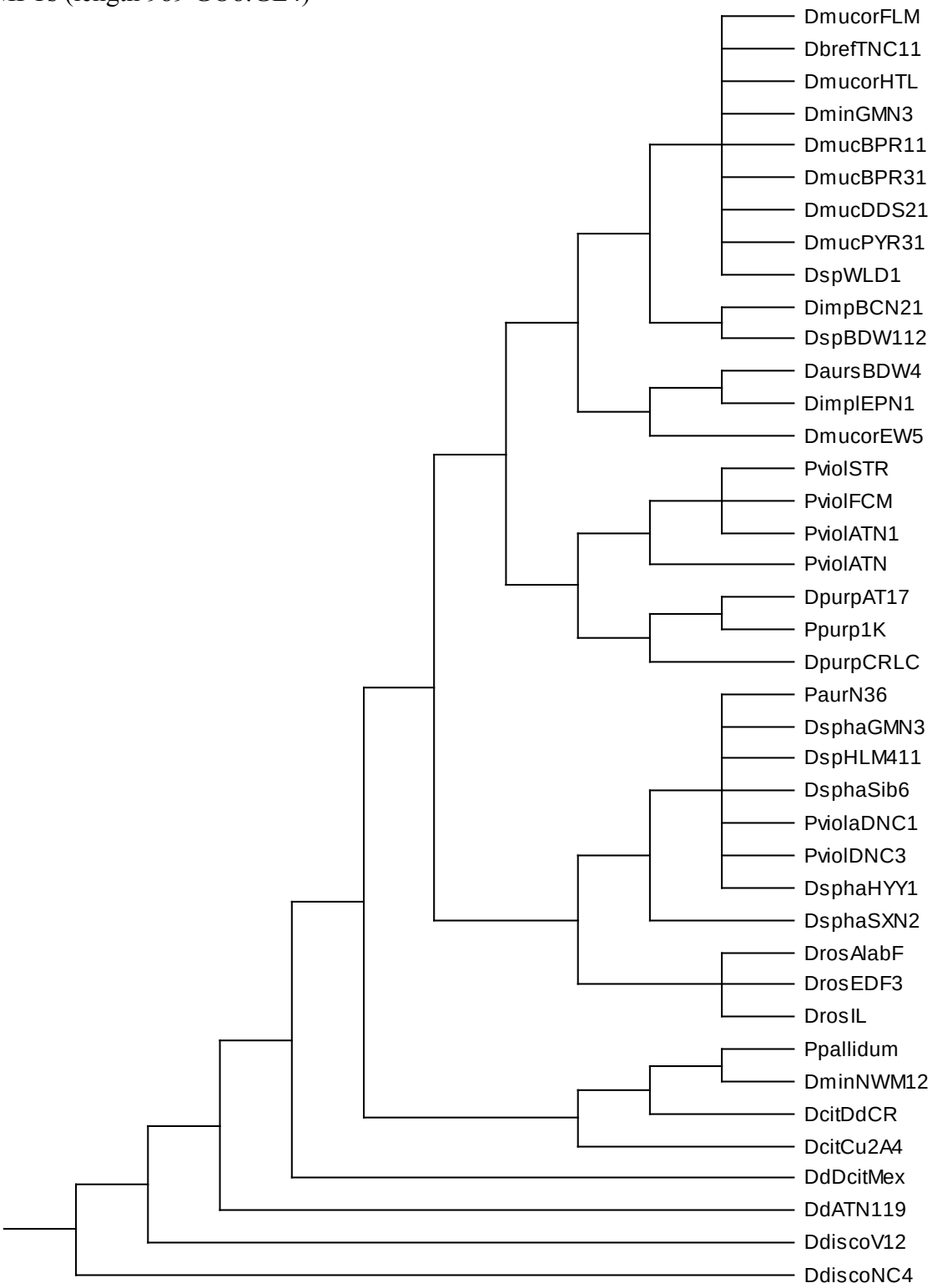


Figure 4.11 ITS 1 Strict Consensus tree of 100 MPTs (length 1024 GO8:GE6)

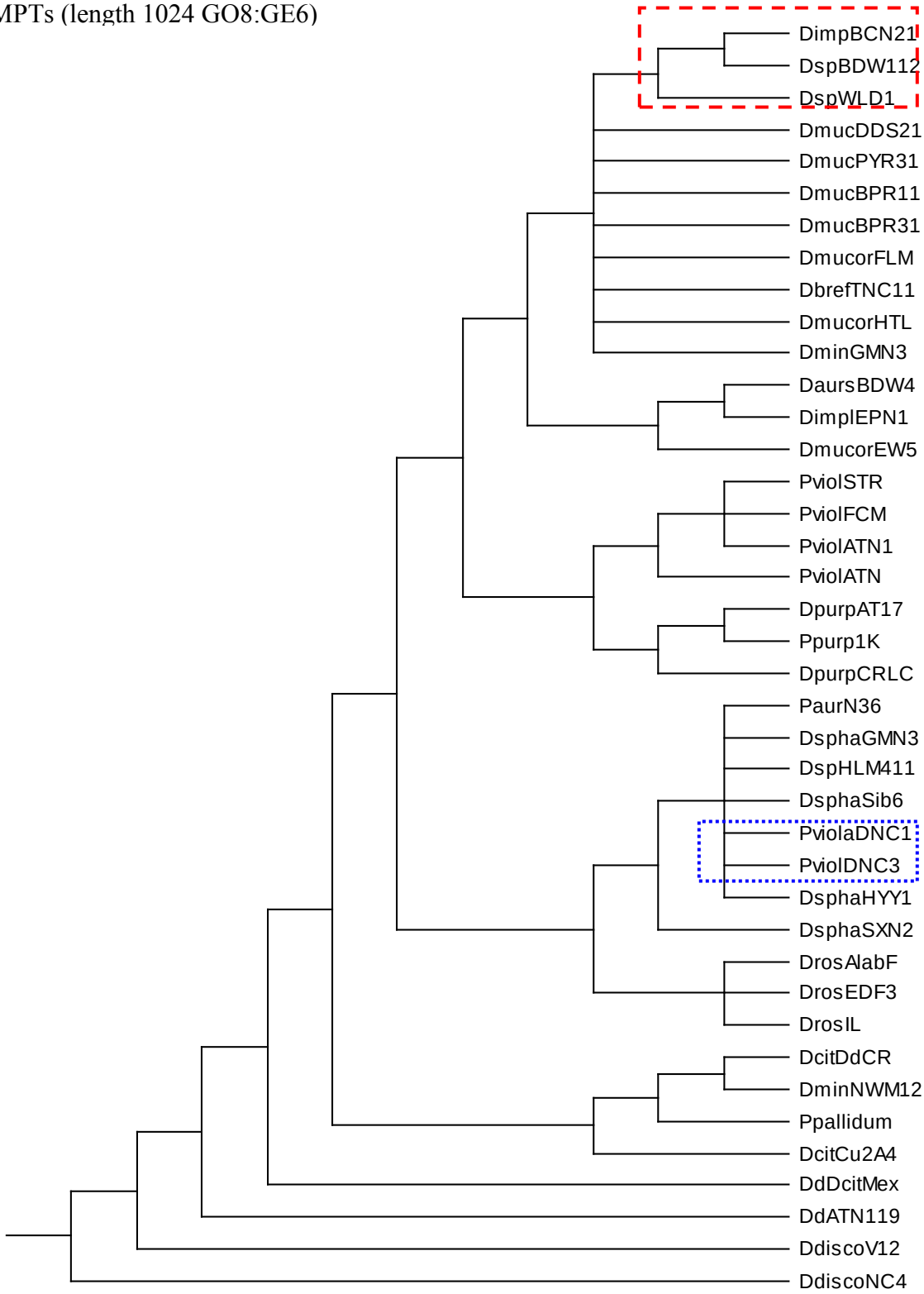


Figure 4.12 ITS 1 Strict Consensus tree of 100 MPTs (length 1027 GO8:GE8)

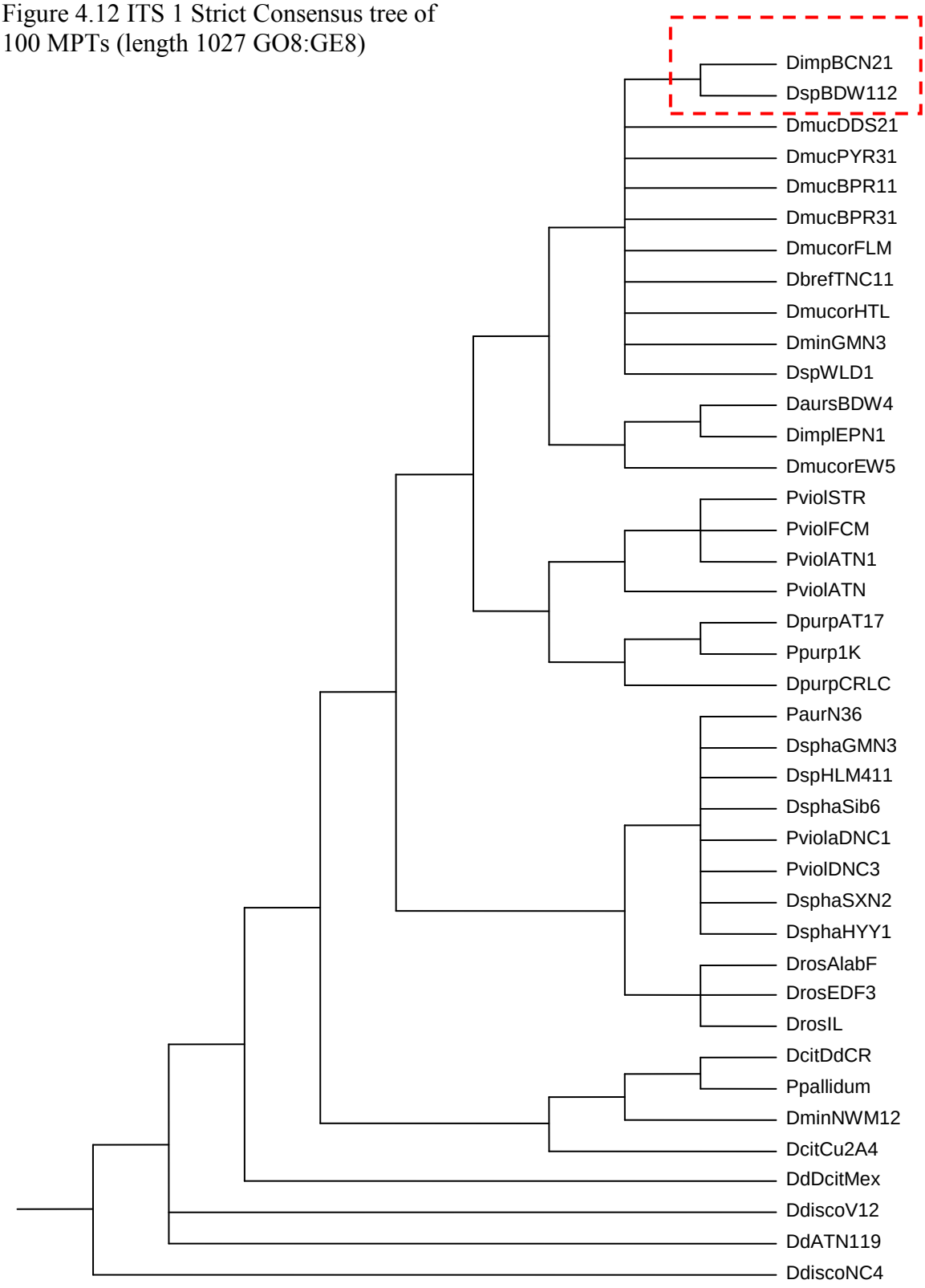


Figure 4.13 ITS1 Bootstrap tree (GO1:GE1)

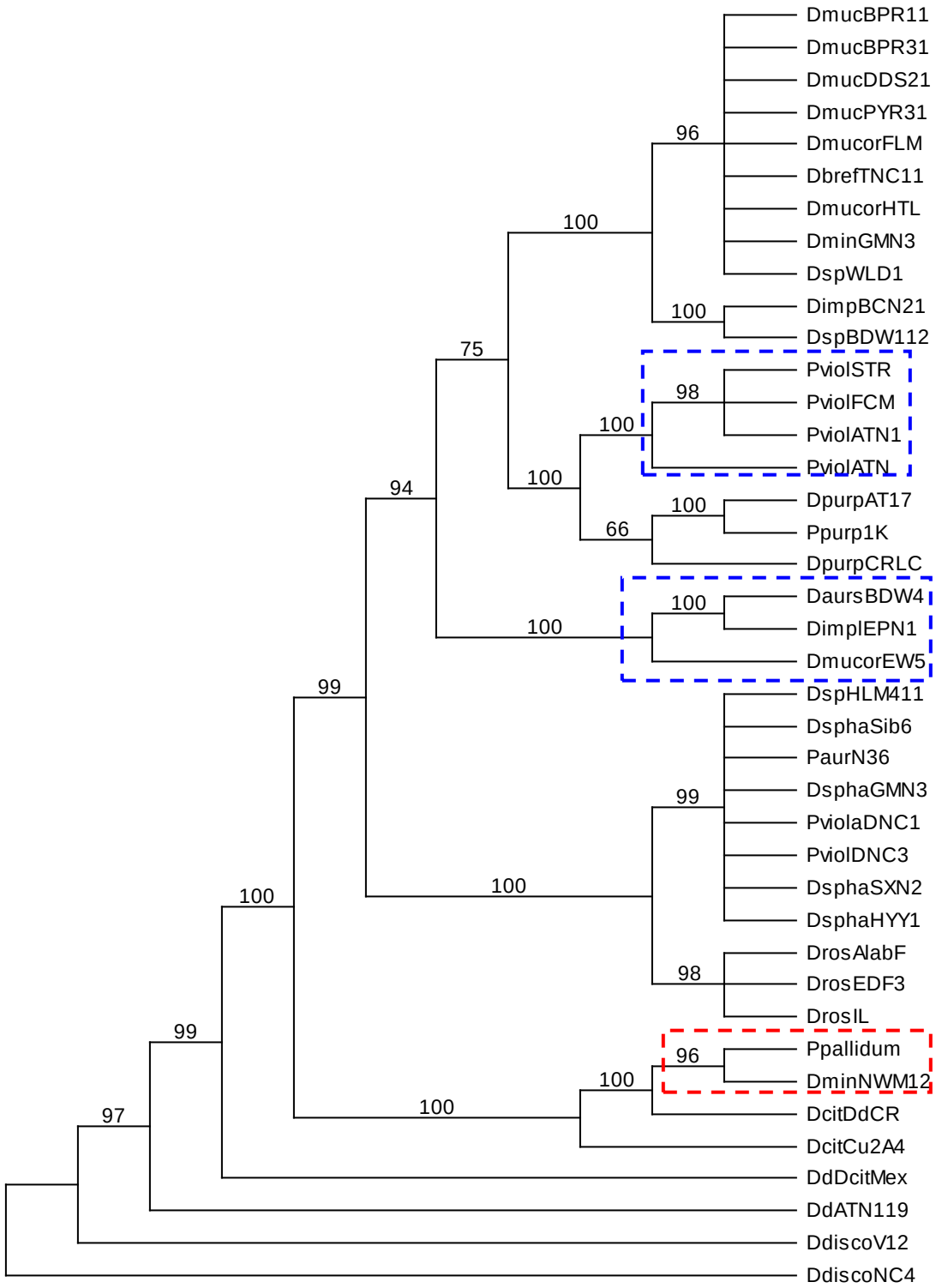


Figure 4.14 ITS1 Bootstrap tree (GO8:GE8)

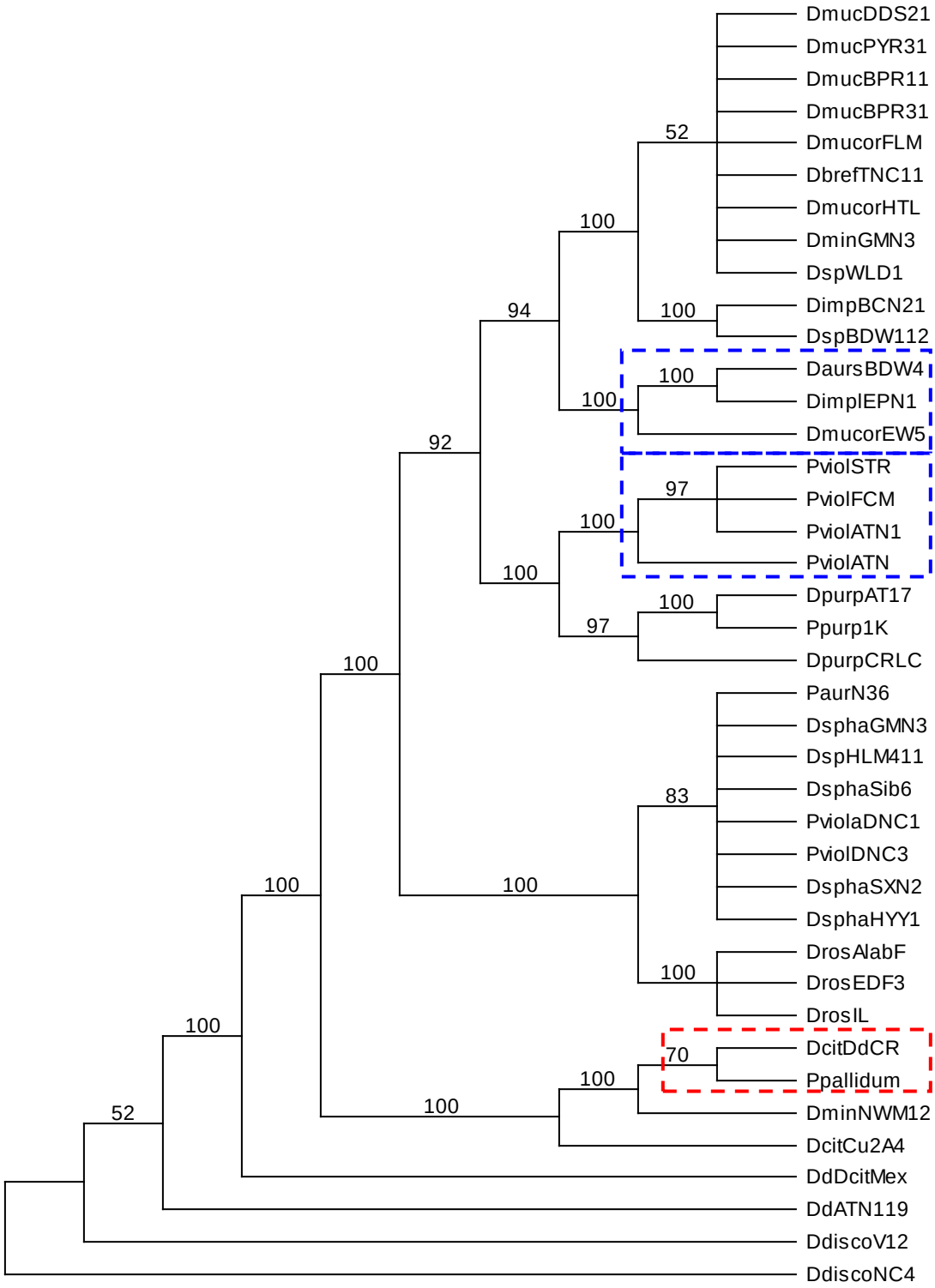


Figure 4.15 ITS2 Bootstrap tree (GO1:GE1)

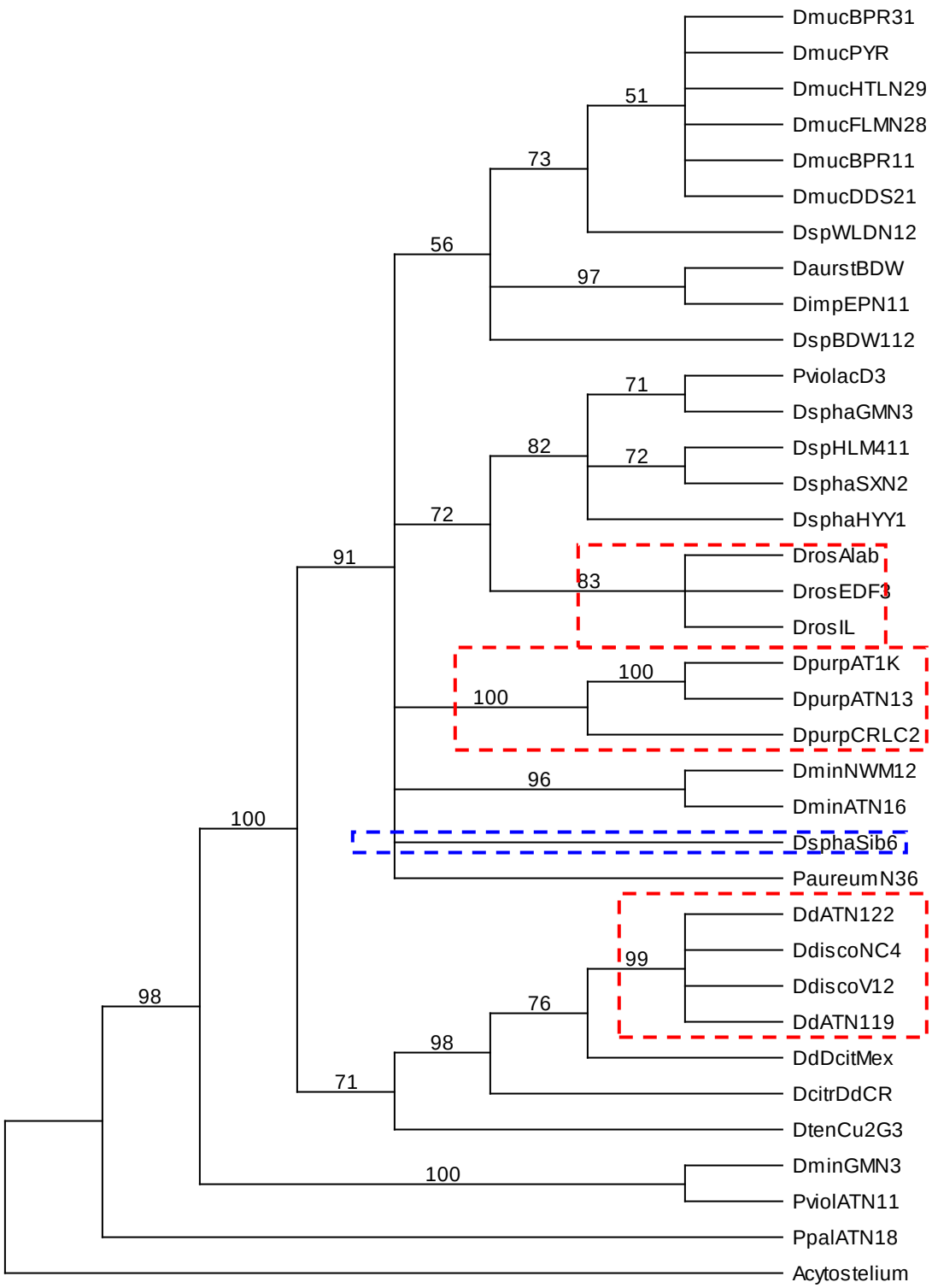


Figure 4.16 ITS2 Bootstrap tree (GO8:GE8)

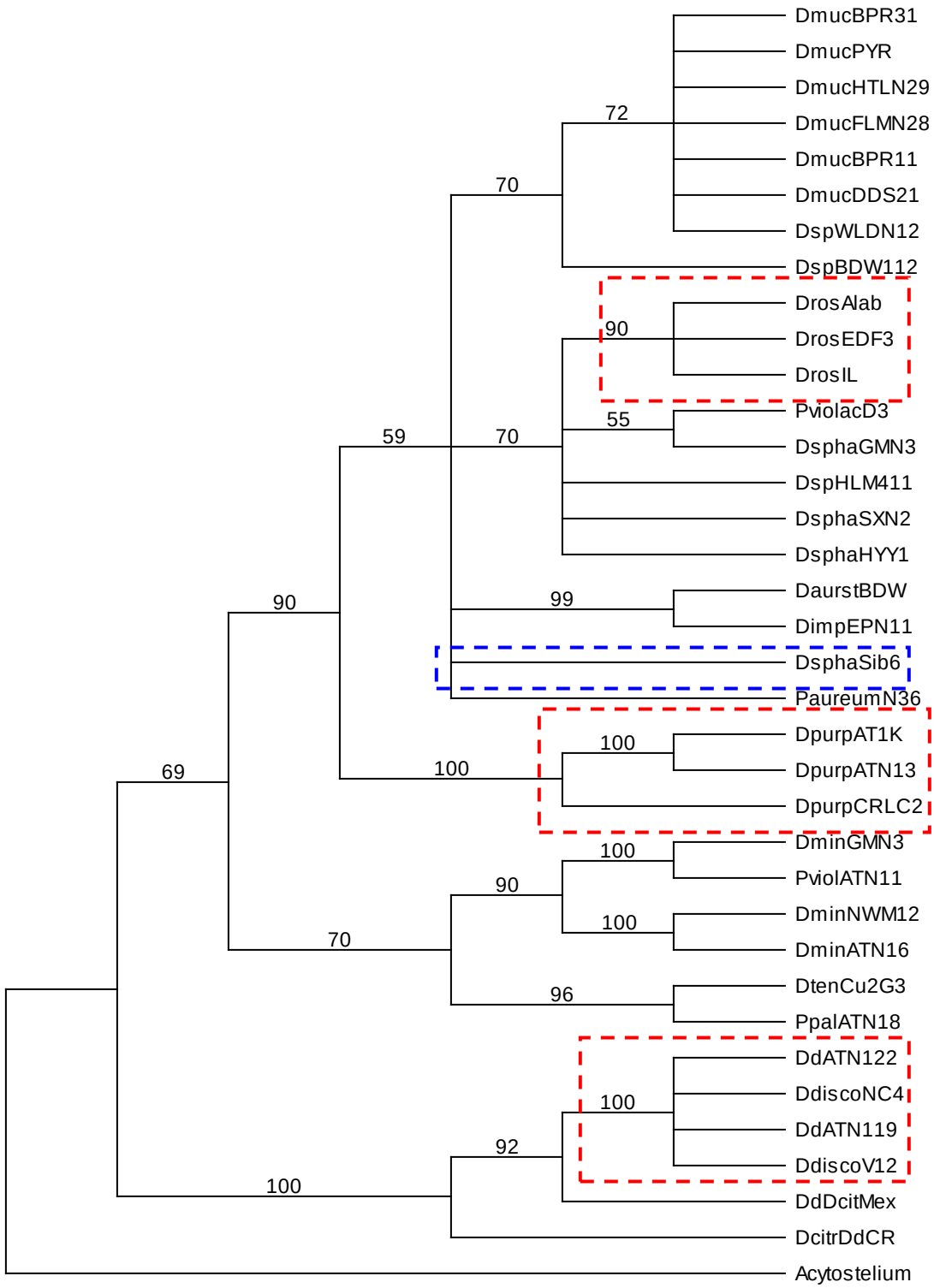


Figure 4.17 Bootstrap tree for the combined ITS1 and ITS 2 (GO1:GE1)

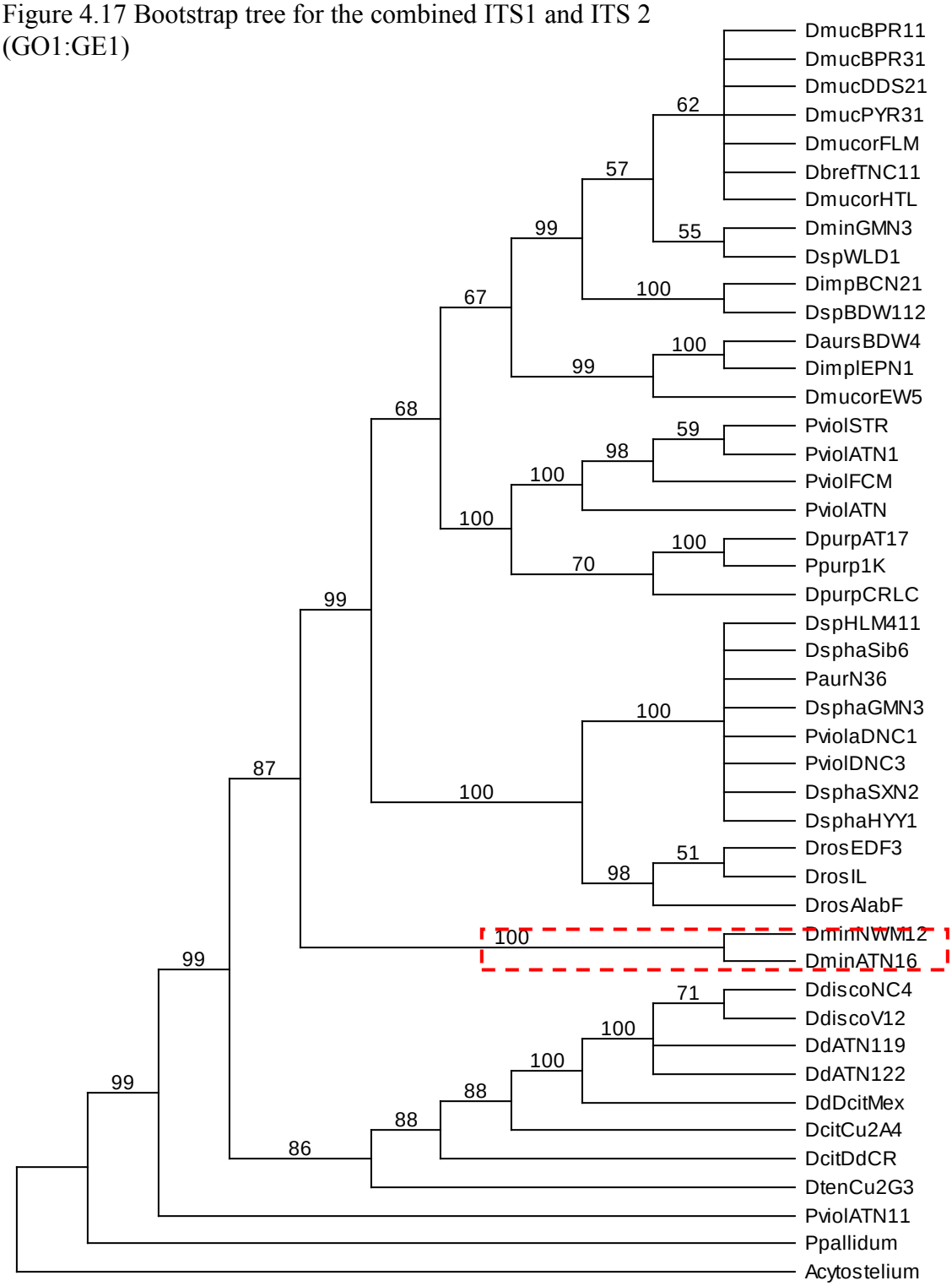


Figure 4.18 Bootstrap tree for the combined ITS 1 and ITS 2; (GO8:GE8)

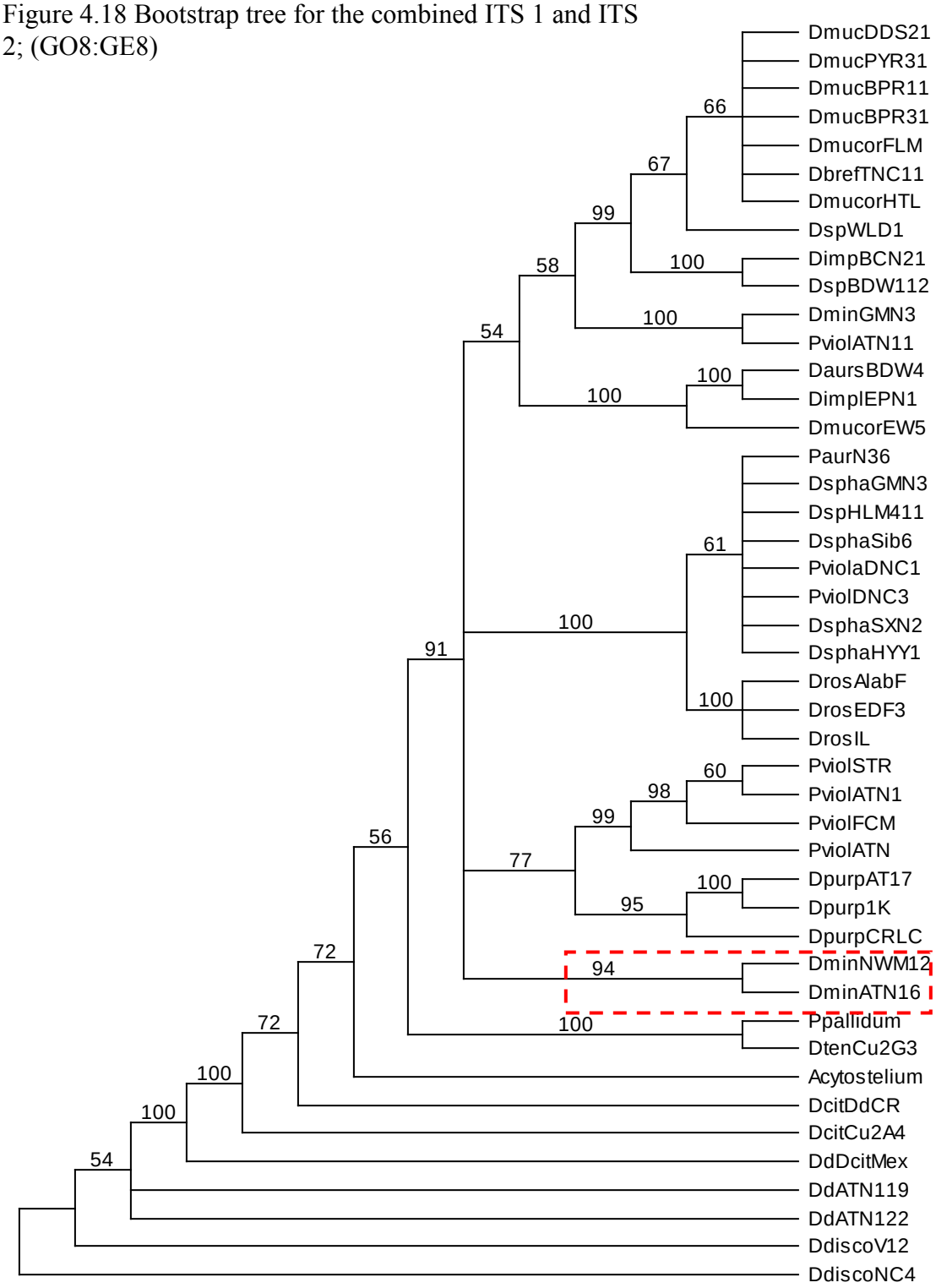
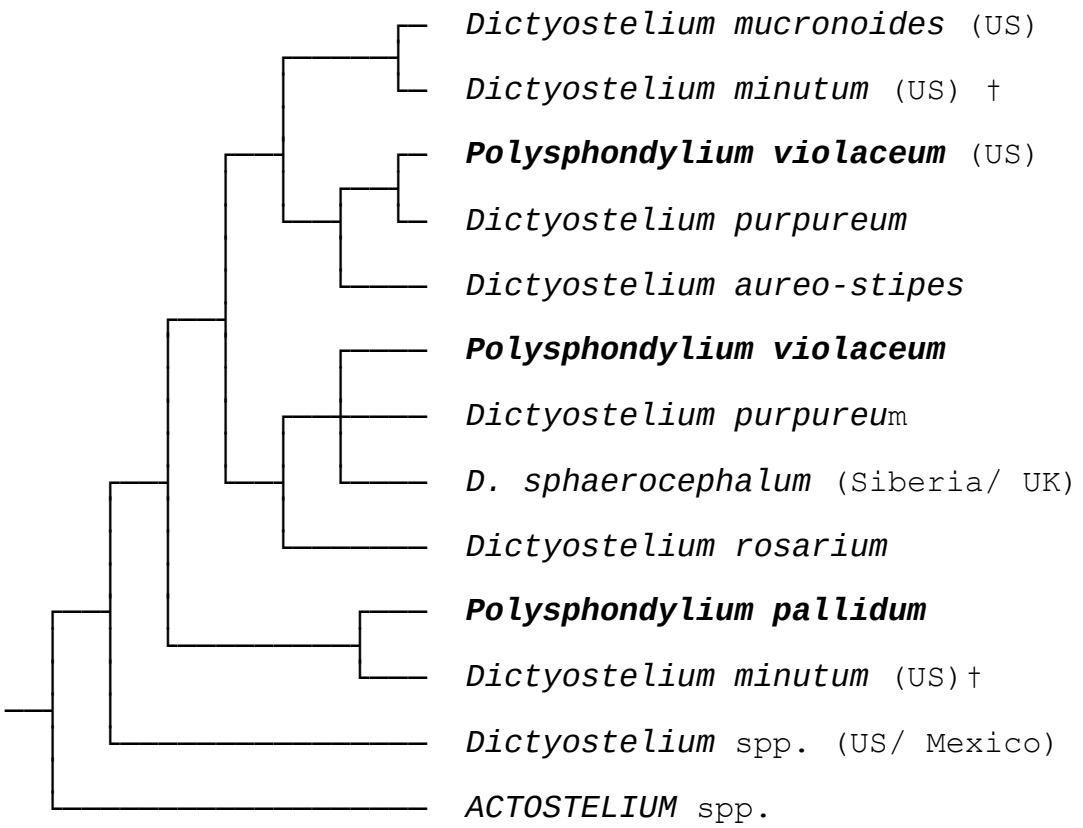


Figure 4.19 A putative ITS1/ ITS2 consensus tree



Notes: ***Polysphondylium* spp. are in bold**, c.f. *Dictyostelium* spp.; *ACTOSTELIUM (OUTGROUP) IS IN UPPER CASE ITALICS.* † represent different isolates.

4.2 DISCUSSION

Isolation and characterisation of overseas and U.K. dictyostelid species and isolates was a research theme at the Department of Life Sciences at Anglia Ruskin University in the late 1990s (Hodgson, 1999), generating a number of clones which formed the basis of the departmental culture collection. Twenty five species and isolates from this collection were used for my research to which I added a further 26 species and isolates, mainly from the USA. This study extracted DNA and sequenced ITS 1 and ITS 2 from these 51 species and isolates to establish phylogenetic relationships between them.

Molecular phylogeny using DNA sequences is a fast developing discipline and has proved a powerful and informative tool to understand relationships between taxa at a variety of levels (Sibley, 1965; Page, 1998). It was hoped that (1) these ITS DNA sequences of cellular slime moulds could be aligned and then subjected to phylogenetic analysis to reflect the true relationships between Dictyostelids; (2) this technique would be sufficiently powerful to reflect not only relationships between species within the genera but also highlight the phylogenetic relationship between *Dictyostelium* and *Polysphondylium* which has remained controversial and largely unresolved; (3) sequencing and subsequent phylogenetic analysis would establish whether *Dictyostelium* and *Polysphondylium* established on the basis of morphological features are indeed valid or whether a third intermediate genus as suggested by some researchers (Traub and Hohl, 1976; Raper, 1984) should be invoked.

Assessing dictyostelid species identification and establishing their systematic position has proven difficult especially if based on morphology. However, the use of the molecular data is no less challenging especially when dealing with closely related taxa and, as here, with one genus of few species (Avise, 1994). I considered multiple isolates from three of the seven known *Polysphondylium* species. Sequenced molecular data requires careful consideration to be given to the region of the genome being examined. It should show sufficient polymorphism to enable effective comparisons as suggested by (Hillis and Dixon1991; Hillis *et al.,* 1996; Hoelzel, 1998).

The ITS 1 and ITS 2 molecular data were analysed both separately and combined. Initial parsimony analysis of ITS 1, performed for eleven gap penalty settings, did not yield fully resolved strict consensus trees (the strict consensus tree is chosen as the groups certainty is considered), yet they revealed a few species-consistent characteristic clades of isolates. These included nearly all *D. mucoroides* isolates (except *D. mucoroides* EW 5), *P. violaceum* isolates, or *D. rosarium* or *D. discoideum (*e.g. see figure 4.4). Interestingly two DNC 1 and DNC 3 isolates of *P.violaceum*, were constant sister taxa and appeared at a separate position relative to other *P.violaceum* isolates (e.g. see figure 4.9). Furthermore, *D. implicatum* BCN 21 always formed a monophyletic group with *D.* sp. BDW 112 and appeared in the same position on the trees despite different gap penalty settings, except for GO8:GE6 and GO8:GE8 where they were resolved as a crown clade (see figure 4.11 and 4.12). This could result from *D.* sp. BDW 112 representing a possible *D. implicatum* isolate. More interesting is the fact that some species were recovered together in all consensus trees but at slightly different positions. For instance, *D. aureostipes* BDW 4 forms a clade with an isolate of *D. implicatum*, EPN 1, to which *D. mucoroides* EW 5 is a sister group that never aligned with *D. mucoroides* isolates. There have been previous difficulties in discriminating *D. mucoroides* from *D. sphaerocephalum* in terms of morphological features as both produce white sori apart from sharing features common to the genus *Dictyostelium* (Hagiwara, 1983).

Interestingly the Siberian *D. sphaerocephalum* Sib6 isolate formed a crown clade with the English *D. sphaerocephalum* SXN 2 isolate, despite their geographic separation. Similarly, in all cases *P. pallidum* formed a clade with *D. minutum* NWM12, results that disagree with established dictyostelid phylogeny, in particular the relationships between *Dictyostelium* species, as the established dictyostelid phylogeny strictly separates the genera *Dictyostelium* and *Polysphondylium* on the basis of different morphological features. More significant is the finding that nowhere, in any of the presented strict consensus trees are the *Polysphondylium* species and *Dictyostelium* species reconciled as discrete generic clades. In all strict consensus trees *Polysphondylium* species are interspersed within the genus *Dictyostelium*. Thus, implies that there is no valid

distinction between the genera *Dictyostelium* and *Polysphondylium* as neither was resolved as being discrete, i.e. monophyletic. Furthermore, no consensus tree showed a strict distinction between American (New World) and English (Old World) taxa collections. This is supported by Swanson *et al.,* (2002) who subjected 19 dictyostelid taxa to parsimony analysis based on 18 chosen morphological features and concluded that neither the two dictyostelid genera nor indeed the family Dictyosteliales appears monophyletic.

The two isolates of *D. sphaerocephalum* (Sib6 and SXN2) mentioned above also formed an interesting clade with the partially resolved *D.* sp. HLM 411. In the majority of consensus trees both *D. sphaerocephalum* isolates were monophyletic forming a sister group with *D.* sp. HLM 411. Yet *D. sphaerocephalum* Sib 6 is from Siberia and the other *Dictyostelium* sp. isolate is from England (a mix up of the labels is a possibility but it seems very unlikely). This finding could suggest that the dictyostelids show limited genetic variability and/or that existing polymorphism has neither phenotypic nor biogeographic significance. It also suggests that *Dictyostelium* is a complex taxon, which would need a much broader genome sampling to explain and confirm monophyletic relationships within *D. sphaerocephalum* isolates (Sib 6 and SXN 2) and other sister taxa. There is also some evidence that *Dictyostelium* may have acquired new genes via horizontal gene transfer from bacteria, a mechanism of genome evolution which is now gaining increased recognition (Eichinger *et al.,* 2005). An alternative hypothesis could be cached in terms of *Dictyostelium* spores dispersal to different habitats via different vectors or dispersal agents; for example, the nematode *C. elegans,* which is not only a *Dictyostelium* food competitor but a *Dictyostelium* amoebae predator (Kessin *et al.,* 1996; 2001); or migrating birds (Feest, 1987). This could explain the close relationship between the Siberian and English *D. sphaerocephalum*.

Parsimony analysis of ITS 2 delivered slightly different results mainly because sequencing *Acytostelium,* the chosen outgroup, was successful and therefore used for alignment in contrast to ITS 1 analysis where unsuccessful sequencing of *Acytostelium,* necessitated the use of *D. discoideum* NC 4 for rooting. Moreover, the majority of ITS 2

strict consensus trees obtained were not fully resolved. However, ITS 2 analysis did recover similar groupings to those obtained from ITS 1. In the ITS 2 case all *D. purpureum* isolates were placed together in one clade as were *D. discoideum* isolates. Interestingly, the poorly resolved cultures of *D. discoideum/ D. citrinum* from Mexico and *D. citrinum/ D. discoideum* from Costa Rica formed sister groups with *D. discoideum* isolates. This grouping was not unexpected as *D. citrinum* and *D. discoideum* share some common morphological features, most notably yellow sori (see paragraph 2.2.2.2). The slightly different appearance of the consensus trees in terms of order of species and isolates provided in the ITS 2 analysis could be explained by the fact that not all species and isolates used for analysis of ITS 1 were successfully sequenced for ITS 2. Some of the sequences were of poor quality and so excluded from ITS 2 analysis. Although a full comparison between ITS 1 and ITS 2 data in relation to the recovered grouping of species and isolates was rather problematic, the recovered ITS 1 and ITS 2 species and isolates clades are in agreement with that actually expected, i.e. isolates of the same species should be grouped together, e.g. *D. discoideum* or *P. violaceum* or close to each other, e.g. *D. citrinum/D discoideum* as it was believed that they would share some common features.

The most interesting outcome of separate ITS 1 and ITS 2 analysis is that *Dictyostelium* and *Polysphondylium* were never resolved as separate clades as should be the case if they do indeed represent different genera. Even more interestingly species and isolates of both genera are interspersed amongst each other e.g. New World/ Old World. Furthermore, there is little evidence of any biogeographic isolation. These findings suggest that dictyostelids, despite displaying different generic morphological features show only limited genetic polymorphism based on ITS 1 and ITS 2 sequencing results. Indeed Traub and Hohl (1976) pointed out that some *Dictyostelium* species possess a number of features in common with the genus *Polysphondylium* suggesting the need for a major dictyostelids revision and proposed a third intermediate genus. These findings of this study corroborate the need for revision. However, rather than two or three separate genera the sequencing results support one major genus with sub-genera to accommodate species sharing common features. Support for the single genus is via the relatively high retention

index (RI) of 0.8-0.9 for ITS 1 and 0.8 for ITS 2, which indicates that the analysed characters are not randomly distributed and so must carry at least a reasonable phylogenetic signal. This implies that the obtained and described ITS 1 and ITS 2 clades are not the result of artefact.

In the hope of obtaining an even better picture of the phylogenetic relationships of the dictyostelids, the ITS 1 and ITS 2 sequencing data considered previously as two separate partitions were combined and analysed together. The combined analysis should reveal a new, more reliable/ valid relationship, obtained by combining the strong signals of both partitions while cancelling out some of their phylogenetic noise. Combining the ITS 1 and ITS 2 analysis proved positive as it delivered better resolved consensus trees comparable to the ITS 1 consensus trees. The main *D. discoideum, P. violaceum, D. mucoroides* clades (obtained in separate analysis) were confirmed and indeed better resolved (see figures 6.15 to 6.25 CD). The position of the *D. purpureum* and three *Dictyostelium* (*D. aureostipes* BDW 4, *D. implicatum* EPN 1 and *D. mucoroides* EW 5) species were also confirmed. The combined analysis also confirmed the lack of any *Dictyostelium*/ *Polysphondylium* species/ isolates distinction both in terms of sequence and biogeographic origin.

In conclusion, the sequencing technique was reliable and powerful enough to reflect the relationships between closely related species. The results obtained by this technique only partly agree with those used so far for dictyostelids systematic analysis, while these results strongly suggest the need for the reclassification of dictyostelids, which is also supported by Schaap *et al.,* (2006) and Romeralo *et al.,* (2007). There is also a need for further work with more focus on *Polysphondylium* species than was performed in this project. New work could focus on other genes, for example α- or β- tubulin or calmodulin frequently used in molecular phylogenetic analysis.

4.3 Defining Dictyostelid genera: phylogenetic analysis of ISSR-PCR Profiles

Phylogenetic analysis of ITS 1 and ITS 2 sequences presented in the previous section showed that the sequencing technique used in this project to explore the phylogenetic relationship of dictyostelids proved sufficiently reliable and powerful to reflect the phylogenetic position between closely related dictyostelid species. The results from these phylogenetic analyses disagree with contemporary established dictyostelids phylogeny, especially the genus *Dictyostelium*, which is currently strictly separated from the genus *Polysphondylium* on the basis of morphological features. The phylogenetic analyses also show that neither *Dictyostelium* or *Polysphondylium* were reconciled as discrete generic clades but that *Dictyostelium* species are often interspersed amongst *Polysphondylium* species. This would suggest the lack of a valid distinction between *Dictyostelium* and *Polysphondylium*, as neither genus was resolved as discrete, i.e. monophyletic. The ITS sequencing results also suggest limited genetic polymorphism of dictyostelid species, despite the fact that they display different morphological features, and therefore would suggest the need for the reclassification of current dictyostelid systematics.

However, to assess further the validity of the above findings it would be appropriate to confirm the results with those obtained by an alternative technique. ISSR-PCR fingerprinting was chosen as an alternative technique as it is a rapid and reproducible technique (described in more detail in chapter 1) as well as capable of discriminating genetic variability at both the intra- and inter-species levels, a key issue in this project. The following section will discuss results obtained by this technique applied to 49 *Dictyostelium* and *Polysphondylium* species/ isolates.

4.3.1 PAUP analysis of ISSR-PCR profiles

The composed data matrix (see appendix VI**),** comprises 60 characters (polymorphic bands produced with four different primers) and analyzed by PAUP. 49 species/isolates, representing both *Dictyostelium* and *Polysphondylium*, were scored for presence/absence of the polymorphic bands. The presence of the band was given a score 1 and the absence of the band scored 0, giving the profile based on binary system e.g. 1001110000. Two species, *Acytostelium* (Protostelidae) and *Amoeba proteus* were used for outgroup-rooting. The data were analysed using a heuristic maximum parsimony (MP) search implemented in PAUP 4.0b4a (Swofford, 1999). The most parsimonious trees (MPTs) were estimated using 10,000 random additions each, holding 10 trees at any one time. Each MPT was then used as a starting tree for TBR (tree-bisection reconnection) branch swapping algorithm, saving only one tree at any one time and with 'maxtrees' set as unlimited. Finally the re-weighting strategy of Quicke *et al*., (2001) was used to search for shorter trees. The search strategy allowed a large area of the tree-scape to be explored and gave a good chance of finding potential multiple islands of MPTs (Maddison, 1991; Quicke *et al*., 2001). To get a subset of more resolved trees by limiting the number of most parsimonious trees the successive approximations weighting strategy was then used with the maximum value of Retention Index (RI) as a re-weighting function (Farris, 1989; Carpenter, 1994; Quicke, 1999; 2001; 2003).

The initial phylogenetic analysis of the data obtained with four primers for 49 dictyostelid species and isolates together with two outgroups, *Acytostelium* sp. and *Amoeba proteus* using 100 random additions, holding only one tree, resulted in three equally parsimonious trees of length 274. The two outgroups were not recovered together: *Amoeba proteus* appeared deep within the slime mould tree when *Acytostelium* was used for rooting. Therefore, *Amoeba proteus* was excluded from further analysis, as it may distort relationships among the in-group taxa, because it is phylogenetically too distant.

The heuristic search yielded 270 most parsimonious trees (MPTs) of length 272, but the strict consensus tree is not well resolved (figure 4.20). Subsequent branch swapping (TBR) on each of the 270 trees in turn, revealed two separate islands of 252 and 16 MPTs. More resolved strict consensus trees constructed from each of these islands are shown in figures 4.21 and 4.22.

Successive approximations weighting (SAW) was used to obtain more fully resolved trees, implementing the retention index (ri) maximum value as a re-weighting function as recommended by Quicke *et al* (2001). Stability was reached after the second round of re-weighting, which yielded 5 trees, and the strict consensus tree of these 5 trees is shown in figure 4.23. Application of SAW to the second 16 trees island yielded a single preferred tree (figure 4.24). The consensus tree obtained from this set of 5 trees shows all *D. mucoroides* isolates (except *D. mucoroides* EW5) in one clade forming a sister group to *D. brefeldianum,* two unidentified *Dictyostelium* (WLD and HLM) two isolates of *D. sphaerocephalum.* However, the position of unidentified *Dictyostelium* HLM was changed in the consensus tree derived from the SAW of island of 16 trees where it was placed as an unresolved basal branch. Moreover, the position of *D. mucoroides* EWS 5 isolate was different in both consensus trees (see figure 4.23), as it formed a sister group with unidentified *Dictyostelium* BDW and *D. aureo-stipes* BDW in the first consensus tree, while the second single tree obtained from the application of SAW to the second island placed *D. mucoroides* EW5 as an unresolved basal branch (see figure 4.24). Interestingly two *Dictyostelium discoideum* isolates (NC4 and V12) formed a sister group with *D. tenue, Polysphondylium pallidum* and with some other species of *Dictyostelium* such as *D. citrinum*, *D. citrinum/ D. discoideum* or *D. rosarium*, from different parts of America. Also the *Polysphondylium* spp. for example *P. violaceum,* and especially its four different isolates (RTN, TDN, STR, FCM) are grouped together in one clade, while another 3 ATN *P. violaceum* isolates **(**see table 2.1**;** *P.violaceum* ATN/1,11/2, culture number 3) showed a difference in its position in both consensus trees. The *P. violaceum* ATN isolate (culture 38; see table 2.1**)** was unresolved in both consensus trees. Although some *P. violaceum* isolates, representing the second smaller genus of *Polysphondylium* are grouped together, the trees clearly show that all fall between the *Dictyostelium* species. The outcome from the present analysis has also shown that

there is no strict distinction between U.K. and American *Dictyostelium* cultures. A similar trend occurs in the strict consensus of 252 trees of the first island and that of 16 from the second island in that both show similar groupings and lack of *Dictyostelium* or *Polysphondylium* monophyly, though both were much less resolved. However, the strict consensus tree constructed of six trees from both 252 SAW and 16 SAW islands delivered only a partly resolved tree (see figure 4.25), which represents a resolution of only 33 of the 50 species and isolates considered. The remaining 17, including all *D. mucoroides* isolates, remained unresolved.

Figure 4.19 Strict Consensus tree from the final 270 MPTs (length 272

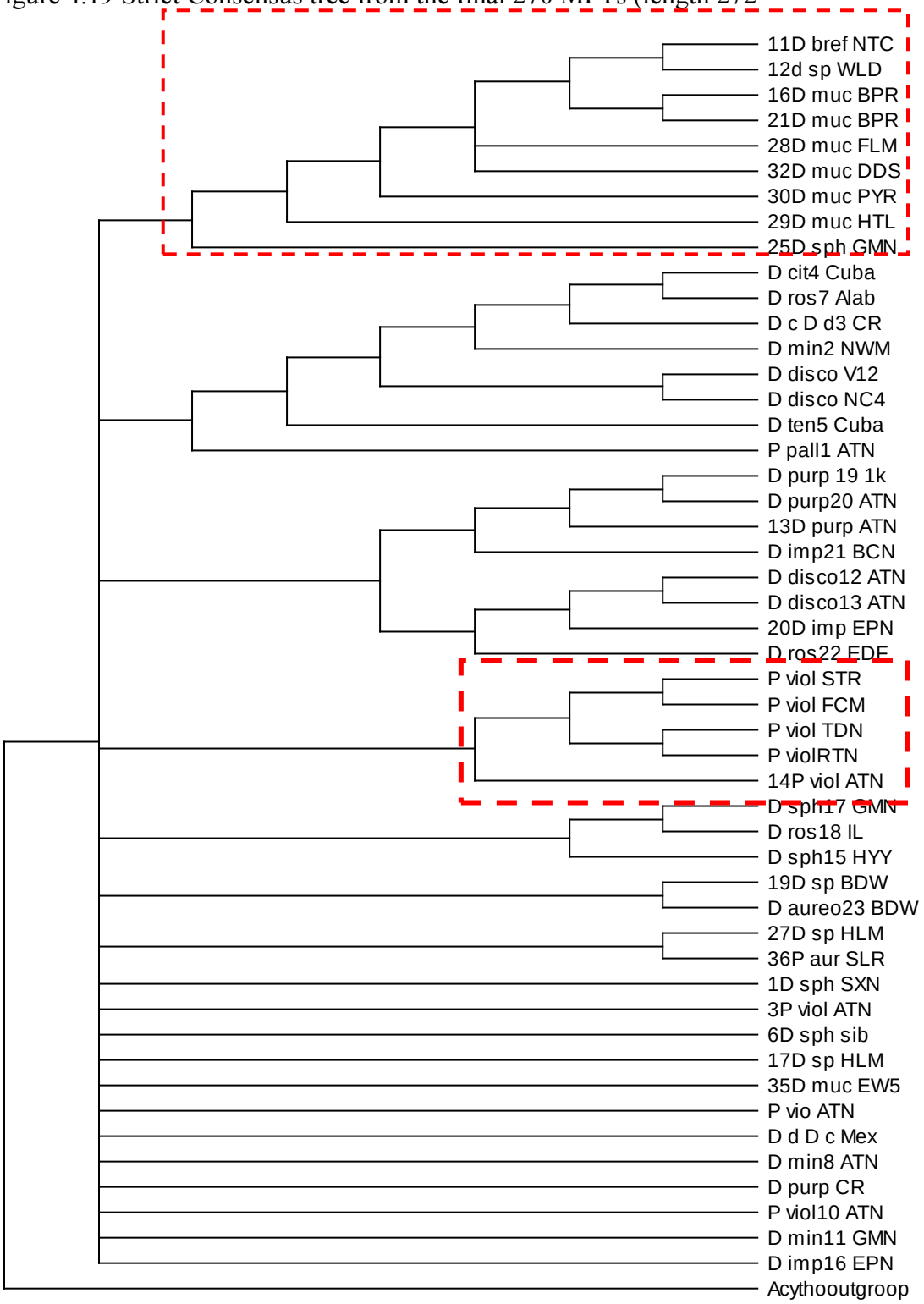


Figure 4.20 Strict Consensus of 252 MPTs island

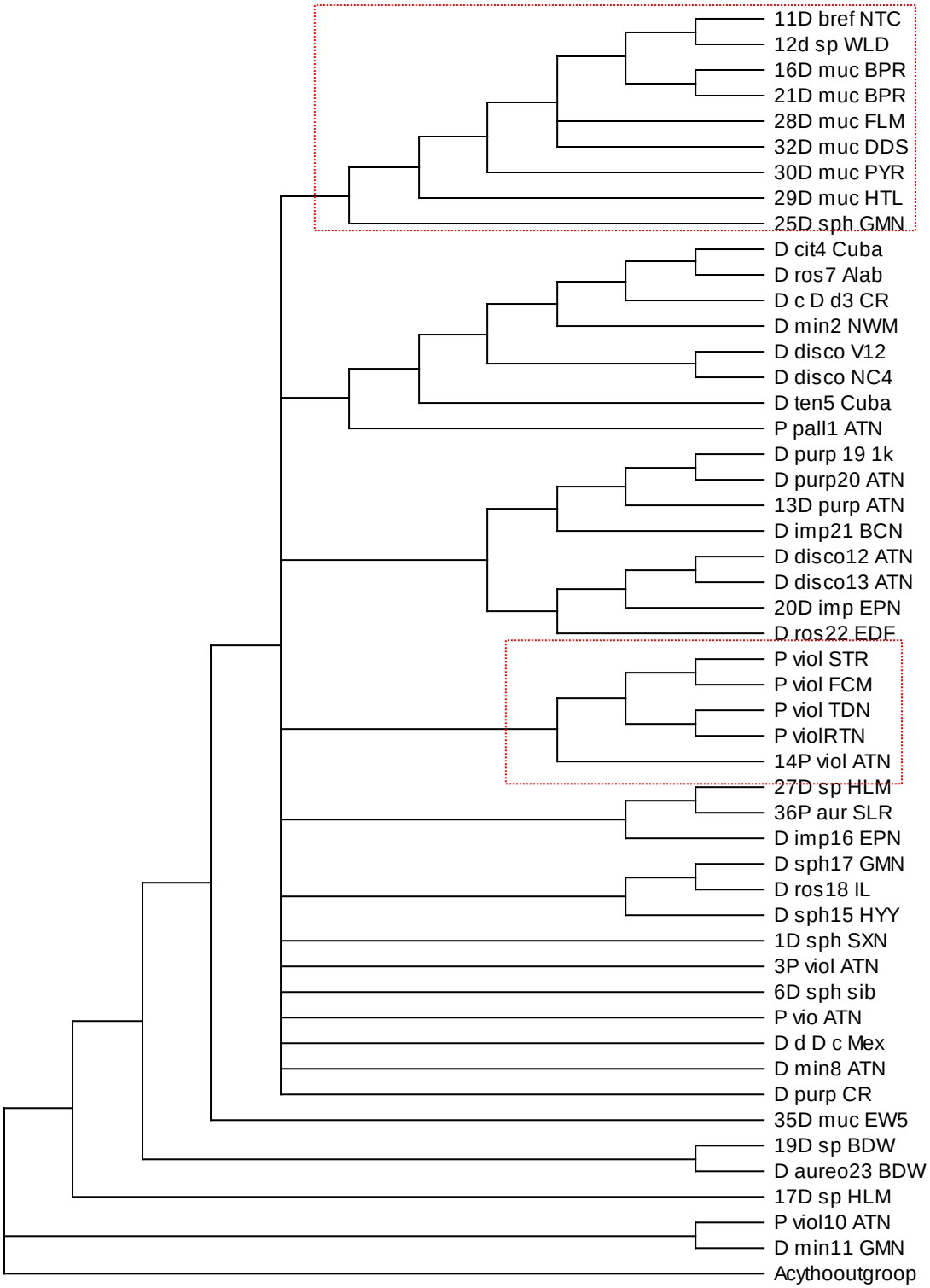


Figure 4.21 Strict Consensus of 16 MPTs island (final 270)

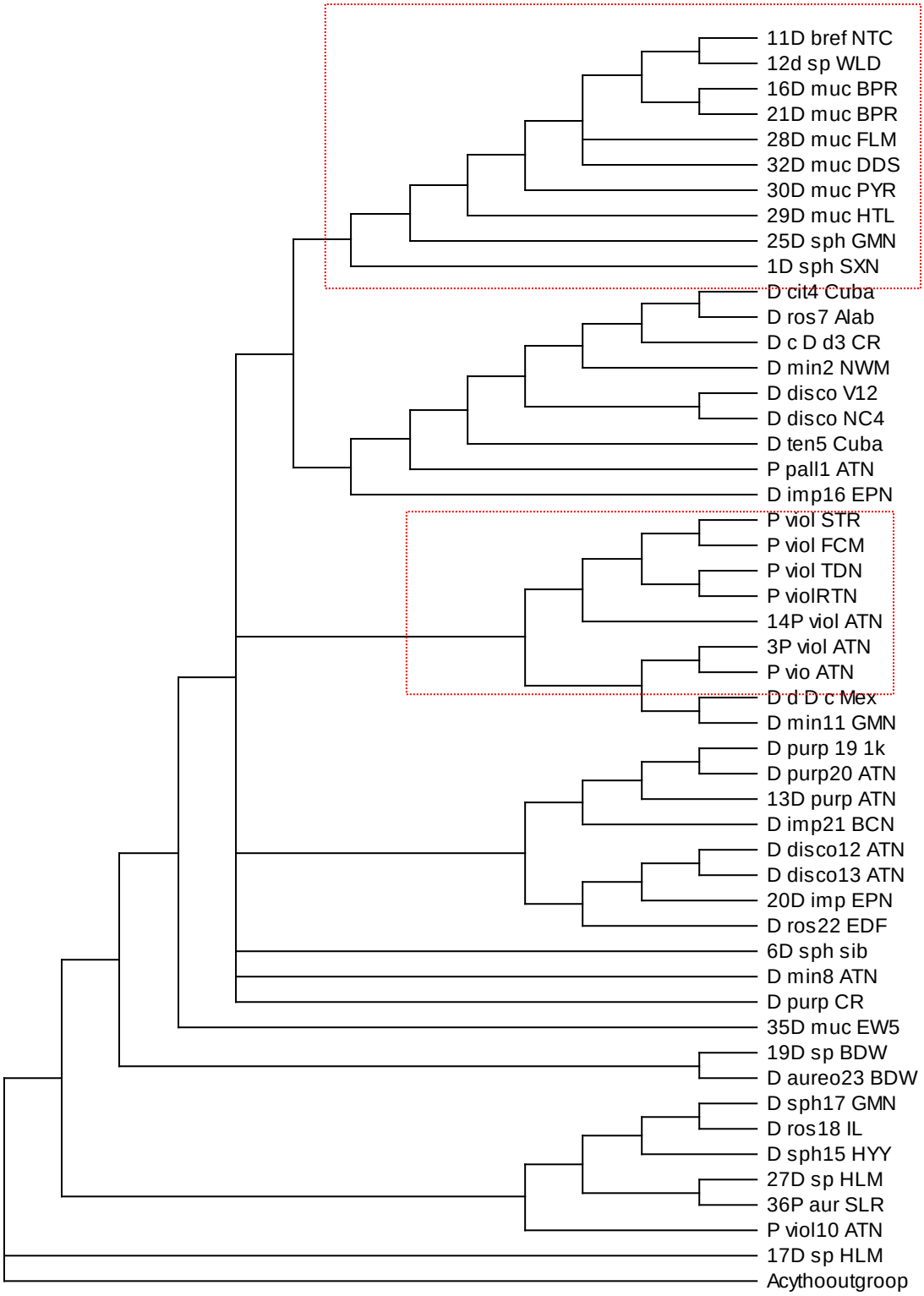


Figure 4.22 Strict Consensus tree of SAW applied to 252 MPTs island

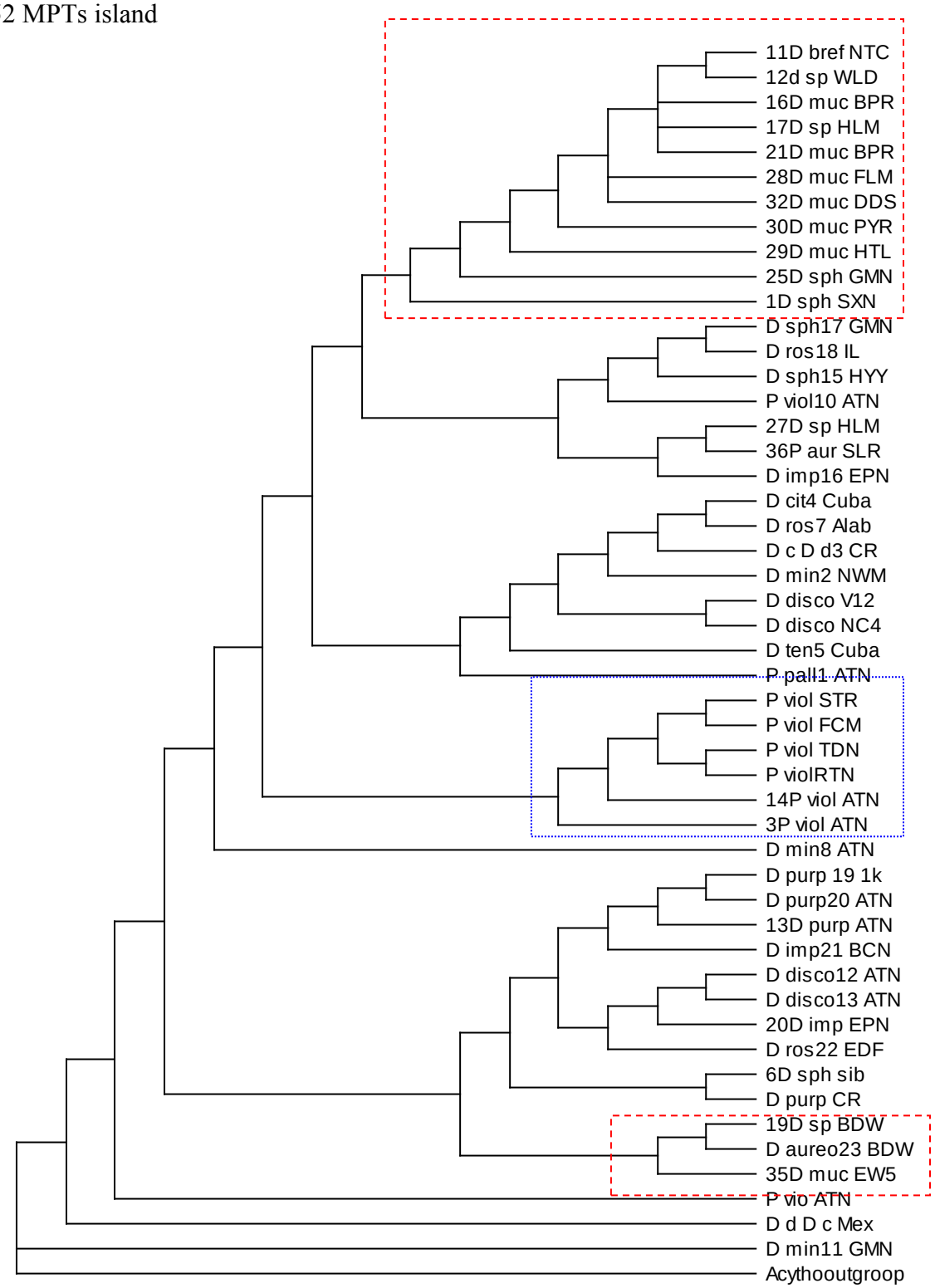


Figure 4.23 Strict Consensus tree of SAW applied to 16MPTs island.

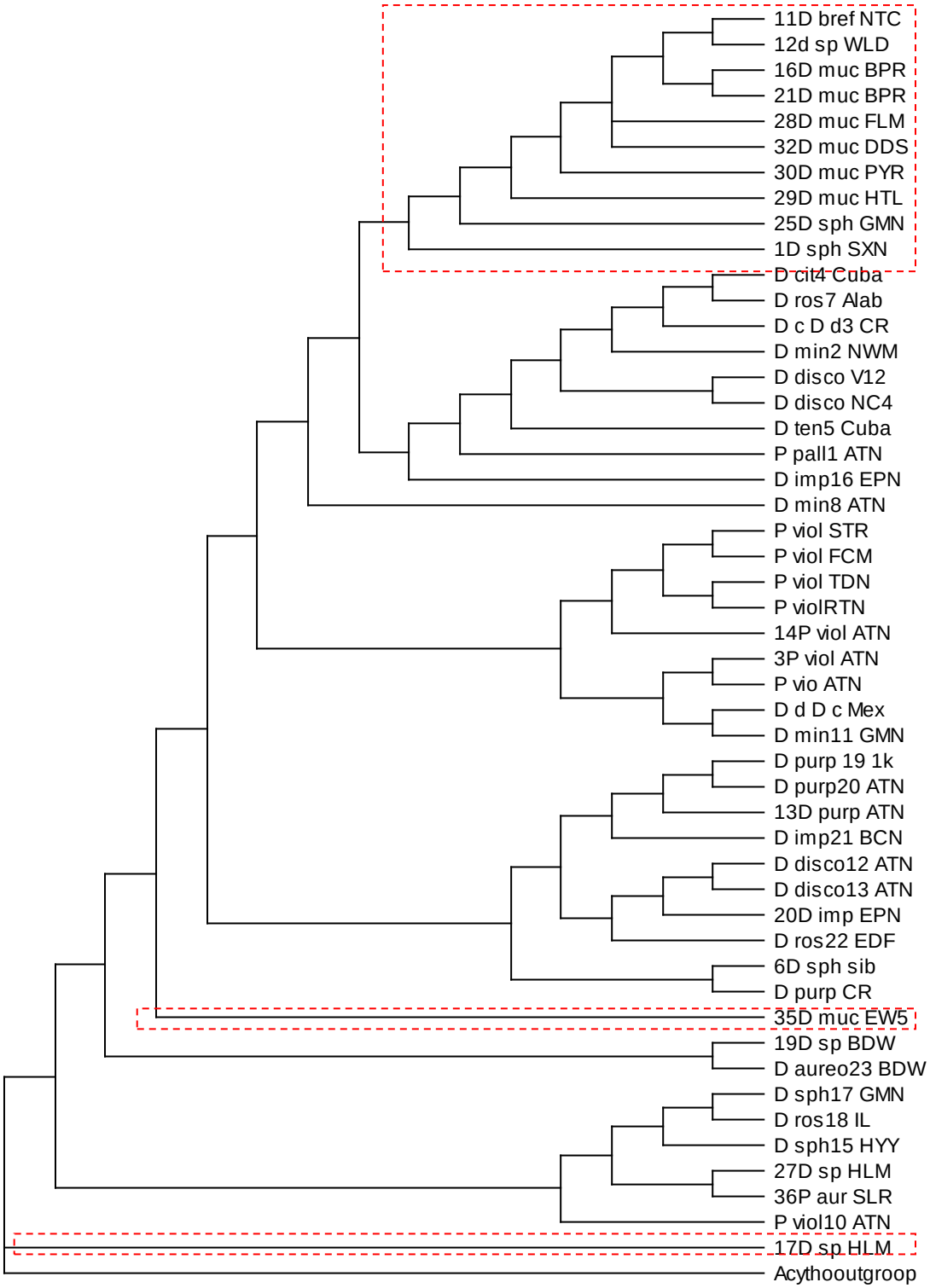
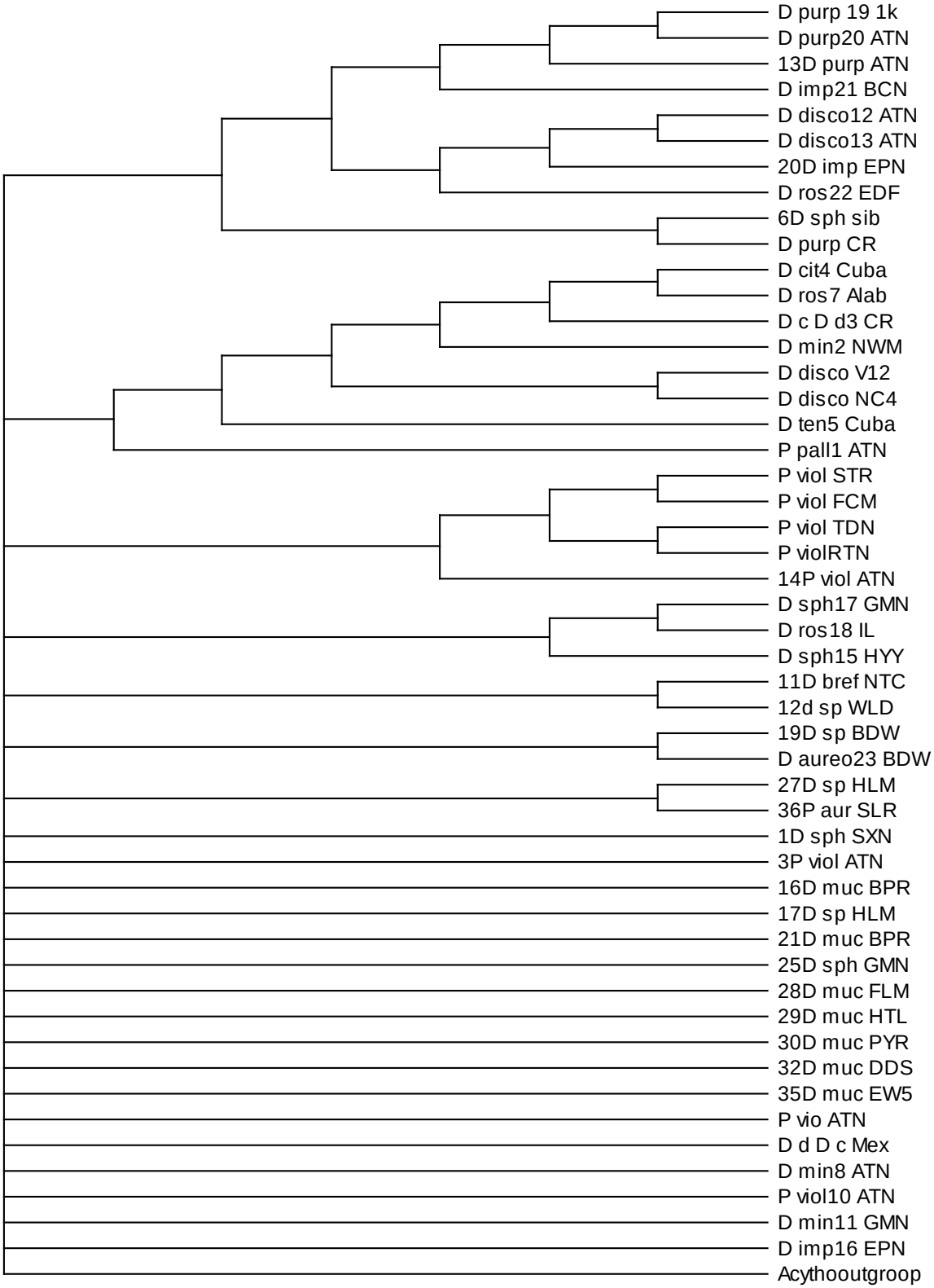


Figure 4.24 Strict Consensus of 6SAW trees.



4.4 Using ISSR-PCR Profiles to Identify Dictyostelid Species/ Isolates

4.4.1 ISSR-PCR fingerprint profile of 27 U.K. Dictyostelid species and isolates

One of the assumptions of ISSR-PCR is that each primer amplifies different target sites and therefore produces different electrophoretic profiles which would vary between different species or even isolates. The following pages focus on the obtained ISSR-PCR profiles obtained in this study and discuss the suitability of using ISSR-PCR to identify unknown, newly isolated dictyostelid species and isolates. As these analyses were performed much earlier in this study, before the New World species and their data were available, therefore, only U.K. species were used and discussed here.

The ISSR-PCR profiles generated by four ISSR-PCR primers were used to compare the relatedness of dictyostelid species and isolates. Individual band presence (1) or absence (0) was scored for each species and isolate with each primer. Only bands present in all three separately performed PCR amplifications, were scored and analysed. Only four of the eight primers (table 2.3) initially examined produced reproducible bands which may, therefore be useful for inter and intra-specific differentiation. Primer one produced 16 polymorphic bands, primer three 18 bands, primer five 14 and primer six 12. Since these four of the original eight primers tested for the production of reproducible bands, generated sixty polymorphic bands, this was considered sufficient for phylogenetic analysis and species identification, and no further primers were screened.

The four primers that proved unsatisfactory for ISSR-PCR were excluded for various reasons. Primer eight, a di-nucleotide AT repeat primer did not perform adequately in all species, producing non-resolvable bands. This poor resolution probably resulted from the extremely A&T rich (ca. 75%) dictyostelids genome. Thus the A&T base sequence used to design the primer probably represented a common genome sequence, which thus resulted in difficult to score multiple bands. Primers two and four tended to produce a different band pattern each time or failed to produce any bands. Primer seven produced a clear banding pattern for American derived species

but with some English species amplified poorly, producing faint often non-repeatable PCR products. Although characteristic band patterns were recognisable, some for particular species and isolates were not reproducible so this primer was also excluded from use.

The electrophoretic profile obtained with the chosen four primers showed that some species and isolates groupings are clearly apparent from directly examining the electrophoresis banding pattern. As primer five and six produced particularly clear patterns, this section will focus on profiles obtained with these two primers highlighting some of the clear species discriminations provided by electrophoresis.

All electropherograms obtained for primer 5 and 6 with the exemplar 27 English species and isolates showed easy to identify banding patterns. The first profiles considered are for primer 5 and consists of two electropherograms (figures 4.26a and 26b), the first one consisting of 14 species and isolates the second the remaining 13 species and isolates. In the first electropherogram, profiles in lanes 1 and 2, (figure 4.26a; table 4.6a) share identical banding patterns comprising three clear bands B, F and H. These belong to two isolates (V12 and NC4) of *Dictyostelium discoideum,* of which NC4 is the wild isolate. Band F is also shared by *D. mucoroides* (lane 21; figure 4.26b). The two *D. discoideum* isolates also share band H with an unidentified species of *Dictyostelium* (lane 12, figure 4.26a) and with four isolates (STR, FCM, TDN, RTN) of *Polysphondylium violaceum* in lanes 24 - 27 (figure 4.26b). These *P. violaceum* isolates represent another group of cultures, which showed characteristic and nearly identical band-patterns. Although, their banding pattern is very similar, it differs from that of other *P. violaceum* isolates e.g. lane 23 (figure 4.26b) or lanes 4 and 9 (figure 4.26a). No *P. violaceum* banding pattern is shared by any other *Polysphondylium* species e.g. *P. aureum* in lane 22 (figure 4.26b).

Figure 4.26a ISSR-PCR electropherogram using primer 5 [DD(CCA)$_5$] (from the left**)** lane 0, 100 bp marker; lanes 1-14, dictyostelid species /isolates as shown in table 4.4.1a

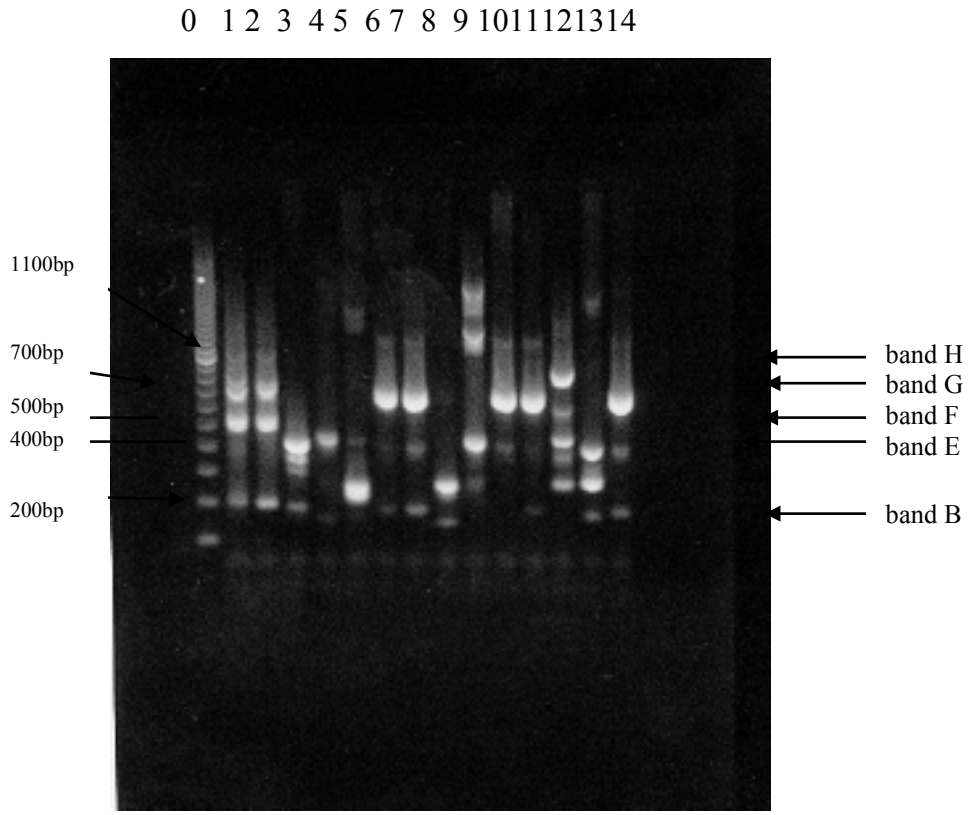


Table 4.4. 1 a Dictyostelid Species/ Isolate Identification for Figure 4.26a

lane	Species /isolates	lane	Species /isolates
1	***D. discoideum* V12 (10)**	8	***D. purpureum* ATN/1,7/1 (13)**
2	***D. discoideum* NC4 (37)**	9	***P. violaceum* ATN /1,8/2 (14)**
3	***D. sphaerocephalum* SXN/2.1/1 (1)**	10	***D. mucoroides* BPR/3,1/21 (16)**
4	***P. violaceum* ATN/1.11/2 (3)**	11	***D. species* HLM/4,2 (17)**
5	***D. sphaerocephalum* Sib (6)**	12	***D. species* BDW /1,1/2 (19)**
6	***D. brefeldianum* TNC115 (11)**	13	***D. implicatum* EPN/1,1/ (20)**
7	***D. species* WLD/1 (12)**	14	***D. mucoroides* BPR/1,1/1 (21)**

Numbers in brackets represent culture number (see table 2.1)

Figure 4.26b ISSR-PCR electropherogram using primer 5 [DD(CCA)$_5$] (from the left**)** lane 0, 100 bp marker; lanes 15-27, dictyostelid species /isolates as shown in table 4.4.1b

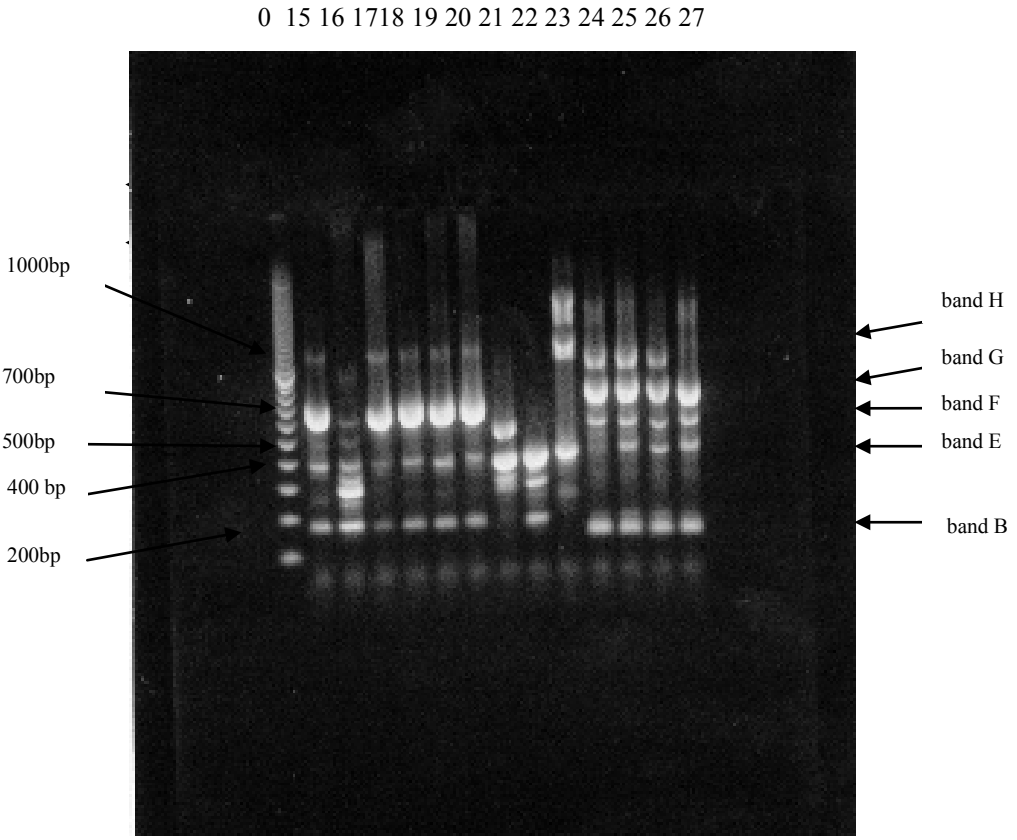


Table 4.4.1b Dictyostelid Species/ Isolate Identification for Figure 4.26b

lane	Species/isolates	lane	Species/isolates
15	***D.sphaerocephalum*** GMN/3,1) (25)	**22**	***P. aureum*** SLR/1,1/1(36)
16	***D. species*** HLM/4,1/1 (27)	**23**	***P. violaceum*** ATN (38)
17	***D. mucoroides*** FLM/1,1/1 (28)	**24**	***P. violaceum*** STR (39)
18	***D. mucoroides*** HTL/2,1/1 (29)	**25**	***P. violaceum*** FCM (40)
19	***D. mucoroides*** PYR/3,1/20 (30)	**26**	***P. violaceum*** TDN (41)
20	***D. mucoroides*** DDS/2,1 (32)	**27**	***P. violaceum*** RTN (42)
21	***D. mucoroides*** EW5,2,7 (35)		

Numbers in brackets represent culture numbers (see table 2.1)

Figure 4.27a ISSR-PCR electropherogram using primer 6 [DHB(CGA)$_5$], (from the left) lanes 0, 100bp marker; lanes 1-13, dictyostelid species/isolates as shown in table 4.4.2a

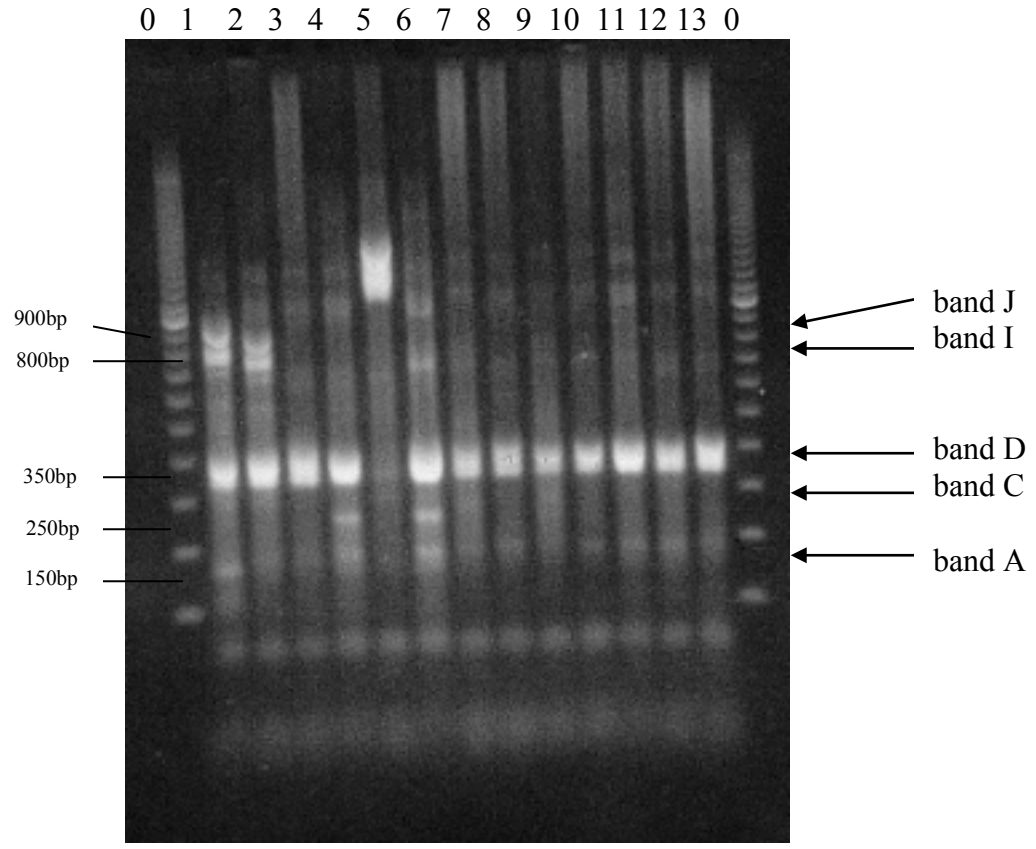


Table 4.4.2a Dictyostelid Species/ Isolate Identification for Figure 4.27a

Lane	Species / isolates	Lane	Species /isolates
1	***Dictyostelium discoideum* V12 (10)**	8	***D. species* HLM/4.2 (17)**
2	***D. discoideum* NC4 (37)**	9	***D. species* BDW/1,1/2 (19)**
3	***D. sphaerocephalum* SXN/2.1/1 (1)**	10	***D. implicatum* EPN /1,1/ (20)**
4	***Polysphondylium violaceum* ATN/1,11/2 (3)**	11	***D. mucoroides* BPR/1,1/1 (21)**
5	***D. sphaerocephalum* Sib (6)**	12	***D. mucoroides* PYR/3,1/20 (30)**
6	***P. violaceum* ATN/1,8/2 (14)**	13	***D. mucoroides* DDS/2,1 (32)**
7	***D. mucoroides* BPR/3,1/21 (16)**		

Numbers in brackets after species/isolates name and code are cultures' numbers

Figure 4.27b ISSR-PCR electropherogram using primer 6 [DHB(CGA)$_5$] (from the left) lanes 0, 100 bp marker; lanes 14-26, species/isolates are shown in table 4.4.2.b

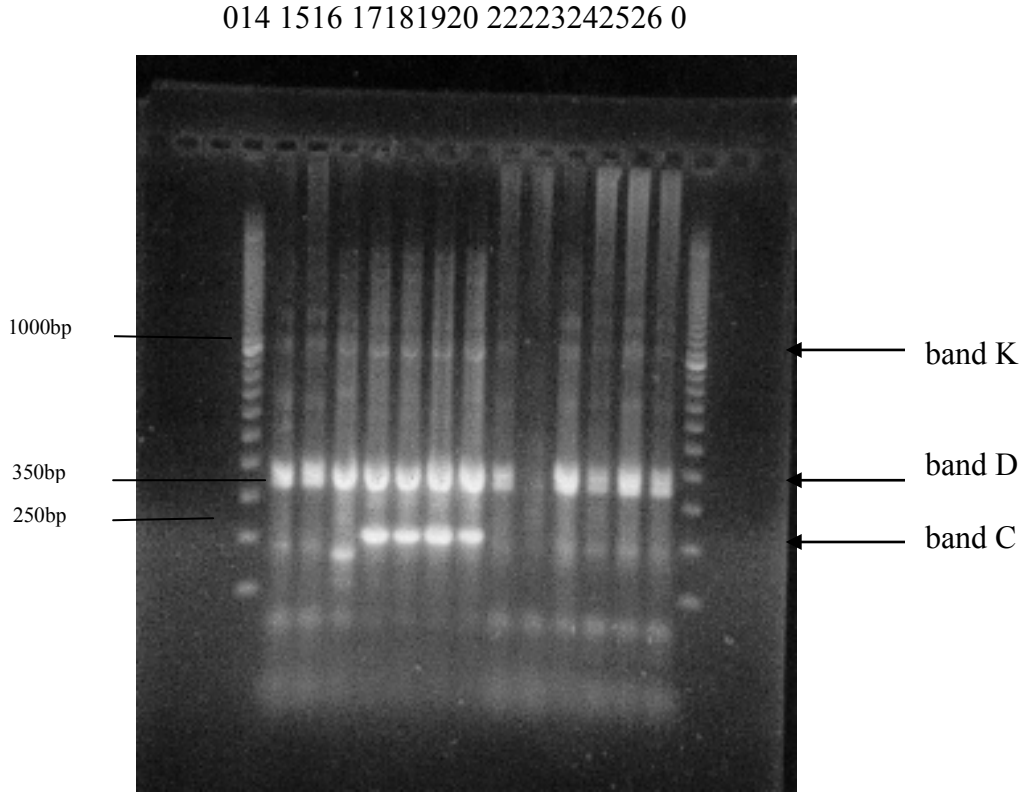


Table 4.4.2b Dictyostelid Species/ Isolate Identification for Figure 4.27b

lane	Species /isolates	lane	Species/isolates
14	***D. mucoroides* EW 5,2,7,92 (35)**	22	***D. species* WLD/1 (12)**
15	***P. aureum* SLR/1,1/1 (36)**	23	***D. sphaerocephalum*GMN/3,1 (25)**
16	***P. violaceum* ATN (38)**	24	***D. species* HLM/4,1/1 (27)**
17	***P. violaceum* STR (39)**	25	***D. mucoroides* FLM/1,1/1 (28)**
18	**P. *violaceum* FCM (40)**	26	***D. mucoroides* HTL/2,1/1 (29)**
19	***P. violaceum* TDN (41)**		
20	***P. violaceum* RTN (42)**		
21	***D. brefeldianum* TNC 115 (11)**		

Numbers in brackets as above

The same banding pattern is also seen in lanes 6, 7, 10, 11 and 14 (Figure. 4.26a); where all five profiles are produced by different *Dictyostelium* species while another, well distinguished grouping occurs in lanes 15, 17, 18, 19 and 20 (Figure. 4.26b) and in lanes 24, 25, 26 and 27 (Figure 4.26b) identifed as *P. violaceum* (STR, FCM, TDN and RTN). All show three, B, E, G sharp bands suggesting, that they may represent the same species. In fact lanes 17-20 were confirmed as four different *D. mucoroides* isolates and the culture in lane 15 as *D. sphaerocephalum.* Moreover, two other *D. mucoroides* isolates in lanes 10 and 14 shared the E band, as did lanes 3, 4, 7, 9, belonging to two different *Dictyostelium* species (lanes 3, 7) and two different *P. violaceum* isolates (lanes 4, 9). What was noticeable was that some bands, for instance B or E, seemed to be present in many species and isolates of *Dictyostelium* and *Polysphondylium* genera. The culture in lane 5 (figure 4.26a) by contrast, showed a unique pattern of two very strong bands close to each other, which are not shared by any other species of *Dictyostelium* or *Polysphondylium*, and this culture represents *D. sphaerocephalum* from Siberia (see Table 2.1). A summary of banding patterns produced by bands B, E, F, G and H with primer 5 is shown in Table 4.5.

Primer 6, by contrast, with its high CG content, produced profiles of fewer bands and less background noise than the other working primers. However, the banding patterns obtained for this primer (Figures 4.27a & b) also showed some characteristics that allowed some grouping. One strong band D and a weaker band A were common for all species and isolates of both *Dictyostelium* and *Polysphondylium*. As with primer 5 (Figure 4.26a), both isolates NH4 and V12 of *D. discoideum* (see lanes 1 and 2 in Figure 4.27a) showed a similar band patterns. These isolates also shared two other exclusive bands, I and J, which were absent in all other species and isolates. Four *P. violaceum* isolates (STR, FCM, TDN, RTN) (lanes 17-20; Figure 4.27b) formed another group, sharing the same banding pattern with exclusive weaker band K. Their band C was also shared by two other *P. violaceum* isolates (lanes 4, 6; Figure 4.27 a), forming a third group with a similar banding pattern. Amplification with primer 6 also revealed a unique band pattern for the lane 5 culture (Figure. 4.27 a) represented by *D. sphaerocephalum* from Siberia, thus mirroring the results obtained with primer 5. For the summary of grouping on the basis of banding patterns for primer 6 see Table 4.6.

Table 4.5 A summary of the banding pattern for ISSR primer 5 (presence of a band is represented by '+'; absence by '–')

Species/ isolate	Lane	Band B	Band E	Band F	Band G	Band H
D.discoideum NC4	1	-	-	+	-	-
D.dicoideum V12	2	-	-	+	-	-
P.violaceum	4	-	+	-	-	-
P.violaceum	9	-	+	-	-	-
P.violaceum	23	-	-	-	-	-
P.violaceum	24	+	+	-	+	+
P.violaceum	25	+	+	-	+	+
P.violaceum	26	+	+	-	+	+
P.violaceum	27	+	+	-	+	+
P.aureum	22	-	-	-	-	-
D.mucoroides	17	+	+	-	+	-
D.mucoroides	18	+	+	-	+	-
D.mucoroides	19	+	+	-	+	-
D.mucoroides	20	+	+	-	+	-
D.mucoroides	21	-	-	+	-	-
D.mucoroides	10	+	+	-	+	-
D.mucoroides	14	+	+	-	+	-
D.sphaerocephalum	3	-	+	-	-	-
D.sphaerocephalum	15	+	+	-	+	-
D.brefeldianum	6	+	+	-	+	-
D.species	7	+	+	-	+	-
D.species	11	+	+	-	+	-
D.species	12	-	-	-	-	+

Table 4.6 A summary of the banding pattern for ISSR primer 6 (presence of a band is represented by '+'; absence by '–')

Species/ isolates	Band A	Band C	Band D	Band I	Band J	Band K
P.violaceum STR	+	+	+	-	-	+
P.violaceum FCM	+	+	+	-	-	+
P.violaceum TDN	+	+	+	-	-	+
P.violaceum RTN	+	+	+	-	-	+
P.violaceum lane 4	+	+	+	-	-	-
P.violaceum lane 6	+	+	+	-	-	-
D.dictyostelium NC4	+	-	+	+	+	-
D.dictyostelium V12	+	-	+	+	+	-

4.5 Discussion

ISSR-PCR profiling was used to reassess the classification of the dictyostelids following its successful use in resolving fungal intra-specific and inter-specific relationships (Zietkiewich *et al*., 1994; Muthumeenakshi *et al*., 2001). The identification and taxonomy of dictyostelids has proven to be a challenging task, especially if focused only on morphological characters or cultural and biochemical characters of the asexual life cycle. The task is often even more difficult when closely related species are considered, especially if those isolates are from similar habitats. The analysis of ISSR-PCR profiles demonstrated that it is a reliable and efficient technique for differentiation of closely related species. In the context of this study, ISSR-PCR data showed clear banding pattern similarities between different isolates of what appeared to be the same species e.g. the four isolates of *P. violaceum* (RTN, TDN, FCN and STR). Moreover, this marker system was also able to identify putative homologous bands between species e.g. band D (figure. 4.27a & b) or H (figure 4.26b). Some 42 of 51 species and isolates showed the presence of band D and 7 of 51 showed band H. In each case the bands were shared by both *Dictyostelium* and *Polysphondylium* species and isolates. Probably the most important outcome of this work was the finding that this DNA based marker system also could not differentiate the genus *Dictyostelium* from *Polysphondylium*. Many bands were shared by a number of *Dictyostelium* and *Polysphondylium* cultures and none were shown to be diagnostic of either *Dictyostelium* or *Polysphondylium*. In fact, all strict consensus trees showed that both *Dictyostelium* and *Polysphondylium* were consistently grouped together, as were different isolates of *P. violaceum* or *D. discoideum*. Thus banding patterns also elucidated inter-specific differences. Greater distinction, in terms of banding pattern was in fact found between groups of different isolates of the same *Dictyostelium* or *Polysphondylium* species (e.g. isolates NC4 and V12 of *D. discoideum* or STR, FCM, TDN, RTN isolates of *P. violaceum*) than between species from the two genera. Interestingly, there was also no clear distinction between English and American cultures, which could indicate that genetic polymorphism is generally limited in the dictyostelids and thus, what little genetic variation there is, does not relate to either phenotype or place of origin. A possible explanation for such low level polymorphism could be that dictyostelids mostly reproduce asexually. Thus, these

findings clearly indicate that a preliminary grouping of species on the basis of the ISSR- PCR profiling is not only possible but may also be useful as a basis for developing a tool for an identification of unknown soil-dwelling dictyostelid species.

Furthermore, parsimony analysis strongly demonstrated that neither *Dictyostelium* nor *Polysphondylium* are monophyletic. All consensus trees, including those obtained from two islands of 252 and 16 trees, as well as the consensus trees obtained from these two islands further subjected to improved-resolution SAW (weighting) strategy, delivered an essentially similar picture. All show similar grouping of different isolates of the same species e.g. *P. violaceum* or *D. discoideum*. However, the position of some different isolates or species differed on different consensus trees. For example, the position of *D. sphaerocephalum* from Siberia was unresolved in the first two consensus trees (figure 4.20 and 4.21) as was the position of *D. mucoroides* EW5. Yet the consensus tree obtained from the first 252 islands subjected to the SAW strategy (figure 4.23) placed *D. sphaerocephalum* Sib together with *D. purpureum* from Costa Rica and *D. mucoroides* EW5 with *D. auro-stipes* BDW and an unidentified species of *Dictyostelium* BDW. However, in the consensus tree obtained from the second island subjected to SAW (figure 4.24), the position of *D. sphaerocephalum* Sib is the same but that of *D.mucoroides* EW5 was separated from the *D. auro-stipes* BDW, which now forms a sister group with unrecognised *Dictyostelium* species. The results from SAW analyses seem to indicate that there are difficulties with the establishing the taxonomic position of the *D. sphaerocephalum* Sib, *D. mucoroides* EW5 or *D. auro-stipes* BDW species on the family tree. As shown above their taxonomic status was unclear in all SAW analyses. The taxonomic status of some other species was left unresolved in all consensus trees.

Ideally, characterisation of dictyostelid species, and therefore their systematics, should consider all aspects of their morphology, development and growth. However, dictyostelid systematics is currently largely based on alpha morphology such as sorocarp dimension, sorus pigmentation and sorophore branching patterns. These characters have been used to differentiate species belonging to the two principal genera of cellular slime moulds, *Dictyostelium* and *Polysphondylium*. Furthermore, there are morphological difficulties in distinguishing *D. mucoroides* from *D. sphaerocephalum* which both display closely related white sori, indeed there is

dispute as to whether *D. sphaerocephalum* is a separate species from *D. mucoroides*, or whether *D. sphaerocephalum* is a synonym of *D. mucoroides* (Hagiwara, 1983). My findings seem to confirm these difficulties in distinguishing both species mentioned above, as both consensus trees obtained from SAW strategy showed that there are noticeable differences connected with the positions of *D. sphaerocephalum* isolates. Not all isolates of this species were consistently grouped together, indeed while, some *D. sphaerocephalum* isolates were grouped with *D. mucoroides* others such as for example Siberian *D. sphaerocephalum* was either grouped with *D. purpureum* from Costa Rica or left unresolved. The *D. sphaerocephalum* HYY isolate was consistently grouped with *D. rosarium*.

Moreover, the aptly named *D.citrinum* produces yellow sori while *D. discoideum* generally produces white to cream sori but under some not fully explained conditions, the latter may also produce bright yellow sori (Raper, 1984). The presented consensus trees showed that not fully resolved cultures such as *D. discoideum /D. citrinum* or *D. citrinum/D. discoideum* (both from America) were neither grouped together nor close to *D. discoideum* as may be expected from morphological criteria. A similar situation occurred in other species, e.g. the ATN isolate of *P. violaceum*, which showed a distinct banding pattern and was never grouped together with other *P. violaceum* isolates such as STR or TDN, which morphologically, all appear to represent the same species. Therefore, my findings could suggest that isolates which failed to group together (as expected in the case of different conspecific isolates) may indeed represent some sort of "morphospecies" composed of heterogenous entities and therefore, may probably be mixtures of cryptic species. In addition, the fact, that all obtained consensus trees (despite their differences in terms of resolution) showed both *Dictyostelium* and *Polysphondylium,* may be interpreted as a confirmation that neither of the SAW trees showed *Dictyostelium* or *Polysphondylium* to be a monophyletic culture and therefore, implying neither represent valid genera.

The appropriateness of ISSR-PCR, as indeed of any DNA fingerprinting technique for phylogenetics, depends upon its ability to clearly reflect species relationship (Soll, 2000). ISSR-PCR appears to achieve this criterion. The ISSR-PCR profiles as well as PAUP analysis showed that it is quick and reliable technique, able to differentiate closely related species and therefore resolve their phylogenetic relationships.

However, when ISSR-PCR profiles are considered caution should be taken to the homology of bands.

4.5.1 Conclusions

The primary aim of this section was to test the suitability of ISSR-PCR markers for establishing the phylogenetic relationship between closely related species of two dictyostelid genera. Phylogenetic analysis provided consistent evidence showing that this technique is suitable for the preliminary grouping of taxa. My findings also suggest that ISSR-PCR profiling is sufficiently powerful for resolving the phylogeny of the dictyostelid taxa, however detailed phylogenetic resolution between and within the taxa to be fully achieved would need much more work with differently designed primers in use.

CHAPTER 5: GENERAL DISCUSSION

5.0 Reflection on the results obtained by ITS 1 and ITS 2 Sequencing and ISSR-PCR

The Department of Life Sciences at Anglia Ruskin University has had a long-standing interest in the dictyostelids or cellular slime moulds which has resulted in a number of research projects, mainly focused on the isolation and characterisation of new dictyostelids edaphic species. These research projects identified two main systematic problems; first, the accurate classification of a large proportion of isolates presently included in the *Dictyostelium mucoroides* complex; second, questioning of the validity of the *Dictyostelium* /*Polysphondylium* generic boundary. These concerns have prompted the application of molecular techniques to dictyostelid systematics, described in this thesis. The aim was to use PCR-based techniques and the application of appropriate phylogenetic methods to explore the relationship between *Dictyostelium* and *Polysphondylium* species. Two different techniques sequencing of two ITS regions of rDNA and ISSR-PCR, were employed for this study.

There have been few attempts to apply modern molecular techniques to the classification of slime moulds; one based on analysis of generic and species specific amino acid composition detected via a high-resolution two dimensional gel technique (Ramagopol and Ennis, 1984); and a second, representing the only previous attempt to establish the systematic relationship between *Dictyostelium* and *Polysphondylium* on the basis of rDNA coding regions and ITS 1/ITS 2 spacer regions via RFLP (Evans *et al*., 1987). Neither study, described in chapter one, used PCR. Much of the early work for this project was spent developing a PCR working regime, as the standard one failed. This phase of the project proved challenging as the extremely A&T rich dictyostelids genome resulted in the time consuming procedure of designing primers, to ensure that they had an acceptable number of G&C bases with no A&T repeats. There then followed experiments to optimize the annealing temperatures. Despite many attempts with different temperatures a final optimum temperature of 55°C at the best produced weak or multiple bands. This problem was finally resolved by an unusual decrease of extension temperature to 65°C as described in chapter three.

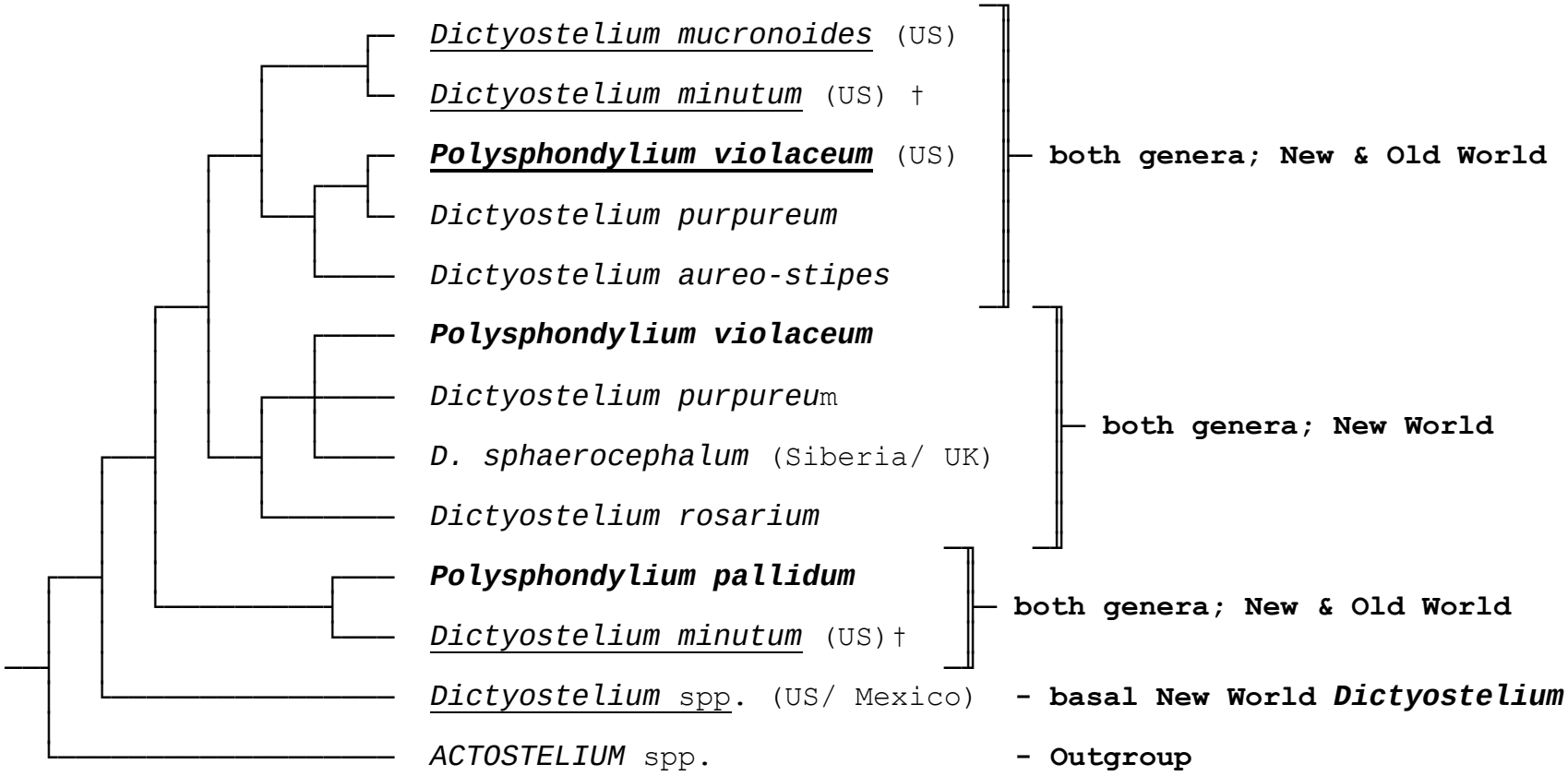
For sequencing purposes the nuclear ribosomal ITS 1 and ITS 2 regions of the rDNA were chosen. These generally evolve much faster than the rRNA coding regions because substitutions which occur in spacer regions do not show lethal effects. As these neutral mutations are carried without any strong constraints the result is a relatively fast evolutionary rate within spacer regions. Over recent years both rDNA spacer regions, i.e. internal generic spacers (IGS) and ITS, have been employed to resolve phylogenetic relationships in lower categorical levels among genera, species and populations (Baldwin, 1992, 1995; Morgen and Blair, 1998; Navajas *et al.,* 1998). The ITS regions proved more popular for that purpose (Baldwin, 1992,1995; Bain and Jansen, 1995), mainly because they are shorter (600-700bp) than IGS regions and because sequence information for design of PCR primers can be easily obtained as ITS regions are flanked by the conserved sequences of the rDNA 18 S or 5.8 S units. Furthermore, on the basis of these conserved sequences universal primers can be developed which can often be applied for cross-species amplifications. Also, and in contrast to IGS, the ITS regions have no internal repetitive structure, making it more accessible for direct sequencing. Moreover, as a non-coding region it is expected that they should contain a relatively high level of polymorphism among congeners. Conversely, lack of variation in spacer sequences such as ITS could suggest a close relationship among the taxa studied. In the latter cases more informative molecular markers would be necessary for further phylogenetic studies. However, direct sequencing proved to be an informative way of estimating variation in specific genes among the examined dictyostelid taxa.

In this study the phylogenetic analysis of data obtained from the direct sequencing of ribosomal ITS 1 and ITS 2 regions of rDNA showed that sequencing is a reliable technique with the power to reveal true species relationships. This was recently confirmed by Romeralo *et al.,* (2007) who used the 5.8 S unit and ITS regions of rDNA to study the molecular systematics of dictyostelids and have proved the utility of these regions for that purpose. In this study ITS 1 and ITS 2 sequencing data were analysed both separately and combined using the maximum parsimony phylogenetic analysis. Both separate and combined analyses yielded identical results in that all consensus trees showed, with few exceptions, that the same isolates consistently grouped together. This was expected as the isolates represent the same species, but such results do serve to validate the technique used in this thesis. Interestingly, some of this study's isolates, e.g. *D.*

mucoroides EW5 which was not grouped together with other *D. mucoroides* isolates, appeared on the tree as a monophyletic group along with the *D. aureostipes* BDW4 and *D. implicatum* EPN1 species. Also two isolates of *P. violaceum* (DNC 1 and DNC 3) often appeared as a monophyletic group but were never grouped together with the other *P. violaceum* isolates.

Although the consensus trees obtained by separate and combined phylogenetic analysis of ITS 1 and ITS 2 were very similar and provided mutual support for each other, there were minor differences in the order of species and isolates on the trees. These differences could be explained by the fact that not all species/ isolates were successfully sequenced for both ITS 1 and ITS 2 analysis and because a different species was used for rooting. In the case of the ITS 2 analysis, the protostelid *Acytostelium* was used as an outgroup, but for ITS 1 the *D. discoideum* NC4 was used as the ITS 1 of *Acytostelium* failed to sequence. Moreover, ITS 2 consensus trees come out as not fully resolved compared with ITS 1 or combined ITS 1/ITS 2. Another factor to consider is that a number of ITS 2 alignment sequences were of poor quality or of short length. A further explanation for the poorly resolved ITS 2 trees could be interference from homoplastic characters. Moreover, some of the species used for analysis could have been erroneously classified as a result of low species diversity. Despite these imperfections with the ITS 2 sequences a clear result was obtained in which species and isolates from both *Dictyostelium* and *Polysphondylium* genera showed very little intra-generic polymorphism and also little inter-specific variability in both ITS 1 and ITS 2 regions. A putative consensus tree derived from ITS 1 and ITS 2 (figure 5.1) clearly showed that *Polysphondylium* species/ isolates fall between *Dictyostelium* species/ isolates, showing a lack of clear distinction between them, suggesting that the morphologically defined genera *Dictyostelium* and *Polysphondylium* could be congeners instead of the two or even three genera proposed by Traub and Hohl (1976). This is a most interesting outcome of this study.

Figure 5.1 The case for a single genus: (derived from putative (simplified) ITS1/ ITS2 consensus tree)



Notes: (1) New World taxa are underlined (c.f. Old World taxa); (2) ***Polysphondylium* spp. are in bold**, c.f. *Dictyostelium* spp.; *ACTOSTELIUM (OUTGROUP) IS IN UPPER CASE ITALICS.* † represent different isolates.

The consequence of a single genus does not necessarily mean that *Dictyostelium* and *Polysphondylium* are a monophyletic clade descending from one common ancestor. It could be that some morphological features, for example the branching fruiting bodies or type of amoeba shown by many species, could represent multiple character analogues (these character analogues are used in current diagnostic of some of existing species that appear to be polymorphic) (Kessin, 2001; Eichinger, *et al.,* 2005). Thus to accommodate species with slightly different or unusual features, one or more subgenera could be proposed. Recently, Schaap *et al.* (2006), similarly suggested that dictyostelids taxonomy requires complete revision and proposed four different taxonomic groups that could accommodate *Dictyostelium* and *Polysphondylium* spp. Some such groups accommodate species from both genera and therefore do not align with the traditional established genera. Indeed, Schaap *et al.* (2006) indicated that fruiting body morphology used in traditional dictyostelid classification is a variable (plastic) trait and therefore of little taxonomic value.

Perhaps the most surprising result of this study is that species and isolates from soil in different parts of the world were grouped together. A good example of such unexpected grouping was *D. sphaerocephalum* (*D.sphaero* Sib) from Siberia and *D. sphaerocephalum* from England. Such observations support the tenet that the dictyostelids show little genetic polymorphism relative to morphological differences. One possible explanation for this may be that dictyostelids mainly reproduce asexually (see section 1.4.1). However, the question remains as to how congeners or even isolates of the same species living in the different parts of the world would be placed together in the phylogenetic trees. This prompts the question of how closely related species managed to get so far apart? Since the dictyostelids do not have any real dispersal mechanism they, or their spores, could be only transferred to their new habitats by a secondary dispersal agent such as on migrating birds, the nematode *Caenorhabditis. elegans*, or in the guts of any motile animals; aerial dispersal (anemochory) of the spores is a further possibility. Both explanations appear likely but probably may only partly explain the problem, suggesting that other unknown mechanisms or explanations should be considered.

If sequences are composed of functioning genes, one would expect to find some similarity between different isolates and species from different parts of the world, especially between

individuals occupying similar habitats and showing similar behaviours. Furthermore, the very few morphological characters used for the dictyostelids classification together with the large variability and adaptability of their fruiting bodies often leads to confusion as to whether two samples really contain conspecifics or not. It is also well established that different genes evolve at different rates (Hillis and Moritz, 1990). Those genes, encoding for proteins, may differ in their tolerance to mutational changes in their amino-acid sequence. As a result, genes that encode more tolerant, and less constrained proteins evolve faster than those which encode more constrained proteins, especially proteins responsible for important, survival related functions. Similar mechanisms might also apply to spacer regions. Yet non-coding regions do not generally possess any constraints and therefore evolve rapidly, accumulating many more mutations over a period of time compared to "functional" genes. In the case of this study which considers closely related species it might be expected that although spacer sequences would display more differences than sequences delivered from genes coding for proteins, they still may show many similarities as their divergence must have occurred in the recent-past when selective forces were similar.

The findings of this study also suggested that the identification of new soil isolates may in fact be better achieved by a quick and reproducible DNA-based technique, which could also screen a large number of newly isolated samples in a short time. The need to employ a different approach was supported by the fact that it was important to see whether data obtained by a different technique would support the phylogenetic conclusion of a single dictyostelid genus made after analysing the sequencing data.

My choice of the simple DNA profiling technique Inter Simple Sequence Repeat-PCR (ISSR-PCR) also known as Randomly Amplified Microsatellite PCR (RAM-PCR), developed by Zietkiewicz (1994) proved successful. ISSR generally presents itself as a good source of molecular markers with high reproducibility and has been more frequently used in screening genetic diversity within species but has been also used for phylogenetic reconstruction at the species level or above (Grunig *et al.,* 2001; Meng & Chen, 2001; Muthumeenakshi *et al.,* 2001). Although ISSR-PCR presents some concerns with respect to non-homology of some co-migrating gel electrophoresis bands, typical of fast-evolving DNA markers, the lack of ITS

divergence in closely related species renders ISSR as a marker of choice (Xu and Sun, 2001). As many researchers suggest it proves successful in phylogenetic studies allowing the differentiation of different isolates as well as taxonomic studies of closely related species (Meyer *et al.,* 1993). An overview of this technique is presented section 1.12.3. As this technique amplifies inter-microsatellite sequences (via single primers), which should vary between species and possibly isolates, it was hoped that it would discriminate different species and isolates. It was also hoped that variable profiles generated for individual species and isolates would yield a clear distinction between the different dictyostelids. However, the ISSR-PCR results were not informative enough to fully resolve the phylogenetic position of dictyostelids but they were reliable and efficient enough to show preliminary groupings of new soil-dwelling species. The main isolate groupings e.g. *P. violaceum* (STR, FCM, TDN) or *D. discoideum* NC4 and V12, obtained while working with different primers, were sustained in all analyses. Furthermore, the similar banding patterns of different isolates grouped together clearly suggested close relationships between them. In these cases ISSR-PCR clearly supported the results obtained by sequencing.

Sequencing the ITS 2 region shows that the position of some species and isolates remains unresolved. *D. sphaerocephalum* Sib isolate from Siberia was grouped with *D. purpureum* from Costa Rica or left unresolved in the consensus trees. These results differed from those obtained by sequencing where this isolate was placed with another *D. sphaerocephalum* SXN2 from England in a monophyletic group. Despite the fact that the position of this isolate was supported by neither technique, in each case the associated isolate or species of *D. sphaerocephalum* Sib came from different parts of the world. Also, the position of *D. mucoroides* EW5, which was never aligned with other *D. mucoroides* isolates, on the consensus trees delivered by sequencing analysis, was mainly left unresolved in ISSR-PCR analysis or placed with *D. aureostipes* BDW4. Thus, both the sequencing and ISSR-PCR analysis confirm that this is a distinctive isolate in need of further consideration.

Although some of the detailed species and isolates groupings examined by the ISSR-PCR technique differed from those examined by sequencing, one important finding of ISSR-PCR is noteworthy, namely that there is no clear differentiation between *Dictyostelium* and *Polysphondylium*. Many bands were shared by species from both genera and there were no bands

unique to either *Dictyostelium* spp. or *Polysphondylium* spp. while parsimony analysis showed neither *Dictyostelium* nor *Polysphondylium* to be monophyletic. In fact, all maximum parsimony (MP) consensus tree phylogenetic analyses showed species from both genera, which were variously grouped together. In this context the ISSR-PCR results supported the sequencing findings. Furthermore, ISSR-PCR showed no distinction between isolates from America and England. This finding agrees with the sequencing results. These two key results are thus strongly supported by two independent techniques, using different molecular markers.

5.1 Dictyostelids: The way Forward

The results obtained from my work suggest that the existing systematics of cellular slime moulds, based on alpha-morphology, needs reassessment. Although morphology strongly suggests the existence of two principal genera, there was, even at the time when they were created, some dispute over a number of species, which could not be neatly assigned to one or the other genus (Troub and Hohl, 1976; Raper, 1984). Indeed many species shared some features with 'the other' genus.

In this study, the molecular data, in the form of sequences of rDNA ITS spacer regions as well as inter-microsatellites variations, suggest that dictyostelids show very little polymorphism, indicative of the existence of only one major genus in some subgenera. If however there is to be a major change to dictyostelid systematic, further work is needed and more genes should be considered for scrutiny. Analysis of a larger number of species and isolates, ideally from other parts of the world, is also strongly recommended to see if the outcome of this study is supported by other species. As this study dealt with a limited number of (three) *Polysphondylium* species, considering other species is strongly recommended to throw more light on their relationships with the genus *Dictyostelium*.

Furthermore, future work could sequence other genes including a selection of coding genes, e.g. calmodulin and beta-tubulin on which some initial work was done in this study but abandoned as a result of time constraints. The preliminary results done on beta-tubulin with a limited number

of species and isolates showed a lack of between-species variability. The preliminary results with calmodulin were more promising as this gene showed more variability than beta-tubulin, suggesting more phylogenetic signals, thus the calmodulin gene is suggested as a better target gene for sequencing. Furthermore, the ITS alignments showed some common nucleotide stretches within ITS region which were characteristic for a particular group of isolates. They could possibly be used to design a probe, which then could be utilized for preliminary identification of any new soil-dwelling species.

5.2 Concluding Comments

The conclusion of this thesis is that the use of morphological criteria to classify the dictyostelids needs reassessing, but before any reclassification takes place, further work with more species, especially from the genus *Polysphondylium,* is needed, as well as with species whose position failed to be resolved by phylogenetic analysis and with isolates considered to be cryptic species, which failed to be grouped with other isolates.

Two different molecular techniques, sequencing of ITS1 and ITS2 regions as well as ISSR-PCR, were employed to reassess the phylogenetic relationships of dictyostelids, previously reconstructed on the basis of their alpha morphology. Only one, sequencing, appeared to be sufficiently powerful to deliver a full phylogenetic resolution of dictyostelid species and isolates. Although the position of some species and isolates failed to be supported by both techniques or their position was left unresolved, the techniques strongly supported the outcome that there is no clear distinction between American and English dictyostelid species and isolates and, most importantly, that there is no consistent distinction between *Dictyostelium* and *Polysphondylium* species, suggesting that *Polysphondylium* is a junior synonym of *Dictyostelium*. There is a case for a single genus.

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APPENDIX 1

Lactose-Peptose Medium (LP)

Lactose 5.0g
Peptose (bacteriological) 5.0g
Agar (tech N°3) 90.0 g
Distilled Water 5 l

Dissolve all ingredients and bottle in 400ml per bottle. Autoclave at 110° C for 20 min.
All ingredients were supplied by Sigma Aldrich Scientific, (Epsom, U.K.).

Hay Infusion Agar (HIA)

40 g Meadow Hay was put in 3 l of distilled water.
Autoclave at 120° C for 20 min and filter through cotton chase cloth.
Add 75 g of Agar (technical N°3) and make up to 4.5 l and then steam to dissolve agar.
Dissolve 7.5 g of Potassium dihydrogen orthophosphate and 3.1g of Di sodium hydrogen orthophosphate heptohydrate in 500 ml of distilled water and add to 4.5 l of Hay-Agar mixture.
Bottle in 400 ml per bottle, then autoclave at 121° C for 15 min.

APPENDIX 2

Extraction by boiling

Suspension of free amoebae roughly 1x10 boil in water for 2 min then use it for PCR.

Extraction by microwaving

Suspension of free amoebae put in 50-100ul of lysis buffer and microwave it for a total of 20 s in segments of 10 s, 5 s and 5 s.
After that immediately add 300-350ul of lysis buffer and incubate in 80°C for 10 min.
After incubation add 400ul of centrifuge mixture 1 and centrifuge at 10,000 g for 15 min. Remove an aqueous phase and put into tube with 0.54ml of isopropanol and 10ul of 5 M sodium acetate (mixture 2). Centrifuge at 10,000 g for 2 min. Pellet wash with 80 % ethanol and then resuspend in 50-150ul of resuspension mixture. Obtained DNA use for PCR.

For buffers and mixtures see appendix 3.

APPENDIX 3

Lysis buffer

50mM TrisHCL (pH 7.2)	70 ml
EDTA	50 mM
Sodium dodecyl sulphate	3 g
2-mercaptoethanol	1 ml

Centrifuge mixture 1

1: 1 chloroform: phenol 12 ml

Centrifuge mixture 2

5M sodium acetate.

0.54ml of isopropanol.

Resuspension mixture

10mM Tris HCL

0.1mM EDTA (pH 8)

All ingredients supplied by Fisher Scientific, (Loughborough, U.K.).

APPENDIX 4

Solution 1

25mM Tris HCL (pH 8)
1M EDTA (pH 8)

Solution 2

0.2N NaOH (freshly diluted from a 10N stock).
1% SDS

Solution 3

5M potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

The resulting solution is 3M with respect to potassium and 5M with respect to acetate
All ingredients supplied by Fisher Scientific, (Loughborough, U.K.).

APPENDIX 5 (CD)

- **ITS 1 STRICT CONSENSUS TREES**
- **ITS 2 STRICT CONSENSUS TREES**
- **COMBINED ITS 1 AND ITS 2 TREES**
- **BOOTSTRAP TREES**

APPENDIX 6

ISSR data matrix

Character No./ Taxon	10 20 30 40 50 60 12345678901234567890123456789012345678901234567890123456789012345678901234567890
D_disco_V12	10011100000001000101000000111100000000000000000001000101100000
D_disco_NC4	00011100000001000101000000111000000000000000000001000101100000
1D_sph_SXN	00010000000001011000000000101010000001000000010010101000000
3P_viol_ATN	01110000000000001000000001000011100000000000000010011100000
6D_sph_sib	00000011000001000000000001000000000000000010000101010000000
11D_bref_NTC	00010000000000000100000001100011000010000000000010000000
12d_sp_WLD	00000000000001000010000000110001100101000000100000100000000
13D_purp_ATN	00010000000010100000000000001001100001000100000011000000000
14P_viol_ATN	01110000100000001000110000101000001100000000000100001000000
16D_muc_BPR	01010000000000000100000000110001100110000000000000100000000
17D_sp_HLM	01010000000000010010000000010001010000110000100000100000000
19D_sp_BDW	00010000000000111001000000010000101000000000000000100000000
20D_imp_EPN	01010000000010110000000000000110000100000000110000001000000
21D_muc_BPR	01010000000001000010000000011001100101000000010000010000000
25D_sph_GMN	00010000000001001010100000001100001000000000010000101000000
27D_sp_HLM	01010000000001111010000000011000000000011000100000000000000
28D_muc_FLM	01010000000001001010100000011000110010000000010000100000000
29D_muc_HTL	00010000000001001010100000011100110001000000011000100000000
30D_muc_PYR	01010000000001001010100000011100110000000000010000100000000
32D_muc_DDS	01010000000001001010100000011000110000000000010000100000000
35D_muc_EW5	00010000000000111100000000000000001010000000010000000000000
36P_aur_SLR	00010000000001111000000000101001000000001000101000001000000
P_vio_ATN	10010000000000001000110000100001011000000000000000001000000
P_viol_STR	01010000100011000011100000000010101000000000100000001000000
P_viol_FCM	01010000100011001011100000100010101000000000100000001000000
P_viol_TDN	01010000100011001011100000100110001000000000100000001000000
P_violRTN	01010000100011001011000000100110001000000000100000001000000
P_pall1_ATN	10000000010001001100000000000000000000000000001000000100000
D_min2_NWM	10010000000001000100000000001001000100000000000010110000100
D_c_D_d3_CR	10010000000001000000010001110000011000000000000010010011000
D_cit4_Cuba	10010000000010100000011000011000100110001000001001001001000
D_ten5_Cuba	10010000000000001100000000010101010000001000000100101000000
D_d_D_c_Mex	00010000000011001000000000001001011001100000001000000000000
D_ros7_Alab	00010000000011010000010000010000101000100000010010010000010
D_min8_ATN	00010000000000001000000000000000000000000000000100010000000
D_purp_CR	00010000000001000100001000000000000000000000000100100000000
P_viol10_ATN	00110000000001111000000100101000101100001100000100001100110
D_min11_GMN	00110000000010001000000100101000111000000100000000000000000
D_disco12_ATN	00010100000011000000000010000110001100001000011000010000000
D_disco13_ATN	00010100000011000000000010000110001100001000011000010000000
D_sph15_HYY	00000000000001111000000000001100100000010000000000110000000
D_imp16_EPN	10010000000001111000000000000000000000000000010000010100000 10
D_sph17_GMN	00000000000001101000000000100001100000010100010101001100000 10
D_ros18_IL	00000000000001100000000001000000100000001000000010000000001000
D_purp_19_1k	10010000000010100000000001000110100001100000000000000000000
D_purp20_ATN	10000000000010100000000001000110100001100000000000100000000 1
D_imp21_BCN	10010000000000100000000000100101010000110000000010010000001
D_ros22_EDF	00000000000000100000000001001010001110000000110000000000001000
D_aureo23_BDW	10010000000001111000000000100001011000000010000000000000000
Acytho-outgroup	10010000010100110000010000010001010000011000000000000000000
Amoeba-outgroup	00000000000110000000000000100101010000011001000000000000010

APPENDIX 7

ITS 1 sequence data (series 1: 1-130)

	Taxa	sequence 1 2 3 4 5 6 7 8 9 10 11 12 13 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	----------------TTTTATCTGT-GG-CAACACCTG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-N-G-GTATCC-GTAG-GT
[2]	'DdiscoV12'	----------------XXXXXXXXXX-XX-XXXCAC-TGTATATA-AAT-TAAAAGTTGAT-TTCAAATC-TCAT-T-GTCTGTATGCAGGTAAGGAGAAGTCGGTATACA-TAG-GTATCC-GTAG-GT
[3]	'DdATN119'	----------------TTTTATCTGT-GG-CAACAC-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[4]	'DdDcitMex'	----------------TTTTATCTGT-NG-CAACAC-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[5]	'DspHLM411'	----------------XTTTATCTTT-GA-CNACAT-TG-ATATA-AAC-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[6]	'DsphaSib6'	----------------XTTTATCTTT-GA-CAACAT-TG-ATATA-AAC-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[7]	'PaurN36'	----------------XTTTATCTT--GG---ACAT-TG-ATATA-AAC-TAAAAGTTGAT-TTCAAATCGTCAT-T-GTCTGTA-G-AGG-AAGGAGAAGTCGGTA-ACANT-GCGTATCCNGTAG-GT
[8]	'DsphaGMN3'	----------------TTTTATCTTT-GG-CAACAT-TG-ATATA-AAC-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[9]	'PviolaDNC1	----------------XXXXXXXXXX-XX-XXXCAT-TG-ATATA-AAC-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[10]	'PviolDNC3'	----------------XXXXXXXXXX-XX-XXXCAT-TG-ATATA-AAC-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[11]	'DsphaSXN2'	----------------XXXXXXXXXX-XX-XXACAT-TG-ATATA-AAC-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[12]	'DrosAlabF'	----------------XXXXXXXXXX-XX-XXXXXX-XX-XXXXX-XXX-XXXXXXXX-XX-XX-XXXXX-XXXX-X-XX-X-XX-X-XXX-XXXXXXXXXXXX-XX-XXA-A-G-GTATCC-GTAG-GT
[13]	'DrosEDF3'	----------------TTTTATCTTT-GG-CAACAT-TG-ATATA-AAC-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[14]	'DsphaHYY1'	----------------XXXXATCTTT-GG-CAACAT-TG-ATATA-AAC-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[15]	'DrosIL'	----------------XXXTATCTTT-GGNNAACAT-TG-ATATA-AAC-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[16]	'DmucBPR11'	----------------XXXXXXXXXX-XX-XXCCAT-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[17]	'DmucBPR31'	----------------XXXXXXXXXX-XX-XXXCAT-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[18]	'DmucDDS21'	----------------XXXXXXXXXX-XX-XXACAT-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[19]	'DmucPYR31'	----------------XXXXXXXXXX-XX-XXNCAT-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[20]	'DmucorFLM'	----------------XXTTATCTTT-GG-CNACAT-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[21]	'DbrefTNC11'	----------------XXXTATCTNT-GG-C-CCNT-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[22]	'DmucorHTL'	----------------XXXTATCTTT-GG-C-NCAT-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[23]	'DminGMN3'	----------------TTTTATCTTT-GG-CAACAT-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[24]	'DspWLD1'	----------------XXXXXXXXXX-XX-CATCATCTGTATATANAATATANAAGTTGAT-TTCAAATAGTCATATAGTCT-TA-GCAGGTAAGGAGAAGTCGGTATACATA-GCGTATCC-GTAGNGN
[25]	'DimpBCN21'	----------------XXXXXXXXXX-XX-XXXXXX-XX-XXXXX-XXX-XXXXXXXX-XX-XX-XXXXXXTCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[26]	'DspBDW112'	----------------XXXXXXXTCT-TG-GAACAT-TG-ATATA-TAA-AAGTTATT-TC-AA-ATCTCAT-AT-A-GTCT-TA-G-AGG-AAGGAGAAGTCGGTA-ACA-A-GCGTATCC-GTAG-GN
[27]	'DaursBDW4'	----------------TTTTATCTTT-GG-CAACAT-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[28]	'DimplEPN1'	----------------XXXXXXXXXX-XX-XXXXXX-XX-XXXXX-XXX-XXXXXGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[29]	'DmucorEW5'	----------------XTTTATCTTT-GG-AAACAT-TG-ATATA-AAC-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[30]	'PviolSTR'	----------------XXXCCTATTG-GG-AAAC-C-AGTATGGA-TTT-TAAAAGTT-GT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[31]	'PviolFCM'	----------------XGTCCTATTG-GG-CAAC-C-AGTATGGA-TTT-TAAAAGTT-GT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[32]	'PviolATN1'	----------------XXXXCTATTG-GG-CAAC-C-AGTATGGA-TTT-TAAAAGTT-GT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[33]	'PviolATN'	----------------NGTCCTATTG-GG-CAAC-C-AGTACGGA-TTT-TAAAAGTT-GT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[34]	'DpurpAT17'	----------------XXXXXXXXTGTGG-NAACAC-AAT-T-AA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[35]	'Ppurp1K'	---------------TAGTTTTTTTGTGG-CAACAC-AAT-T-AA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[36]	'DpurpCRLC'	---------------TCGTTTTTTTGTGG-CAACAC-AAT-T-AA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[37]	'DcitCu2A4'	----------------NNTTATCTGT-GG-CAACAC-TG-ATATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[38]	'DcitDdCR'	----------------XXXXXXXXXX-XX-XXXXXX-XXXXTATA-AAT-TAAAAGTT-AT-TT-AAATC-TCAT-T-GT-T-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-NTAN-GT
[39]	'Ppallidum'	----------------TTTTATCTGT-GG-CAACAC-TG-ATATA-AAT-TAAAAGTT-ATCTT-AATTC-TCAT-T-GTCT-TA-G-AGG-AAGGAGAAGTCG-TA-ACA-A-G-GTATCC-GTAG-GT
[40]	'DminNWM12'	----------------XXXXXXXXXXXXX-XXXXXXXXXXXXXXX-XXX-XXXXXXXXXXX-XXXXXXXXXXXXX-X-XXXXXXX-XXXXX-XXXXXXXXXXXXXXXXXXXXX-XXXXXXXX-XXXG-GT
[41]	'DdATN122'	----------------xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[42]	'DtenCu2G3'	----------------xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[43]	'PviolATN11'	----------------xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[44]	'Acytostelium'	----------------xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[45]	'DminATN16'	----------------xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Notes, xxx = missing sequence or part thereof; --- = gaps in the sequence (e.g. due to sequences alignment)

Table: ITS 1 sequence data (series 2: 131-260)

	Taxa	sequence 14 15 16 17 18 19 20 21 22 23 24 25 26 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--TAATN-TCAT-A-AAC-TTTATGTT-TTTTTGGAT-AATGGAAC-TG-C--T-T-GCGTATAAAGAAATT---AG-GCTTTTTTATTAAGG-TTT-
[2]	'DdiscoV12'	GCAACC-TGCCGGATGGATCATTTTTTATC-T--T-ATA-TCAT-A-AAC-TTTATGTT-TTTTTGGAT-AATGGAAC-TG-C--T-T-GCGTATAAAGAAATT---AG-GCTTTTTTATTAAGG-TTT-
[3]	'DdATN119'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--TAATA-TCAT-A-AAC-TTTATGTT-TTTTTGGAT-AATGGAAC-TG-C--T-T-GCGTATAAAGAAATT---AG-GCTTTTTAATTAAGG-TTT-
[4]	'DdDcitMex'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--TAATT-T-AC-A-AACATTTATGTTCTTTTTGGAT-AATAGAGC-TT-C--T-T-GCGTATAAAGAAATT---AG-GCTTTTTTATTAAGG-TTT-
[5]	'DspHLM411'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--C-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTGTTAA-GC-----AATTA--G-CGA-
[6]	'DsphaSib6'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--C-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTACTAA-GC-----AATTA--G-TGA-
[7]	'PaurN36'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--C-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTGTTAA-GC-----AATTA--G-CGA-
[8]	'DsphaGMN3'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--C-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTGTTAA-GC-----AATTA--G-CGA-
[9]	'PviolaDNC1'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--C-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTGTTAA-GC-----AATTA--G-CGA-
[10]	'PviolDNC3'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--C-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTGTTAA-GC-----AATTA--G-CGA-
[11]	'DsphaSXN2'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--C-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTATTAA-GC-----AATTA--G-TAA-
[12]	'DrosAlabF'	G-AACC-T--CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--T-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTRCCAA-GC-----GATTG--A-TAG-
[13]	'DrosEDF3'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--T-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTACCAA-GC-----GATTG--A-TAG-
[14]	'DsphaHYY1'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--C-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTGTTAA-GC-----GATTA--G-CGA-
[15]	'DrosIL'	G-AACC-TG-CGGATGGATCATTTTTTATC-T--T-AAT-T-AT-A-ATC--ATA-GAT-GATA-G--T-----GT-C-TG-G--AGT-AAGTTGAAC-AAGTTACCAA-GC-----GATTG--A-TAG-
[16]	'DmucBPR11'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTATTAAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TACAATTTGAA-AAGT----AT-GCN----GAAAG-TT-TTG-
[17]	'DmucBPR31'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTATTAAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TACAATTTGAA-AAGT----AT-GCN----GAAAG-TT-TTG-
[18]	'DmucDDS21'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTATTAAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TACAATTTGAA-AAGT----AT-GCN----GAAAG-TT-TTG-
[19]	'DmucPYR31'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTATTAAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TACAATTTGAA-AAGT----AT-GC-----GAAAG-TT-TTG-
[20]	'DmucorFLM'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTATTAAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TACAATTTGAA-AAGT----AT-GCN----GAAAG-TT-TTG-
[21]	'DbrefTNC11'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTATTAAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TACAATTTGAA-AAGT----AT-GCN----GAAAG-TT-TTG-
[22]	'DmucorHTL'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTATTAAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TACAATTTGAA-AAGT----AT-GC-----GAAAG-TT-TTG-
[23]	'DminGMN3'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTATTAAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TACAATTTGAA-AAGT----AT-GCG----GAAAG-TT-TTG-
[24]	'DspWLD1'	C-AANCGTGCCGGATGGATCATTTTTTATC-TTATTAAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TACAATTTGAA-AAGT----AT-GC-----GAAAG-TT-TTG-
[25]	'DimpBCN21'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTAT-AAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TATAATTTGAA-AAGT----AT-GC-----GAAAG-CC-CTG-
[26]	'DspBDW112'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTAT-AAA-T-AT-A-ATT--AAA-GAT-AATATG--T--A--AT-C-TA-T--A-TATAATTTGAA-AA-T----AT-GC-----GAAAG-CC-CTG-
[27]	'DaursBDW4'	G-AACC-TG-CGGATGGATCATTTTTTATC-T-ACCCAT-T-AT-A-ATC--ATA-GAT-AATTTA--T--A--AG-C-TA----A-TTCAATT-GAA-ATGTC---AA-GC-----TAAA-----ATGA
[28]	'DimplEPN1'	G-AACC-TG-CGGATGGATCATTTTTTATC-T-ACCCAT-T-AT-A-ATC--ATA-GAT-AATTTA--T--A--AG-C-TA----A-TTCAATT-GAA-ATGTC---AA-GC-----TAAA-----ATGA
[29]	'DmucorEW5'	G-AACC-TG-CGGATGGATCATTTTTTATC-TAACCCAT-T-AT-A-ATC--GTA-GGT-AATTTA--T--G--CA-C-TAGT--T-TTAAATT-GAA-ATATC---ATCGC-----TAAA-----ATGA
[30]	'PviolSTR'	G-AACC-TG-CGGATGGATCATTTTTCAAC-T----CGA------A-A-C---TT-GAT-ACAAGA--TTGA--AC-C-TT-TG-A-T-CTGTTTTT---T-T----CT-ACA----GAACG-GG-TT--
[31]	'PviolFCM'	G-AACC-TG-CGGATGGATCATTTTTCAAC-T----CGA------A-A-C---TT-GAT-ACAAAA--TTGA--AC-C-TT-TG-A-T-CTGTTTTT---T-T----CT-ACA----GAACG-GG-TT--
[32]	'PviolATN1'	G-AACC-TG-CGGATGGATCATTTTTCAAC-T----CGA------A-A-C---TT-GAT-ACAAGA--TTGA--AC-C-TT-TG-A-T-CTGTTTTT---T-T----CT-ACA----GAACG-GG-TT--
[33]	'PviolATN'	G-AACC-TG-CGGATGGATCATTTTTCATC-T----CGA------A-A-C---TT-GAT-ACAAAA--TTGA--AT-C-TT-TG-A-T-CTGTTTTTC--T-T----TT-AAAATA-GAACG-GG-TT--
[34]	'DpurpAT17'	G-AACC-TG-CGGATGGATCATTTTTTATC-TATTAAGA-TTATTA-ATC--ATT-GAT-AGGTGG--TTGTTTAAACGTA-T-AA-T--AATTAAAA-GT-T---GAT-TCATTG-GATTGATAATT--
[35]	'Ppurp1K'	G-AACC-TG-CGGATGGATCATTTTTTATC-TATTAAGA-TTATTA-ATC--ATT-GAT-AGGTGG--TTGTTTAAACGTA-T-AA-T--AATTAAAA-GT-T---GAT-CCATTG-GATTGATAATT--
[36]	'DpurpCRLC'	G-AACC-TG-CGGATGGATCATTTTTTATC-TTTTTAGA-TTATTA-ACC--ATT-GAT-AAGAGG--TCGATTTT-CGGA-TGTA-T-GTGTCGCTG-GT-TC-AGCC-CCTTTG-GGG-GAGGAAT--
[37]	'DcitCu2A4'	G-AACC-TG-CGGATGGATCATTTTTTATCATACT-AATCT-AT-ACATTTTCTG-GAT-AATA-G--A-----G--C-TT----C-TAGCGTATAAAGAAATT---AG-ACT----GTTAAAGG-TCT-
[38]	'DcitDdCR'	G-AACC-TG-CGGATGGATCATTTTTTATCATACT-AATCT-AT-ACATT-TTTTGGAT-AATA-G--A-----G--C-TT----C-TAGCGTATAAAGAAATT---AG-ACTGT--GAAAA-GG-TTT-
[39]	'Ppallidum'	G-AACC-TG-CGGATGGATCATTTTTTATCATACT-AATCT-AT-ACATT-TTTTGGAT-AATA-G--A-----G--C-TT----C-TAGCGTATAAAGAAATT---AG-ACTGTT-AAAAG--G-TTT-
[40]	'DminNWM12'	G-AACC-TG-CGGATGGATCATTTTTTATCATACT-AATCT-AT-ACATT-TTTTGGAT-AATA-G--A-----G--C-TT----C-TAGCGTATAAAGAAATT---AG-ACTGTT-AAAAG--G-TTT-
[41]	'DdATN122'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[42]	'DtenCu2G3'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[43]	'PviolATN11'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[44]	'Acytostelium'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[45]	'DminATN16'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Table: ITS 1 sequence data (series 3: 261-390)

	Taxa	sequence 27 28 29 30 31 32 33 34 35 36 37 38 39 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	A-AGAGTAAGTTGGCTTGTCAGAT-TTATTGTCTCGATG--CTATTAATAT--GTTTTC-A-TAA-GACAAAT-TGTTTATAGT-ATTTT--GAT------AAAA-AAAG--T-CAA----TTGGGTAAC
[2]	'DdiscoV12'	A-AGAGTAAGTTGGCTTGTCAGAT-TTATTGTCTCGATG--CTATTAATAT--GTTTTC-A-TAA-GACAAAT-TGTTTATAGT-ATTTT--GAT------AAAA-AAAG--T-CAA----TTGGGTAAC
[3]	'DdATN119'	A-AGAGTAAGTTGGCTTGTCAGAT-TTATTGTCTTGATG--CTATTAATAT--GTTTT--T-TAA-GACAAAT-TGTTTATAGT-ATTTT--GAT------AAGA-AAAG--T-CAA----TTGGGTAAC
[4]	'DdDcitMex'	A-AGANTAAGTTGGCTTGTAAGAT-TTGTTATCTCGATG--CGATTAATTT---TGTT----TAA-GACAAAAATGTTTGAA-T-TTTTT--GAT------AAAG-AAA---T-CAA----TTGAGTAAT
[5]	'DspHLM411'	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGTCTTGATG--TTTCTATTAC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTTAAA-GGT------AATAAAATG--T-CAA----CT--TTAGT
[6]	'DsphaSib6'	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGTCTTGATG--TTTCTATTAC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTTAAA-GGT------AATAAAATG--T-CAA----CT--TTAGT
[7]	'PaurN36'	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGTCTTGATG--TTTCTATTAC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTTAAA-GGT------AATAAAATG--T-CAA----CT--TTAGT
[8]	'DsphaGMN3'	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGTCTTGATG--TTTCTATTAC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTTAAA-GGT------AATAAAATG--T-CAA----CT--TTAGT
[9]	'PviolaDNC1	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGTNTTGATG--TTTCTATTAC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTTAAA-GGT------AATAAAATG--T-CAA----CT--TTAGT
[10]	'PviolDNC3'	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGTNTTGATG--TTTCTATTAC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTTAAA-GGT------AATAAAATG--T-CAA----CT--TTAGT
[11]	'DsphaSXN2'	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGTCTTGATG--TTTCTATTAC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTTAAA-GAT------AATAAAATG--T-CAA----CT--TTAGT
[12]	'DrosAlabF'	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGTCTTGATG--TTTCTATTGC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTAAAA-GGT------ATTAAAATG--T-CAA----CT--TTAGT
[13]	'DrosEDF3'	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGTCTTGATG--TTTCTATTGC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTAAAA-GGT------ATTAAAATG--T-CAA----CT--TTAGT
[14]	'DsphaHYY1'	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGGCTTGATG--TTTCTATTAC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTTAAA-GGT------AANAAAATG--T-CAA----CT--TTAGT
[15]	'DrosIL'	C-CTAGTAAACTAG--C-TCAAGTCCTGTTGTCTTGATG--TTTCTATTGC--AATTT--A--TT-GT-GGTT-TATAA-T--TATTAAAA-GGT------ATTAAAATG--T-CAA----CT--TTAGT
[16]	'DmucBPR11'	CNTTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATG---TAAT-TTAC--CCTAT--A--AA-GT-AAAT-TGTAT-T--T-AGCGA--GGT------ATTA-AATG--T-TTG----AT--TTCG-
[17]	'DmucBPR31'	CNTTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATG---TAAT-TTAC--CCTAT--A--AA-GT-AAAT-TGTAT-T--T-AGCGA--GGT------ATTA-AATG--T-TTG----AT--TTCG-
[18]	'DmucDDS21'	C-TTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATG---TNNT-TTAC--CCTCT--A--AA-GT-AAAT-TGTAT-T--T-AGCGA--GGT------ATTA-AATG--T-TTG----AT--TTCG-
[19]	'DmucPYR31'	C-TTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATG---TAAT-TTAC--CCTCT--A--AA-GT-AAAT-TGTAT-T--T-AGCGA--GGT------ATTA-AATG--T-TTG----AT--TTCG-
[20]	'DmucorFLM'	CNTTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATG---TAAT-TTAC--CCTAT--A--AA-GT-AAAT-TGTAT-T--T-AGCGA--GGT------ATTA-AATG--T-TTG----AT--TTCG-
[21]	'DbrefTNC11'	CNTTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATG---TAAT-TTAC--CCTCT--A--AA-GT-AAAT-TGTAT-T--T-AGCGA--GGT------ATTA-AATG--T-TTG----AT--TTCG-
[22]	'DmucorHTL'	C-TTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATG---TAAT-TTAC--CCTAT--A--AA-GT-AAAT-TGTAT-T--T-AGCGA--GGT------ATTA-AATG--T-TTG----AT--TTCG-
[23]	'DminGMN3'	C-TTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATG---TAAT-TTAC--CCTAT--A--AA-GT-AAAT-TGTAT-T--T-AGCGA--GGT------ATTA-AATG--T-TTG----AT--TTCG-
[24]	'DspWLD1'	C-TTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATG---TAAT-TTAC--CCTAT--A--AA-GT-AAAT-TGTAT-T--T-AGCGA--GGT------ATTA-AATG--T-TTG----AT--TTCG-
[25]	'DimpBCN21'	C-TTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATGT-TTAAT-TTG----CT-T--T--TA-GT-AAAT-TGTGT-T--T-T-AAA--GGT------ATTA-AATG--T-TTG----ATC-TTCC-
[26]	'DspBDW112'	C-TTAGTAAAATAA-TA-TAGGTTATTATTGTCTTGATGT-TTAAT-TTG----CT-T--T--TA-GT-AAAT-TGTGT-T--T-T-AAA--GGT------ATTA-AATG--T-TTG----AT--TTCG-
[27]	'DaursBDW4'	C-AAAGTAAATTGG-AT-TAGAGTATGATTATCTTGATG---TTAT-TT------TTT--A--AT--T-GAAT-AGTAA-TGGT-A-CAA--GGT------ATTA-AATG--T-AAG----GT--TTAG-
[28]	'DimplEPN1'	C-AAAGTAAATTGG-AT-TAGAGTATGATTATCTTGATG---TTAT-TT------TTT--A--AT--T-GAAT-AGTAA-TGGT-A-CAA--GGT------ATTA-AATG--T-AAG----GT--TTAG-
[29]	'DmucorEW5'	T-AGAGTAGGTCGA-AC-TAGAGTACGATTATCTTGATGC--ACAT-TT------TCT--T--AT--T-GAAT-AATAA-TGGT-A-CAA--GTT------ATTA-AATG--T-AAG----GTC-TTAG-
[30]	'PviolSTR'	C-TTTTTTG-TTCC--A-TCTG---CAATT-TCCA--AA---CACA-TT-----GTTT--AC-AA--A-TGAT-T-TC--T--T-T-CTTT--AT--------T---TTA--T-AA------T--AA---
[31]	'PviolFCM'	C-TTTTTTG-TTCC--A-TCTG---CAATT-TCCA--AA---CACA-TT-----GTTT--AC-AA--A-TGAT-T-TC--T--T-T-CTTT--AT--------T---TTA--T-AA------T--AA---
[32]	'PviolATN1'	C-TTTTTTG-TTCC--A-TCTG---CAATT-TCCA--AA---CACA-TT-----GTTT--AC-AA--A-TGAT-T-TC--T--T-T-CTTT--AT--------T---TTA--T-AA------T--AA---
[33]	'PviolATN'	C-TTTTTTG-TTCC--A-TCTG---CAATT-TCCA--AA---CACA-TT-----GTTT--AC-AT--T-TGAT-T-TC--T--T-A-CTTT--ATTATTTTTTT---TTA--T-AA------T--AA---
[34]	'DpurpAT17'	A-ATTATTG-TTTAGTA--CT--T-CCCTTATCATGGTG---TACT-TT-T-TCAATT-AACGAT-GAGTGAT-AATCTAT--TTA--AAT-GAT------AATA-AATG--TCGAG---TGTA-AAAA-
[35]	'Ppurp1K'	A-ATTATTG-TTTAGTA--CT--T-CCCTTATCATGGTG---CACT-TT-T-TCAATT-AACGAT-GAGTGAT-AATCTAT--TTA--AAT-GAT------AATA-AATG--TCGAG---CGT--AAAA-
[36]	'DpurpCRLC'	A-AGGGCAGGTTCCGAA-TCTGCTGCCCTTATCATGGTG---TACT-TTATATTATTTTTAATAT-GAGTGAT-AATCGCT--TGA-CAAA-GAT------ACT--AATG--TCAAGGCCCCT--AGGG-
[37]	'DcitCu2A4'	A-AGAGTAAGTTGGCTTGTAAGAT-TTATTATCTTGATGCGATTAA-TTA---ATTTT--A--AACGA-GTATATAAAG-T--TGATAAAAAGGT-----CAATT-GGTG--C-CAG--TAAT--ATGGT
[38]	'DcitDdCR'	A-AAAGTAAGTTGGCTTGTAAGAT-TTATTATCTCGATGCGATTAA-TTA---ATTTT--A--AACGA-GTATATAAAAGT--TGATAAAAAGGT-----CAATT-GGTGCAT-CAG-----T-AATGGT
[39]	'Ppallidum'	A-AAAGTAAGTTGGCTTGTMTGAT-TTATTATCTCGATGCAATTAA-TTA---ATTTT--A--AACGA-GTATATAAAAGT--TGATAAAAAGGT-----CAATT-GGTGCAT-CAG-----T-AATGGT
[40]	'DminNWM12'	A-AAAGTAAGTTGGCTTGTAAGAT-TTATTATCTCGATGCGATTAA-TTA---ATTTT--A--AACGA-GTATATAAAAGT--TGATAAAAAGGT-----CAATT-GGTGCAT-CAG-----T-AATGGT
[41]	'DdATN122'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[42]	'DtenCu2G3'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[43]	'PviolATN11'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[44]	'Acytostelium'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[45]	'DminATN16'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Table: ITS 1 sequence data (series 4: 391-520)

	Taxa	sequence 40 41 42 43 44 45 46 47 48 49 50 51 52
		1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	A-C-CAA-TTG-AATCCAT-CTA-GCAA--TATGTTACAC-ATGTTA-C-GACATAAAATCC--C--G--ACT-C----------------------A-A-A---AA-----TTTA--CA--T-C--AC-
[2]	'DdiscoV12'	A-C-CAA-TTGGAATCCAT-CTA-GCAA--TATGTTACAC-ATGTTA-C-GACATAAAATCC--C--G--ACT-C----------------------A-A-A---AA-----TTTA--CA--T-C--AC-
[3]	'DdATN119'	A-C-CAA-TTG-AATCCAT-CTA-GCAA--TATGTTACAC-ATGTTA-C-GACATAAAATCC--C--G--ACT-C----------------------A-A-A---AA-----TCTA--CA--T-C--AC-
[4]	'DdDcitMex'	A-T-CAA-TTG-AATCCAT-CTA-GCAA--TATGTTACAC-ATGTTA-C-GAC-TAATATCC--CT-G--ACT-C----------------------A-A-A---A------TCTA--TA--T-C--AC-
[5]	'DspHLM411'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTTACAC-ATGTTATC-GAC-TG--ATCC--C--G--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[6]	'DsphaSib6'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTTACAC-ATGTTATC-GAC-TG--ATCC--C--G--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[7]	'PaurN36'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTTACAC-ATGTTATC-GAC-TG--ATCC--C--G--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[8]	'DsphaGMN3'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTTACAC-ATGTTATC-GAC-TG--ATCC--C--G--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[9]	'PviolaDNC1'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTTACAC-ATGTTATC-GAC-TG--ATCC--C--G--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[10]	'PviolDNC3'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTTACAC-ATGTTATC-GAC-TG--ATCC--C--G--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[11]	'DsphaSXN2'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTTACAC-ATGTTATC-GAC-TA--ATCC--T--G--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[12]	'DrosAlabF'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTTACAC-ATGTTATC-GAC-TG--ATCC--T--A--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[13]	'DrosEDF3'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTTACAC-ATGTTATC-GAC-TG--ATCC--T--A--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[14]	'DsphaHYY1'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTCACAC-ATGTTATC-CAC-TG--ATCC--A--G--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[15]	'DrosIL'	G---T---T-G-AATCCAT-CTA-GCAA--T--GTTACAC-ATGTTATC-GAC-TG--ATCC--T--A--ACT-C----------------------A-A-T---T------ACA---CA----C--A--
[16]	'DmucBPR11'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCATATGTTA-C-TTC-TA--ATCC--C--A--AT--C---------------------TTTACT-CGA------ACAG--CG--G-C--AA-
[17]	'DmucBPR31'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCATATGTTA-C-TTC-TA--ATCC--C--A--AT--C---------------------TTTACT-CGA------ACAG--CG--G-C--AA-
[18]	'DmucDDS21'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCATATGTTA-C-TTC-TA--ATCC--C--A--AT--C---------------------TTTACT-CGA------ACAG--CG--G-C--AA-
[19]	'DmucPYR31'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCATATGTTA-C-TTC-TA--ATCC--C--A--AT--C---------------------TTTACT-CGA------ACAG--CG--G-C--AA-
[20]	'DmucorFLM'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCATATGTTA-C-TTC-TA--ATCC--C--A--AT--C---------------------TTTACT-CGA------ACAG--CG--G-C--AA-
[21]	'DbrefTNC11'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCATATGTTA-C-TTC-TA--ATCC--C--A--AT--C---------------------TTTACT-CGA------ACAG--CG--G-C--AA-
[22]	'DmucorHTL'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCATATGTTA-C-TTC-TA--ATCC--C--A--AT--C---------------------TTTACT-CGA------ACAG--CG--G-C--AA-
[23]	'DminGMN3'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCATATGTTA-C-TTC-TA--ATCC--C--A--AT--C---------------------TTTACT-CGA------ACAG--CG--G-C--AA-
[24]	'DspWLD1'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCATATGTTA-C-TTC-TA--ATCC--C--A--AT--C---------------------TTTACT-CGA------ACAG--CG--G-C--AA-
[25]	'DimpBCN21'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCA-ATGTTA-C-TTC-TG--ATCC--C--A--AT--C---------------------AA-ACT-CGA------ACTG--TGTAG-C--AA-
[26]	'DspBDW112'	G-T-C---A-A-AATCCAT-CTA-GCAA--TT-GTTTCCA-ATGTTA-C-TTC-TG--ATCC--C--A--AT--C---------------------AA-ACT-CGA------ACTG--TGTAG-C--AA-
[27]	'DaursBDW4'	G-C-C---T-T-AATCCAT-CTACGCAA--T--GTTTCTT-TTGTTA-C-TTC-TA--ATCC--T--G--AT-TC----------------------TTA-T-TGA------ACTG--CA--T-C--AC-
[28]	'DimplEPN1'	G-C-C---T-T-AATCCAT-CTA-GCAA--T--GTTTCTT-TTGTTA-C-TTC-TA--ATCC--T--G--AT-TC----------------------TTA-T-TGA------AC-G--CA--T-C--AC-
[29]	'DmucorEW5'	G-AGC---T-T-AATCCAT-CTTAGCAA--T--GTTTCACCTTGTTA-CCTTC-TA--ATCC--C--GA-ATCTCCCGCCGAGAGGATAAATAAATATTA-TATGTGTGCGCAC-G--CA--T-CT-AC-
[30]	'PviolSTR'	----C---T-G-AATCTTT--T---CAA--T--GT------TTG-AT-A-ATC-TC--AAAAAAC-----AC--C----------------------T-A-----A------T-T---TG--T----T--
[31]	'PviolFCM'	----C---T-G-AATCTTT--T---CAA--T--GT------TTG-AT-A-ATC-TC--AAAA--C-----AC--C----------------------T-A-----A------T-T---TG--T----T--
[32]	'PviolATN1'	----C---T-G-AATCTTT--T---CAA--T--GT------TTG-AT-A-ATC-TC--AAAA--C-----AC--C----------------------T-A-----A------T-T---TG--T----T--
[33]	'PviolATN'	----C---T-G-AATCTTT--T---CAA--T--G-------TTG-AA-A-ATC-TC--AAAA--C-----AC--C----------------------T-A-----A------TCT---TA--A----T--
[34]	'DpurpAT17'	G---C---TTG-AATCCAT-CT--GCAT--T--AA--CAA-TT--AA-A-AAC-TG--TTCA--CTTGTTACAAC----------------------TGA-T-CTA------ACTC--TA--TATATTA-
[35]	'Ppurp1K'	G---C---TTG-AATCCAT-CT--GCAT--T--AA--CAA-TT--AA-A-AAC-TG--TTCA--CTTGTTACAAC----------------------TGA-T-CTA------ACTC--TA--TAT--TA-
[36]	'DpurpCRLC'	GCTTC---TTG-AATCCATCCT--GCTTG-T--TATTCAC-TTGTTA-C-GTC-TG--AACC--C--G--ACT-C----------------------TGA-T--AA------TATA--TA--TACA-TA-
[37]	'DcitCu2A4'	G-T-CTAATTG-AATCCAT-CTA-GCAAAAT--GTTACAC-ATGTTA-CTGAC-TG--AACC--CT-A--ACT-C---------------------AATAATCATATTGGTCACAC--CTTTTTA--ACT
[38]	'DcitDdCR'	GTGGCTAATTG-AATCCAT-CTA-GCAAAAT--GTTACAC-ATGTTA-CTGAC-TG-AATCC--T--A--ACT-C---------------------AATACT-CAT------ATTGTTCA--TACCTTT-
[39]	'Ppallidum'	GTGTCTAATTG-AATCCAT-CTA-GCAAAAT--GTTACAC-ATGTTA-CTGAC-TG-AATCC--T--A--ACT-C---------------------AATACT-CAT------ATNGTTCA--TACCTTA-
[40]	'DminNWM12'	GTGTCTAATTG-AATCCAT-CTA-GCAAAAT--GTTACAC-ATGTTA-CTGAC-TG-AATCC--T--A--ACT-C---------------------AATACT-CAT------ATTGTTCA--TACCTTT-
[41]	'DdATN122'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[42]	'DtenCu2G3'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[43]	'PviolATN11'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[44]	'Acytostelium'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[45]	'DminATN16'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Table: ITS 1 sequence data (series 5: 521-650)

	Taxa	sequence 53 54 55 56 57 58 59 60 61 62 63 64 65 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	AGGC--TTG---CC--AGTGATAT----TTTTCTAGTACGTCAAACAA-AA-CATTTAAAATATG-TCTTT-AGATTTT-AAATCT-TTAAGCATAAACGGTGAATACCCTCGACTCCTAAATCGATGAA
[2]	'DdiscoV12'	AGGC--TTG---CC--AGTGATAT----TTTTCTAGTACGTCAAACAA-AA-CATTTAAAATATG-TCTTT-AGATTTT-AAATCT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[3]	'DdATN119'	TGGC--TTG---CC--AGCG--AT----TTTTCTAGTACGTCAAACAA-AA-CATTTAAAATATG-TCTTT-AGATTTT-AAATCT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[4]	'DdDcitMex'	TGGG--TTT---CC--AGCG--AT----TTTTCTAGTACGTCAAACAA-AA-CATTTAAAATATG-TCTTT-AGATTTT-AAATCT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[5]	'DspHLM411'	TGGT--ACT---TG--TACC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATTT-TTAAGCATAAACGGTGGATACC-TCGACTCCTAAATCGATGAA
[6]	'DsphaSib6'	TGGT--ACT---TG--TACC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATTT-TTAAGCATAAACGGTGGATACC-TCGACTCCTAAATCGATGAA
[7]	'PaurN36'	TGGT--ACT---TG--TACC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATTT-TTAAGCATAAACGGTGGATACC-TCGACTCCTAAATCGATGAA
[8]	'DsphaGMN3'	TGGT--ACT---TG--TACC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATTT-TTAAGCATAAACGGTGGATACC-TCGACTCCTAAATCGATGAA
[9]	'PviolaDNC1'	TGGT--ACT---TG--TACC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATTT-TTAAGCATAAACGGTGGATACC-TCGACTCCTAAATCGATGAA
[10]	'PviolDNC3'	TGGT--ACT---TG--TACC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATTT-TTAAGCATAAACGGTGGATACC-TCGACTCCTAAATCGATGAA
[11]	'DsphaSXN2'	TGGT--ACT---TG--TACC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATTT-TTAAGCATAAACGGTGGATACC-TCGACTCCTAAATCGATGAA
[12]	'DrosAlabF'	TGGC--ACT---TG--TGCC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATCT-TTAAGCATAAACGGTGGATACC-TCGACTCCTAAATCGATGAA
[13]	'DrosEDF3'	TGGC--ACT---TG--TGCC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATCT-TTAAGCATAAACGGTGGATACC-TCGACTCCTAAATCGATGAA
[14]	'DsphaHYY1'	TGGC--ACT---TG--TACC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATTT-TTAAGCATAAACGGTGGATACC-TCGXXXXXXXXXXXXXXXXX
[15]	'DrosIL'	TGGC--ACT---TG--TGCC--AT----TTTTCTAGTACGACAAACAC-AA-CATTTAAAATATG-T-TTACAGAATTTTAAATCT-TTAAGCATAAACGGTGGATACC-TCGACTCCTAAATCGATGAA
[16]	'DmucBPR11'	TAGC-CAAAG-TTC--AAAA--ACC-ATTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATG-T-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[17]	'DmucBPR31'	TAGC-CAAAG-TTC--AAAA--ACC-ATTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATG-T-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[18]	'DmucDDS21'	TAGC-CAAAG-TTC--AAAA--ACC-ATTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATG-T-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[19]	'DmucPYR31'	TAGC-CAAAG-TTC--AAAA--ACC-ATTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATG-T-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[20]	'DmucorFLM'	TAGC-CAAAG-TTC--AAAA--ACC-ATTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATG-T-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[21]	'DbrefTNC11'	TAGC-CAAAG-TTC--AAAA--ACC-ATTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATG-T-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[22]	'DmucorHTL'	TAGC-CAAAG-TTC--AAAA--ACC-ATTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATG-T-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[23]	'DminGMN3'	TAGC-CAAAG-TTC--AAAA--ACC-ATTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATG-T-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[24]	'DspWLD1'	TAGC-CAAAG-TTC--AAAA--ACC-ATTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATG-T-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[25]	'DimpBCN21'	TAGCGAAAAG-TTC--ACAA--A---TTTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATGTT-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[26]	'DspBDW112'	TAGCGAAAAG-TTC--ACAA--A---TTTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATGTT-TTT-A-TATTT-AAATAT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[27]	'DaursBDW4'	TATT--ATT--ATA--TGTA--ATAGTTTTTTCTAGTATGTCAAACAAGAC-CATTTAAAATATGTT-TTT-AGTATTT-AAATCT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[28]	'DimplEPN1'	TATT--ATT--ATA--TGTA--ATAGTTTTTTCTAGTATGTCAAACAA-AC-CATTTAAAATATGTT-TTT-AGTATTT-AAATCT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[29]	'DmucorEW5'	TATT-TATTT-CTC--TTTT--TTTTTTTTTTCTAGTATGCCAAACAA-AC-CATTTAAAATATGTT-TTT-AGTATTT-AAATCT-TTAAGCATAAACGGNGAATACC-TNGACTCCTAAATCGATGAA
[30]	'PviolSTR'	TAGC--AAA---T---T-----AT----TTTTCTAGTATGTCAAACAA--C-CATTTAAAG-ATG-T-TTA-A-GCTTT-AAATCACTAAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[31]	'PviolFCM'	TAGC--AAA---T---T-----AT----TTTTCTAGTATGTCAAACAA--C-CATTTAAAG-ATG-T-TTA-A-GCTTT-AAATCACTAAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[32]	'PviolATN1'	TAGC--AAA---T---T-----AT----TTTTCTAGTATGTCAAACAA--C-CATTTAAAG-ATG-T-TTA-A-GCTTT-AAATCACTAAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[33]	'PviolATN'	T-G-----A---T---T-----AT----TTTTCTAGTATGTCAAACAA--C-CATTTAAAG-ATG-T-TTA-A-GCTTT-AAATCACTAAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[34]	'DpurpAT17'	TAAT--TAA---TC--TCTA--AT----TTTTCTAGTATGACAAACAA--AACATTTAAAATATG-C-TTT-A--TTTT-AAAAAAATTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[35]	'Ppurp1K'	TAAT--TAA---TC--TCTA--AT----TTTTCTAGTATGACAAACAA--AACATTTAAAATATG-C-TTT-A--TTTT-AAAAAAATTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[36]	'DpurpCRLC'	TTGT--AAA---TC--TCAA--ATC--TTTTTCTAGTACGACAAACAA--T-CATTTAAAATATG-C-TTT-A--TTTT-AACAAAACTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[37]	'DcitCu2A4'	TTGTTAATTGGTTG--ATCA--AAC--CTTTTCTAGTACGTCAAACAA-TA-CATTTAAAATATG-T-TTTTAGAATTTTAAATCT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[38]	'DcitDdCR'	TGGT--TTT--ATCAATACC--TT--TTTTTTCTAGTACGTCAAACAA-TA-CATTTAAAATATG-T-TTTTAGAATTTTAAATCT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[39]	'Ppallidum'	TGGT--TTG--ATCAATACC--TT--TTTTTTCTAGTACGTCAAACAA-TA-CATTTTAAATATG-T-TTTTAGAATTTTAAATCT-TTAAGCATAAACGGTGAATACC-TCGACTCCTAAATCGATGAA
[40]	'DminNWM12'	TGGT--TTG--ATCAATACC--TT--TTTTTTCTAGTACGTCAAACAA-TA-CATTTAAAATATG-T-TTTTAGAATTTTAAATCT-TTAAGCATAAACGGGGAATAXX-XXXXXXXXXXXXXXXXXXXX
[41]	'DdATN122'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[42]	'DtenCu2G3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[43]	'PviolATN11'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[44]	'Acytostelium'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[45]	'DminATN16'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

Table: ITS 1 sequence data (series 6: 651-780)

	Taxa	sequence 66 67 68 69 70 71 72 73 74 75 76 77 78 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATCGGTCCTTTCNG-ATTANGTGTTATACT-TGG--G-T--GAGAGT-GGTCA
[2]	'DdiscoV12'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATCGGTCCTT-CGGGATTAGGTG-TAT-CTGANN--N-N--GXXXXX-XXXXX
[3]	'DdATN119'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--X-X--XXXXXX-XXXXX
[4]	'DdDcitMex'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATCGGTCCTTTCGG-ATTAGGTGTTATACT-TGG--G-T--GAXXXX-XXXXX
[5]	'DspHLM411'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTACGG-ATTAAATGTTATACT-TGGGNG-G--NGGGXX-XXXXX
[6]	'DsphaSib6'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTACGG-ATTAAATGTTATACT-TGG--G-G--NGGGGG-GXXXX
[7]	'PaurN36'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTACGG-ATTAAATGTTAT-CT--GN--A-N--NGGGXX-XXXXX
[8]	'DsphaGMN3'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTACGG-ATTAAATGTTAXXXX-XXX--X-X--XXXXXX-XXXXX
[9]	'PviolaDNC1'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTACGG-ATTAAATGTTATACT-TGG--G-T--NGAGTN-GGXXX
[10]	'PviolDNC3'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTACGG-ATTAAATGTTATACT-TGG--G-T--NGGNNN-NGXXX
[11]	'DsphaSXN2'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTACGG-ATTAAATGTTATACT-TGG--G-NGGNGGGXX-XXXXX
[12]	'DrosAlabF'	GCCCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTNAACG-CACATGAXXXXXXXXXXXXXXXXXX-XXXXXXXXXXXXXXX-XXX--X-X--XXXXXX-XXXXX
[13]	'DrosEDF3'	GACCGTAGCAAACXXXGATAATTCACATGAATTGCANCCTACTGGGATAGTTGAAATGTTXXXXXXCACATGXXXXXXXXXXXXXXXXXXX-XXXXXXXXXXXXXXX-XXX--X-X--XXXXXX-XXXXX
[14]	'DsphaHYY1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX-XXXXXXXXXXXXXXX-XXX--X-X--XXXXXX-XXXXX
[15]	'DrosIL'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTACGG-ATTAAATGTTATACT-TGG--G-T--GAGAGT-GGCXX
[16]	'DmucBPR11'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTTATACT-TGG--G-A--GANNGX-XXXXX
[17]	'DmucBPR31'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTTATACT-TGG--G-T--NANGGG-NGXXX
[18]	'DmucDDS21'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTTATACT-TGG--G-T--G-NGGGTNGGXX
[19]	'DmucPYR31'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTTATACT-TGG--G-T--XXXXXX-XXXXX
[20]	'DmucorFLM'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTTATACT-TGG--G-N--GGNGGG-XXXXX
[21]	'DbrefTNC11'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTTATACT-TGG--G-N--GANGGG-XXXXX
[22]	'DmucorHTL'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTTATACT-TGG--GGN--GANGGG-XXXXX
[23]	'DminGMN3'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTTATACT-TGG--GXX--XXXXXX-XXXXX
[24]	'DspWLD1'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTT-T-CT--GA--N-N--GGGGXX-XXXXX
[25]	'DimpBCN21'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTXXXXXX-XXXXXXXXXXXXXXXXXXXXXXXXX-XXXXXXXXXXXX-XX-XXX--X-X--XXXXXX-XXXXX
[26]	'DspBDW112'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTTAT-CT-GGA--G-G--GGGGXX-XXXXX
[27]	'DaursBDW4'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAACGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGXXXXXXX-XXX--X-X--XXXXXX-XXXXX
[28]	'DimplEPN1'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAACGTTGAACG-CACATGATGACATTGGTCCTTNNGG-ATTAAXXXXXXXXXX-XXX--X-X--XXXXXX-XXXXX
[29]	'DmucorEW5'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAACGTTGAACG-CACATGATGACATTGGTCCTTTCGG-ATTAAGTGTXXXXXX-XXX--X-X--XXXXXX-XXXXX
[30]	'PviolSTR'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGGCATTGGCCCTTT-GG-GTTAAGTGTTAT-CT-TXX--X-X--XXXXXX-XXXXX
[31]	'PviolFCM'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGGCATTGGCCCTTT-GG-GTTAAGTGTTAT-CT-TGX--X-X--XXXXXX-XXXXX
[32]	'PviolATN1'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGGCATTGGCCCTTT-GG-GTTAAGTGTTATACT-TGG--G-T--XXXXXX-XXXXX
[33]	'PviolATN'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGGCATTGGCCCTTT-GG-GTTAAGTGTTATACT-TGG--G-T--AXXXXX-XXXXX
[34]	'DpurpAT17'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATAGCATTGGTCCTTTCGG-ATTAAGTGTTATACT-TGG--G-T--GAGAGX-XXXXX
[35]	'Ppurp1K'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTNAACG-CACATGNNNGCATTGGTCCXXXXXX-XXXXXXXXXXXXXXX-XXX--X-X--XXXXXX-XXXXX
[36]	'DpurpCRLC'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[37]	'DcitCu2A4'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACG-CACATGATGACATCGGTCCTTTTGG-ATTAGGTGTTATACT-TGG--G-T--XXXXXX-XXXXX
[38]	'DcitDdCR'	AACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGAAATGTTGAACC-CACATGATGACATCGGTXXXXXXX-XXXXXXXXXXXXXXX-XXX--X-X--XXXXXX-XXXXX
[39]	'Ppallidum'	GACCGTAGCAAACTGCGATAATTCACTTGAATTGCAGCCTACTGGGATAGTTGTARTXXXXXXXX-XXXXXXXXXXXXXXXXXXXXXXXXX-XXXXXXXXXXXXXXX-XXX--X-X--XXXXXX-XXXXX
[40]	'DminNWM12'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX-XXXXXXXXXXXXXXX-XXX--X-X--XXXXXX-XXXXX
[41]	'DdATN122'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[42]	'DtenCu2G3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[43]	'PviolATN11'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[44]	'Acytostelium'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[45]	'DminATN16'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

Table: ITS 1 sequence data (series 7: 781-910)

	Taxa	sequence 79 80 81 82 83 84 85 86 87 88 89 90 91 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	ANGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATGGTATCCGCTCCCAATTCNCCCACATACAGCCC
[2]	'DdiscoV12'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[3]	'DdATN119'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[4]	'DdDcitMex'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[5]	'DspHLM411'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[6]	'DsphaSib6'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[7]	'PaurN36'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[8]	'DsphaGMN3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[9]	'PviolaDNC1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[10]	'PviolDNC3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[11]	'DsphaSXN2'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[12]	'DrosAlabF'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[13]	'DrosEDF3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[14]	'DsphaHYY1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[15]	'DrosIL'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[16]	'DmucBPR11'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[17]	'DmucBPR31'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[18]	'DmucDDS21'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[19]	'DmucPYR31'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[20]	'DmucorFLM'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[21]	'DbrefTNC11'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[22]	'DmucorHTL'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[23]	'DminGMN3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[24]	'DspWLD1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[25]	'DimpBCN21'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[26]	'DspBDW112'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[27]	'DaursBDW4'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[28]	'DimplEPN1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[29]	'DmucorEW5'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[30]	'PviolSTR'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[31]	'PviolFCM'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[32]	'PviolATN1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[33]	'PviolATN'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[34]	'DpurpAT17'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[35]	'Ppurp1K'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[36]	'DpurpCRLC'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[37]	'DcitCu2A4'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[38]	'DcitDdCR'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[39]	'Ppallidum'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[40]	'DminNWM12'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[41]	'DdATN122'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[42]	'DtenCu2G3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[43]	'PviolATN11'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[44]	'Acytostelium'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[45]	'DminATN16'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

Table: ITS 1 sequence data (series 8: 911-976)

		sequence							
		92	93	94	95	96	97		
	Taxa	12345678901234567890123456789012345678901234567890123456789012345678901234567							
[1]	'DdiscoNC4'	GGAGCATAAGTGTAAGCCTGGGTGCCTATGAGTGGCTACTACATTAATTGCGTGCGCNCCTGC----							
[2]	'DdiscoV12'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX----							
[3]	'DdATN119'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX----							
[4]	'DdDcitMex'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[5]	'DsphLM411'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX----							
[6]	'DsphaSib6'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX-----							
[7]	'PaurN36'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[8]	'DsphaGMN3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[9]	'PviolaDNC1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX----							
[10]	'PviolDNC3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[11]	'DsphaSXN2'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX----							
[12]	'DrosAlabF'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[13]	'DrosEDF3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[14]	'DsphaHYY1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[15]	'DrosIL'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[16]	'DmucBPR11'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[17]	'DmucBPR31'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX----							
[18]	'DmucDDS21'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[19]	'DmucPYR31'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[20]	'DmucorFLM'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[21]	'DbrefTNC11'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[22]	'DmucorHTL'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[23]	'DminGMN3'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[24]	'DspWLD1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[25]	'DimpBCN21'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX							
[26]	'DspBDW112'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX----							
[27]	'DaursBDW4'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[28]	'DimplEPN1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[29]	'DmucorEW5'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[30]	'PviolSTR'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[31]	'PviolFCM'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[32]	'PviolATN1'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[33]	'PviolATN'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[34]	'DpurpAT17'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX---							
[35]	'Ppurp1K'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[36]	'DpurpCRLC'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX-							
[37]	'DcitCu2A4'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[38]	'DcitDdCR'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX--							
[39]	'Ppallidum'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX-							
[40]	'DminNWM12'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX-							
[41]	'DdATN122'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx							
[42]	'DtenCu2G3'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx							
[43]	'PviolATN11'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx							
[44]	'Acytostelium'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx							
[45]	'DminATN16'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx							

Table: ITS 2 sequence data (series 1: 1-130)

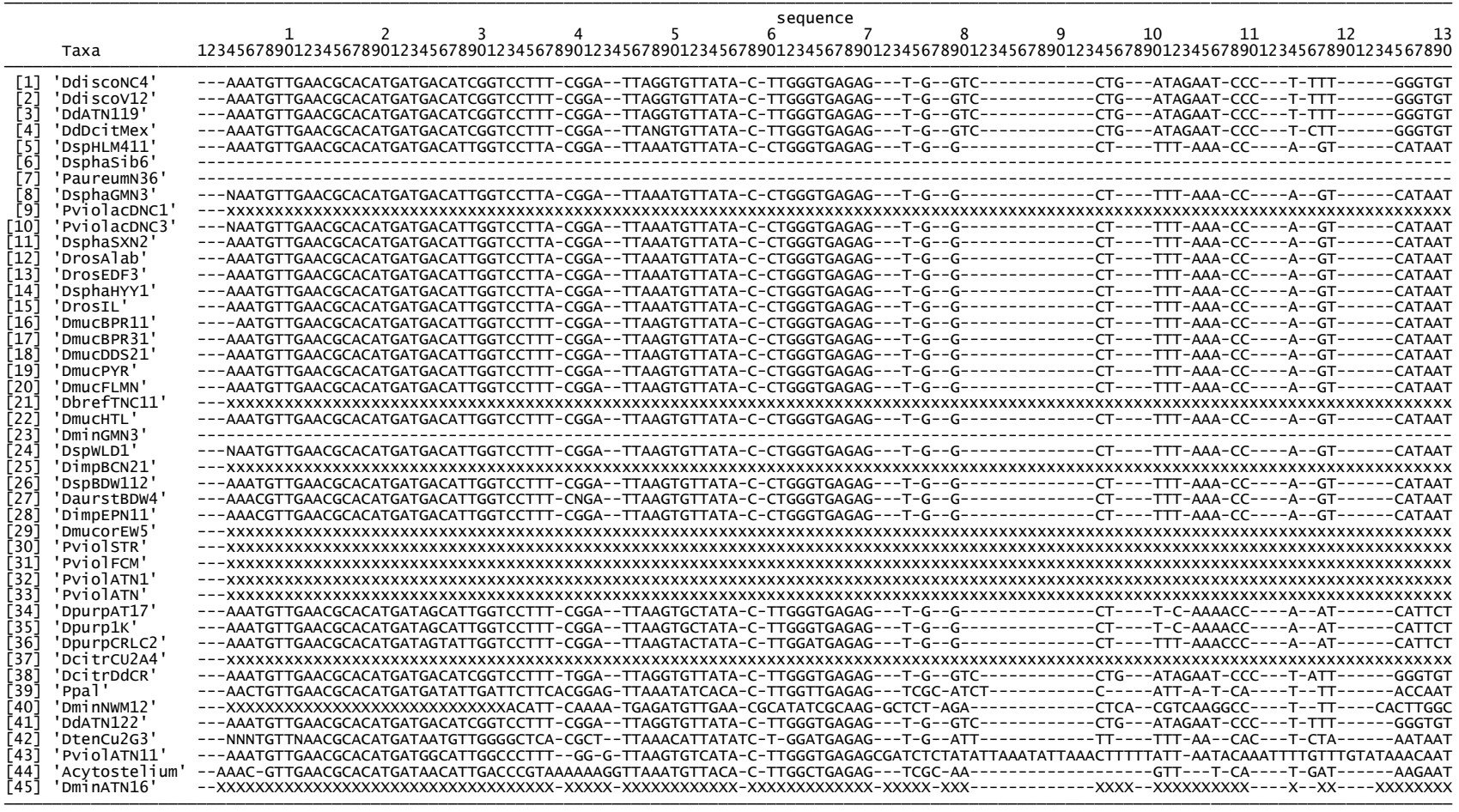

Table: ITS 2 sequence data (series 2: 131-260)

	Taxa	sequence 14 15 16 17 18 19 20 21 22 23 24 25 26 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	---GTCT-ATTG-AACTT--G--AT---TA-G---ATG-GT----G-GT-AAAAAG-TG----CG-CGAGT-GCAT--AGCAG-CT-TCGTCCTAATAAACTATG---CGAGG--AC-----CG--CC-C
[2]	'DdiscoV12'	---GTCT-ATTG-AACTT--G--AT---TA-G---ATG-GT----G-GT-AAAAAG-TG----CG-CGAGT-GCAT--AGCAG-CT-TCGTCCTAATAAACTATG---CGAGG--AC-----CG--CC-C
[3]	'DdATN119'	---GTCT-ATTG-AACTT--G--AT---TA-G---ATG-GT----G-GT-AAAAAG-TG----CG-CGAGT-GCAT--AGCAG-CT-TCGTCCTAATAAACTATG---CGAGG--AC-----CG--CC-C
[4]	'DdDcitMex'	---GTCT-ATTG-AACTT--G--AT---TA-G---ATG-GT----G-GT-AAAAAG-TG----CG-CGAGT-GCAT--AGCAG-CT-TCGTCCTAATAAACTATG---CGAGG--AC-----CG--CC-C
[5]	'DspHLM411'	--CAAATGATTT--TCAA--ACACT---TAGG----TG--C----GCG--AATCTC-TT----TG---A---GAAT---G-A---T-T--T-GTATAGCG-AGT----T-A----AC-----TG--CA--
[6]	'DsphaSib6'	--
[7]	'PaureumN36'	--
[8]	'DsphaGMN3'	--CAAATGATTT--TCAA--ACACT---TAGG----TG--C----GCG--AATCTC-TT----TG---A---GAAT---G-A---T-T--T-GTATAGCG-AGT----T-A----AC-----TG--CA--
[9]	'PviolacDNC1'	--xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[10]	'PviolacDNC3'	--CAAATGATTT--TCAA--ACACT---TAGG----TG--C----GCG--AATCTC-TT----TG---A---GAAT---G-A---T-T--T-G--TAGCG-AGT----T-A----AC-----TG--CA--
[11]	'DsphaSXN2'	--CAAATGATTT--TCAA--ACACT---TAGG----TGA-A----GCG--AATCTC-TT----TG---A---GAAT---G-A---T-T--T-GTATAGCG-AGT----T-A----AC-----TG--TA--
[12]	'DrosAlab'	--CAAATGATTT--TCAA--ACGCT---TGCG----TG--------CG--AATCTC-TT----TG---A---GAAT---G-A---T-T--T-GTATGGCG-AGT----C-A----AC-----GG--CA--
[13]	'DrosEDF3'	--CAAATGATTT--TCAA--ACGCT---TGCG----TG--------CG--AATCTC-TT----TG---A---GAAT---G-A---T-T--T-GTATGGCG-AGT----C-A----AC-----GG--CA--
[14]	'DsphaHYY1'	--CAAATGATTT--TCAA--ACACT---TAGG----TG--C----GCG--AATCTC-TT----TG---A---GAAT---G-A---T-T--T-GTATAGCG-AGT----T-A----AC-----TG--CA--
[15]	'DrosIL'	--CAAATGATTT--TCAA--ACGCT---TGCG----TG--------CG--AATCTC-TT----TG---A---GAAT---G-A---T-T--T-GTATGGCG-AGT----C-A----AC-----GG--CA--
[16]	'DmucBPR11'	--CAAATGATTT--TATT--A--CT---TTCG----AG--T----G-GT-AATATC-TT----TG---A---GAAT---G-A---T-T--T-GTATTTAG-AAT----TCAG---AC-----C---CTTC
[17]	'DmucBPR31'	--CAAATGATTT--TATT--A--CT---TTCG----AG--T----G-GT-AATATC-TT----TG---A---GAAT---G-A---T-T--T-GTATTTAG-AAT----TCAG---AC-----C---CTTC
[18]	'DmucDDS21'	--CAAATGATTT--TATT--A--CT---TTCG----AG--T----G-GT-AATATC-TT----TG---A---GAAT---G-A---T-T--T-GTATTTAG-AAT----TCAG---AC-----C---CTTC
[19]	'DmucPYR'	--CAAATGATTT--TATT--A--CT---TTCG----AG--T----G-GT-AATATC-TT----TG---A---GAAT---G-A---T-T--T-GTATTTAG-AAT----TCAG---AC-----C---CTTC
[20]	'DmucFLMN'	--CAAATGATTT--TATT--A--CT---TTCG----AG--T----G-GT-AATATC-TT----TG---A---GAAT---G-A---T-T--T-GTATTTAG-AAT----TCAG---AC-----C---CTTC
[21]	'DbrefTNC11'	--xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[22]	'DmucHTL'	--CAAATGATTT--TATT--A--CT---TTCG----AG--T----G-GT-AATATC-TT----TG---A---GAAT---G-A---T-T--T-GTATTTAG-AAT----TCAG---AC-----C---CTTC
[23]	'DminGMN3'	--
[24]	'DspWLD1'	--CAAATGATTT--TATT--A--CT---TTCG----AG--T----G-GT-AATATC-TT----TG---A---GAAT---G-A---T-T--T-GTCTTTAG-AAT----TCAG---AC-----C---CTTC
[25]	'DimpBCN21'	--xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[26]	'DspBDW112'	--CAAATGATTT---CTA--AG-CT---T-CG-----G--T----G-GT-GATTTC-TT----TG---A---GAAT---G-A---T-T--T-GTATTGCG-GAT----CAAG---AC-----C---CTT-
[27]	'DaurstBDW4'	--CAAATGATTT--TATT--AA-AT---TTCG-----GTTT----A-GT-AATTTC-TT----TG---A---GAGT---G-A---T-T--T-GTATTT-G-GAT----T-TG---AA-----T---CTTT
[28]	'DimpEPN11'	--CAAATGATTT--TATT--AA-AT---TTCG-----GTTT----A-GT-AATTTC-TT----TG---A---GAGT---G-A---T-T--T-GTATTT-G-GAT----T-TG---AA-----T---CTTT
[29]	'DmucorEW5'	--xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[30]	'PviolSTR'	--xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[31]	'PviolFCM'	--xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[32]	'PviolATN1'	--xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[33]	'PviolATN'	--xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[34]	'DpurpAT17'	GACTGAATATTT---TAA--A--AT---A--G--------T----A-AT-ATTTT--AA----AGTA-G---GCATAGGG-A---TGA--T-TTGTATAGTTAT----G-AC---TT-----GGGGCTAG
[35]	'Dpurp1K'	GACTGAATATTT---TAA--A--AT---A--G--------T----A-AT-ATTTT--AA----AGTA-G---GCATAGGG-A---TGA--T-TTGTATAGTTAT----G-AC---TT-----GGGGCTAG
[36]	'DpurpCRLC2'	--CAAAACCTGT---CTC--T--CT---A--G-----G--G----A-GC-AGTTT--TT----GG---G---GC-T---G-A---T-A--T-CCGTCTC--TAT------AC---CC-----AG--C--G
[37]	'DcitrCU2A4'	--xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[38]	'DcitrDdCR'	---GTTT-ATTG-AGCTT--G--AT---TA-G---ATG-GT----G-GT-AAAAAG-TG----CG-CGAGT-GCAT--AGCAG-CT-TCGTCCTAATATACTATG---CGAGG--AC-----CG--CC-C
[39]	'Ppal'	--AGTTT-TTTT--TCG---A--AA---AA-G---CT--ATT---G-GC-AAAGTA-TT-----G---G---G-A---A--AGT-T-T--T-CTAT-G-ACAATT---CGGGATTAC--TTTC---TT-T
[40]	'DminNWM12'	TGAGAGCGGTTTTAACAA--A--AT---TTAA---CAA--A----AAGTTCTTTTTTTT----TAATTA---AAAA--AGTT--CTTT--TACTATTCGTTAATGATGTAAAA--AC-----AC--CTCG
[41]	'DminNWM12'	---GTCT-ATTG-AACTT--G--AT---TA-G---ATG-GT----G-GT-AAAAAG-TG----CG-CGAGT-GCAT--AGCAG-CT-TCGTCCTAATAAACTATG---CGAGG--AC-----CG--CC-C
[42]	'DtenCu2G3'	---G-AT-ATTT-TTCAT--T--CT---TT-----ATG-AA----T-AT-TAATA--T-----C--C-A-T----T--A--T---T-T--T--TTTTGGACT-T----CTA----AC-----A---TT-T
[43]	'PviolATN11'	--CGTTTGATATAATAGTTTA--ATTTTTA-GTCTCTG-ATTAAAA-GTTAAATTATTTTTATAGTCTGATCG-AT--AGTAGTCT-T--TGCAGTGGTATAATCGACCTTGGTTTCAATTTCG--AT-C
[44]	'Acytostelium'	--C-TCT-ATTT--------A--TT---TA-G-------A-----G-G----AG---TT-----G---A---G-A---G--AG--A-T--T-CTA--GTACAAT----CTGGAGTTC--TTTC---TT-C
[45]	'DminATN16'	--XXXXXXXXXXXXXXXX--X--XX---XXXX---XXX--X----XXXXXXXXXXXXXX----XXXXXX---XXXX--XXXX--XXXX--XXXXXXXXXXXXXXXXXXXXXXX--XX-----XX--XXXX

Table: ITS 2 sequence data (series 3: 261-390)

	Taxa	sequence 27 28 29 30 31 32 33 34 35 36 37 38 39 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	GAA-------ATGAGCC--AAATGTTAATCA--AGGGAG--ATAGAT-AACG--C-----------CTG-A-T--TGGA-C--GTATGC-CTAGGGT--AT---CTTTC-CGTCTG-T-TG--CCCC-G-
[2]	'DdiscoV12'	GAA-------ATGAGCC--AAATGTTAATCA--AGGGAG--ATAGAT-AACG--C-----------CTG-A-T--TGGA-C--GTATGC-CTAGGGT--AT---CTTTC-CGTCTG-T-TG--CCCC-G-
[3]	'DdATN119'	GAA-------ATGAGCC--AAATGTTAATCA--AGGGAG--ATAGAT-AACG--C-----------CTG-A-T--TGGA-C--GTATGC-CTAGGGT--AT---CTTTC-CGTCTG-T-TG--CCCC-G-
[4]	'DdDcitMex'	GAA-------ATGAGCC--AAATGTTAATCA--AGGGAG--ATAGAT-AACG--C-----------CTG-A-T--TGGA-C--GTATGC-CTAGGGT--AT---CTTTC-CGTCTG-T-TG--CCCC-G-
[5]	'DspHLM411'	AAG-------GT-A-CC-----T-TCC--CG---G-------TACTC-G-CG-CA-----------CTG-A-C--AG-A-C---TATAC-AT--------T----TATA-CG---G-T-A---CTCC-G-
[6]	'DsphaSib6'	--
[7]	'PaureumN36'	--
[8]	'DsphaGMN3'	AAG-------GT-A-CC-----T-TCC--CG---G-------TACTC-G-CG-CA-----------CTG-A-C--AG-A-C---TATAC-AT--------T----TATA-CG---G-T-A---CTCC-G-
[9]	'PviolacDNC1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[10]	'PviolacDNC3'	AAG-------GT-A-CC-----T-TCC--CG---G-------T-CTC-G-CG-CA-----------CTG-A-C--AG-A-C---TATAC-AT--------T----TATA-CG---G-T-----CTCC-G-
[11]	'DsphaSXN2'	AAG-------GT-A-CC-----T-TCC--CG---G-------TACTC-G-CG-CA-----------CTA-A-C--GG-A-C---TATAC-AT--------T----TATA-CG---G-T-A---CTCC-G-
[12]	'DrosAlab'	AAG-------GT-A-CC-----T-TCC--CG---G-------TACTC-G-CG-CT-----------CTG-A-C--GG-A-C---TATAC-AT--------T----TATA-CG---G-T-A---CTCC-G-
[13]	'DrosEDF3'	AAG-------GT-A-CC-----T-TCC--CG---G-------TACTC-A-CG-CT-----------CTG-A-C--GG-A-C---TATAC-AT--------T----TATA-CG---G-T-A---CTNC-G-
[14]	'DsphaHYY1'	AAG-------GT-A-CC-----T-TCC--CG---G-------TACTC-G-CG-CA-----------CTG-A-C--GG-A-C---TATAC-AT--------T----TATA-CG---G-T-A---CTCC-G-
[15]	'DrosIL'	AAG-------GT-A-CC-----T-TCC--CG---G-------TACTC-G-CG-CT-----------CTG-A-C--GG-A-C---TATAC-AT--------T----TATA-CG---G-T-A---CTCC-G-
[16]	'DmucBPR11'	AAG-------CTGA-CC-----T--CA--CG---G------TTAGTTTGTCG--A-----------CTAGA-T--TC-T-C---TATAC-AT--------T----TGTA-CG---G-T-A---CTCC-G-
[17]	'DmucBPR31'	AAG-------CTGA-CC-----T--CA--CG---G------TTAGTTTGTCG--A-----------CTAGA-T--TC-T-C---TATAC-AT--------T----TGTA-CG---G-T-A---CTCC-G-
[18]	'DmucDDS21'	AAG-------CTGA-CC-----T--CA--CG---G------TTAGTTTGTCG--A-----------CTAGA-T--TC-T-C---TATAC-AT--------T----TGTA-CG---G-T-A---CTCC-G-
[19]	'DmucPYR'	AAG-------CTGA-CC-----T--CA--CG---G------TTAGTTTGTCG--A-----------CTAGA-T--TC-T-C---TATAC-AT--------T----TGTA-CG---G-T-A---CTCC-G-
[20]	'DmucFLMN'	AAG-------CTGA-CC-----T--CA--CG---G------TTAGTTTGTCG--A-----------CTAGA-T--TC-T-C---TATAC-AT--------T----TGTA-CG---G-T-A---CTCC-G-
[21]	'DbrefTNC11'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[22]	'DmucHTL'	AAG-------CTGA-CC-----T--CA--CG---G------TTAGTTTGTCG--A-----------CTAGA-T--TC-T-C---TATAC-AT--------T----TGTA-CG---G-T-A---CTCC-G-
[23]	'DminGMN3'	--C-G-AATAATGAA-CTAGTTTCGTATAGGTTTAATTTACAAACTCTTATAATATGG-CTC---T
[24]	'DspWLD1'	AAG-------CTGA-CC-----T--CA--CG---G------TTAGTTTGTCG--A-----------CTAGA-T--TC-T-C---TATTC-AT--------T----TGTC-CG---G-T-T---CTCC-G-
[25]	'DimpBCN21'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[26]	'DspBDW112'	TT--------C-GA-CC-----T--CA--CA---G------TT-GAAT--CG--A-----------CTAGA-T---C-TTC---TTTAC-AT--------T----TGTA-CG---G-T-A---CTCC-G-
[27]	'DaurstBDW4'	ATGG------TTAT-CC-----T--CA--CG---GA-----TTTCAATATCG--A-----------CCA-A-T--GA-A-T---TATAC-AT--------T----TGTA-CG---G-T-A---CTCC-G-
[28]	'DimpEPN11'	ATGG------TTAT-CC-----T--CA--CG---GA-----TTTCAATATCG--A-----------CCA-A-T--GA-A-T---TATAC-AT--------T----TGTA-CG---G-T-A---CTCC-G-
[29]	'DmucorEW5'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[30]	'PviolSTR'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[31]	'PviolFCM'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[32]	'PviolATN1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[33]	'PviolATN'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[34]	'DpurpAT17'	ATTGG----CTTGA-CCA----T-ACC--CG---G-------AAC-C---C---C-----------CTG-G-A--TT-T-C---TATAC-AT--------T----TTTT-TG---G-T-A---CTCC-A-
[35]	'Dpurp1K'	ATTGG----CTTGA-CCA----T-ACC--CG---G-------AAC-C---C---C-----------CTG-G-A--TT-T-C---TATAC-AT--------T----TTTT-TG---G-T-A---CTCC-A-
[36]	'DpurpCRLC2'	ATT-------TT---CAA----T--CA--CA---G-------TGG---------T-----------ATG-G-------T-C---CCGG--AT--------T----TTTT-TG---G-T-A---CTCC-A-
[37]	'DcitrCU2A4'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[38]	'DcitrDdCR'	GAA-------ATGAGCC--AAATGTTAGTCA--AGCAAG--ATAAAT-AACG--C-----------CTG-A-T--AGGA-C--GTATGC-CTAGGGT--AT---CTTAC-CGTTTG-T-TG--CCCC-G-
[39]	'Ppal'	AAA------TATTAAAA--CATTGTCAGGTA--TGAAAA--TTAGTT-GTC--TCA----------C-G-ACT--T----CTAATATCCAATAG-----ATT--CAAAATTATTTAAT-TGGTTTCCAGT
[41]	'DminNWM12'	GTA-------GTGA-CC-----T-TTA--AG--AGA-----TTAACCTATTG-AA-----------ATA-A-T--TGTAAAATTTATAG-TTTTATA--GTAAACAATT-TG---GTTATG-TAACTTT-
[40]	'DminNWM12'	GAA-------ATGAGCC--AAATGTTAATCA--AGGGAG--ATAGAT-AACG--C-----------CTG-A-T--TGGA-C--GTATGC-CTAGGGT--AT---CTTTC-CGTCTG-T-TG--CCCC-G-
[42]	'DtenCu2G3'	ATA-------ATGT-----TGTTGTCATACAC-AGGGAA--GT-GAT-ATC---C-----------C---A-T--AA-A-C--TTAAG--AGAGAGA--AG---AACGA-CATCT--T-AG---TCG-G-
[43]	'PviolATN11'	AAACCATACTATTTGATGGCGTT-TCTTTTACCTGAAACTTTTAATC-AACATTCAATTGAAAAAGC-G-AATAATGAA-CTAGTTTCGTATAGGTTTAATTTACAAACTCTTATAATATGG-CTC---T
[44]	'Acytostelium'	AAA------TAAT--AA--CA-----A---A---G------TTAG--------------------C-G-G-------------TAT----T-G-----AT---CAAAA--A---AAT-----------
[45]	'DminATN16'	XXX-------XXXX-XX-----X-XXX--XX--XXX-----XXXXXXXXXXX-XX-----------XXX-X-X--XXXXXXXXXXXXXX-XXXXXXX--XXXXXXAATT-TG---GTTATG-TAACTTT-

Table: ITS 2 sequence data (series 4: 391-520)

	Taxa	sequence 40 41 42 43 44 45 46 47 48 49 50 51 52 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	-CAA-GGGGGGGCTTA-CTGGGTGACTTAGGCTACT-ATGTTGT-AAT--TCT--CTCGA-T--TT--GTC---AC--T-G-T-CATTCG-GCTAGCATT--TAGAGGTT-AAC-GTAATCA--ATATAC
[2]	'DdiscoV12'	-CAA-GGGGGGGCTTA-CTGGGTGACTTAGGCTACT-ATGTTGT-AAT--TCT--CTCGA-T--TT--GTC---AC--T-G-T-CATTCG-GCTAGCATT--TAGAGGTT-AAC-GTAATCA--ATATAC
[3]	'DdATN119'	-CAA-GGGGGGGCTTA-CTGGGTGACTTAGGCTACT-ATGTTGT-AAT--TCT--CTCGA-T--TT--GTC---AC--T-G-T-CATTCG-GCTAGCATT--TAGAGGTT-AAC-GTAATCA--ATATAC
[4]	'DdDcitMex'	-CAA-GGGGGGGCTTA-CTGGGTGACTTAGGCTACT-ATGTTGT-AAT--TCT--CTCGA-T--TT--GTC---AC--T-G-T-CATTCG-ACTAATGGT--TATGTATT-AAC-GTAATCA--ATATAC
[5]	'DspHLM411'	--TA--G------TAA-TTG-----C-TCA----C--A-GT--T---T--TCT--CAC---T--GA-CCAC---TT--T-A-T-TAAACT-ACTTCCATA--TGGATTTTAGTC-ATTAAGG--GTCG-C
[6]	'DsphaSib6'	--
[7]	'PaureumN36'	--
[8]	'DsphaGMN3'	--TA--G------TAA-TTG-----C-TCA----C--A-GT--T---T--TCT--CAC---T--GA-CCAC---TT--T-A-T-TAAACT-ACTTCCATA--TGGATTTTAGTC-ATTAAGG--GTCG-C
[9]	'PviolacDNC1	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[10]	'PviolacDNC3'	--TN--G------TAA-TTG-----C-TCA----C--A-GT--T---T--TCT--CAC---T--GA-CCAC---TT--T-A-T-TAAACT-ACTTCCATA--TGGATTTTAGTC-ATTAAGG--GTCG-C
[11]	'DsphaSXN2'	--TA--G------TAA-TTG-----C-TCA----C--A-GT--T---T--TCT--CAC---T--GA-CCAC---TT--T-A-T-TAAACT-ACTTCCATA--TGGATTTTAGTC-ATTAAGG--GTCG-C
[12]	'DrosAlab'	--TA--G------TAA-TTG-----C-TCA----C--A-GT--T---T--TCT--CAC---T--GA-CCAC---TT--T-A-T-TAAACT-ACTTCCATA--TGGATTTTAGTC-ATTAAGG--GTCG-C
[13]	'DrosEDF3'	--TA--N------TAA-TTG-----C-TCA----C--A-GT--T---T--TCT--CAC---T--GA-CCAC---NN--N-A-T-TAAACT-ACXXXXXXX--TGGATNTTMGTC-ATTAAGG--GTCG-C
[14]	'DsphaHYY1'	--TA--G------TAA-TTG-----C-TCA----C--A-GT--T---T--TCT--CAC---T--GA-CCAC---TT--T-A-T-TAAACT-ACTTCCATA--TGGATTTTAGTC-ATTAAGG--GTCG-C
[15]	'DrosIL'	--TA--G------TAA-TTG-----C-TCA----C--A-GT--T---T--TCT--CAC---T--GA-CCAC---TT--T-A-T-TAAACT-ACTTCCATA--TGGATTTTAGTC-ATTAAGG--GTCG-C
[16]	'DmucBPR11'	--TA--G------TAA-TTG-----C-TCA----C--A-GTC-A---T--TCT--TTA---T--AT-CGTC---TC--TGA-T-TAATCT-ATTTTCGTA--TAGTTTTT--TC-AACGTGG--GAC--C
[17]	'DmucBPR31'	--TA--G------TAA-TTG-----C-TCA----C--A-GTC-A---T--TCT--TTA---T--AT-CGTC---TC--TGA-T-TAATCT-ATTTTCGTA--TAGTTTTT--TC-AACGTGG--GAC--C
[18]	'DmucDDS21'	--TA--G------TAA-TTG-----C-TCA----C--A-GTC-A---T--TCT--TTA---T--AT-CGTC---TC--TGA-T-TAATCT-ATTTTCGTA--TAGTTTTT--TC-AACGTGG--GAC--C
[19]	'DmucPYR'	--TA--G------TAA-TTG-----C-TCA----C--A-GTC-A---T--TCT--TTA---T--AT-CGTC---TC--TGA-T-TAATCT-ATTTTCGTA--TAGTTTTT--TC-AACGTGG--GAC--C
[20]	'DmucFLMN'	--TA--G------TAA-TTG-----C-TCA----C--A-GTC-A---T--TCT--TTA---T--AT-CGTC---TC--TGA-T-TAATCT-ATTTTCGTA--TAGTTTTT--TC-AACGTGG--GAC--C
[21]	'DbrefTNC11'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[22]	'DmucHTL'	--TA--G------TAA-TTG-----C-TCA----C--A-GTC-A---T--TCT--TTA---T--AT-CGTC---TC--TGA-T-TAATCT-ATTTTCGTA--TAGTTTTT--TC-AACGTGG--GAC--C
[23]	'DminGMN3'	TTAAT-GT-T---AAT-TTG-ATTTTTAAAA------TTGTTAC-TAT--TCTT--TTG--T--AT-ATTCCAAACACT-GAT-AAAGAA-GC-AACATTA-TGAA-CATCAACGATAAAGTTTATCTTT
[24]	'DspWLD1'	--TA--G------TTA-TTG-----C-TCA----C--A-GTC-A---T--TCT--TTA---T--NT-CGTC---TC--TGA-T-TAATCT-ATTTCCGTC--TNGTTTTT--TC-AACGTGG--GAC--C
[25]	'DimpBCN21'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[26]	'DspBDW112'	--TA--G------TAA-TTG-----C-TCA----C--A-GTC-A---T--TCT--TTA---T--ATTTGTC---TC--TGA-T-TAACCT-ATTTTCGCA--TAGTTTTT--TC-AACGTGG--GAC--C
[27]	'DaurstBDW4'	--TA--G------TAA-TTG-----C-TCA----C--A-GTC-A---T--TGA--GGA---CCCAAACCTT---TT--TTA-T-TAAACT-ATTTTCTTA--TAGGTTAA--TC-ATCAAGG--GTT--T
[28]	'DimpEPN11'	--TA--G------TAA-TTG-----C-TCA----C--A-GTC-A---T--TGA--GGA---CCCAAACCTT---TT--TTA-T-TAAACT-ATTTTCTTA--TAGGTTAA--TC-ATCAAGG--GTT--T
[29]	'DmucorEW5'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[30]	'PviolSTR'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[31]	'PviolFCM'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[32]	'PviolATN1'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[33]	'PviolATN'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[34]	'DpurpAT17'	--TC--GG-----CAC-AT--------TTA----C--A-GT---------TCA--TAA---C---T--ATT---AT--T-A-T-TATATTTAACT-CTTAGTTAATTATT--TT-ATAGTTA--AACG-C
[35]	'Dpurp1K'	--TC--GG-----CAC-AT--------TTA----C--A-GT---------TCA--TAA---C---T--ATT---AT--T-A-T-TATATTTAACT-CTTAGTTAATTATT--TT-ATAGTTA--AACG-C
[36]	'DpurpCRLC2'	--TC--GG-----CAC-AT--------TTA----C--A-GT------G--TCA--AAA---A--GT--ATC---AT--T-G-A-TAGATT-CCCGGCG-AG-TCCTTCTT-GAT-ATTCTT---AACG-C
[37]	'DcitrCU2A4'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[38]	'DcitrDdCR'	-AAA-GGGGGGGCTTA-CTGGGTGACTTAGGCTACT-ATGAAGT-AAT--TCT--CTCGAGT--CC--TTT---AC--T-G-T-TGTTCA-ATTAGCATT--TATTAGTT-AAT-GTAATCA--ATATAC
[39]	'Ppal'	TTACTGGAGTGGCCAG-TTGGATTTTTTAAAGT-CT-TTGTT-CGTTTCGACTAAATT---T--TT-AGTG-GAACG-G-GAT-TGGACT-GA-AGCATT--TGCTTCTTTTTC-ATATAAA--AACTTT
[40]	'DminNWM12'	--TA--G------TAATCTA-----G-TAA----CATAGGT--T---TGATCA--TAG---TG-AT-GCTAT--TC--TAG-TGTTTTGT-AAAGACATTGAAAGTAGAAACTCTTTAGTAA--GATT-C
[41]	'DminNWM12'	-CAA-GGGGGGGCTTA-CTGGGTGACTTAGGCTACT-ATGTTGT-AAT--TCT--CTCGA-T--TT--GTC---AC--T-G-T-CATTCG-GCTAGCATT--TAGAGGTT-AAC-GTAATCA--ATATAC
[42]	'DtenCu2G3'	-CGA-GGGGGAGGTAA-CT----AACGTAATC--CT-A-GAC-C-AAA--CCTT-ATCG--T--CA--TTC---A---T-A-T-CG--CG-GC-ACCA----TACCGCAA-ACC-G-ATTGT--ATGTAC
[43]	'PviolATN11'	TTAAT-GT-T---AAT-TTG-ATTTTTAAAA------TTGTTAC-TAT--TCTT--TTG--T--AT-ATTCCAAACACT-GAT-AAAGAA-GC-AACATTA-TGAA-CATCAACGATAAAGTTTATCTTT
[44]	'Acytostelium'	---C--GA-T----A--TTG---------AA---------TT-C------ACT---TT---T--AT---T-----C------T-T----T--A-A-CAT-----C--CTTAATG-ATATTG-----C---
[45]	'DminATN16'	--TA--G------TAATCTA-----G-TAA----CATAGGT--T---TGATCA--TAG---TG-AT-GCTAT--TC--TAG-TGTTTTGT-AAAGACATTGAAAGTAGAAACTCTTTAGTAA--GATT-C

Table: ITS 2 sequence data (series 5: 521-650)

	Taxa	sequence 53 54 55 56 57 58 59 60 61 62 63 64 65 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	--A-TT-GG-TCA---CAAAA-AACTT--T---T-T-A----ATG----G---TGG--AAGG--
[2]	'DdiscoV12'	--A-TT-GG-TCA---CAAAA-AACTT--T---T-T-A----ATG----G---TGG--AAGG--
[3]	'DdATN119'	--A-TT-GG-TCA---CAAAA-AACTT--T---T-T-A----ATG----G---TGG--AAGG--
[4]	'DdDcitMex'	--A-CT-GG-TCA---CAAAA-AACTT--T---T-T-A----ATG----G---TGG--AAAG--
[5]	'DspHLM411'	--T-TT-AG--CG---AGTTT-AACTT--T---AGT-A----AT-----C---TAAC-AAT---
[6]	'DsphaSib6'	--
[7]	'PaureumN36'	--
[8]	'DsphaGMN3'	--T-TT-AG--CG---AGTTT-AACTT--T---AGT-A----AT-----C---TAAC-AAT---
[9]	'PviolacDNC1'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[10]	'PviolacDNC3'	--T-TT-AG--CG---AGTTT-AACTT--T---AGT-A----AT-----N---TAAC-AAT---
[11]	'DsphaSXN2'	--T-TT-AG--CG---AGTTT-AACTT--T---AGT-A----AT-----C---TAA--AAT---
[12]	'DrosAlab'	--T-TT-AG--CG---AGTTT-AACTT--T---AGT-A----AT-----C---TAAC-AAT---
[13]	'DrosEDF3'	--T-CT-AG--CG---AGTTT-AACTT--T---AGT-A----AT-----C---TAAC-AAT---
[14]	'DsphaHYY1'	--T-TT-AG--CG---AGTTT-AACTT--T---AGT-A----AT-----C---TAAC-AAT---
[15]	'DrosIL'	--T-TT-AG--CG---AGTTT-AACTT--T---AGT-A----AT-----C---TAA--AAT---
[16]	'DmucBPR11'	--TAAT-AG--AA---AGTCT-AACTT-GT-CAAGT-A----GA-----A---AACCGAAT---
[17]	'DmucBPR31'	--TAAT-AG--AA---AGTCT-AACTT-GT-CAAGT-A----GA-----A---AACCGAAT---
[18]	'DmucDDS21'	--TAAT-AG--AA---AGTCT-AACTT-GT-CAAGT-A----GA-----A---AACCGAAT---
[19]	'DmucPYR'	--TAAT-AG--AA---AGTCT-AACTT-GT-CAAGT-A----GA-----A---AACCGAAT---
[20]	'DmucFLMN'	--TAAT-AG--AA---AGTCT-AACTT-GT-CAAGT-A----GA-----A---AACCGAAT---
[21]	'DbrefTNC11'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[22]	'DmucHTL'	--TAAT-AG--AA---AGTCT-AACTT-GT-CAAGT-A----GA-----A---AACCGAAT---
[23]	'DminGMN3'	--A-TT-GGTTCAA--CAGTT-GTATA--T-NNT-TCG--
[24]	'DspWLD1'	--TATT-NN--AA---AGTTT-AACTCCGT-CAAGN-N----NA-----A---AACCGATT---
[25]	'DimpBCN21'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[26]	'DspBDW112'	--TTAT-TG--AA---AGTCT-AACTT-GT-CAAGT-A----GA-----A---AACCGAAT---
[27]	'DaurstBDW4'	G-TCAA-TG--GA---A-TTT-AAATT-GTACAA-T-A-----C-----A---AACCGAAT---
[28]	'DimpEPN11'	G-TCAA-TG--GA---A-TTT-AAATT-GTACAA-T-A-----C-----A---AACCGAAT---
[29]	'DmucorEW5'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[30]	'PviolSTR'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[31]	'PviolFCM'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[32]	'PviolATN1'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[33]	'PviolATN'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[34]	'DpurpAT17'	----AT-AG--GT---TC-CT-AACCT--T-C-A-A-A----C----------TAAC---T---
[35]	'Dpurp1K'	----AT-AG--GT---TC-CT-AACCT--T-C-A-A-A----C----------TAAC---T---
[36]	'DpurpCRLC2'	--T-AA-AG--G----TCTCTGAACCT--T-C-A-A-A----CC-----C---TTAC--AT---
[37]	'DcitrCU2A4'	--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[38]	'DcitrDdCR'	--A-TT-GG-TCA---CAAAA-AACTT--T---T-T-A----NTG----G---TNG--AAGA--
[39]	'Ppal'	--A-ATTGGTTAAACCCAGTA--ACTA--T-AGT-T-G----ATAAATCGACTTAGC-TATTA---
[40]	'DminNWM12'	ATTAATTTG--AA---ACCCA-AACAT--T--GA-T-T----CT-----C---TTTT-TTTGG---
[41]	'DminNWM12'	--A-TT-GG-TCA---CAAAA-AACTT--T---T-T-A----ATG----G---TGG--AAGG--
[42]	'DtenCu2G3'	--A-------TCT------TA-TCCTT--T---A-A-A----AAG----GG--TGG--TGTG--
[43]	'PviolATN11'	--A-TT-GGTTCAA--CAGTT-GTATA--T-GTT-TCGCCTCATXXXXXXXXXXXXX-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[44]	'Acytostelium'	--A-AT-G--------CAGTT--CCT-------T-T-G----AT-----G--TT----T-TT--
[45]	'DminATN16'	ATTAATTTG--AA---ACCCA-AACAT--T--GA-T-T----CT-----C---TTTT-TTTGG---

Table: ITS 2 sequence data (series 6: 651-780)

	Taxa	sequence 66 67 68 69 70 71 72 73 74 75 76 77 78 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	--TAAA---T
[2]	'DdiscoV12'	--TAAA---T
[3]	'DdATN119'	--TAAA---T
[4]	'DdDcitMex'	--TAAA---T
[5]	'DspHLM411'	--TGA----T
[6]	'DsphaSib6'	--
[7]	'PaureumN36'	--
[8]	'DsphaGMN3'	--TGA----T
[9]	'PviolacDNC1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[10]	'PviolacDNC3'	--TGA----T
[11]	'DsphaSXN2'	--TGA----T
[12]	'DrosAlab'	--TGA----T
[13]	'DrosEDF3'	--TGA----T
[14]	'DsphaHYY1'	--TGA----T
[15]	'DrosIL'	--TGA----T
[16]	'DmucBPR11'	--TGA----T
[17]	'DmucBPR31'	--TGA----T
[18]	'DmucDDS21'	--TGA----T
[19]	'DmucPYR'	--TGA----T
[20]	'DmucFLMN'	--TGA----T
[21]	'DbrefTNC11'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[22]	'DmucHTL'	--TGA----T
[23]	'DminGMN3'	--
[24]	'DspWLD1'	--TGA----T
[25]	'DimpBCN21'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[26]	'DspBDW112'	--TGA----T
[27]	'DaurstBDW4'	--TGA----T
[28]	'DimpEPN11'	--TGA----T
[29]	'DmucorEW5'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[30]	'PviolSTR'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[31]	'PviolFCM'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[32]	'PviolATN1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[33]	'PviolATN'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[34]	'DpurpAT17	--TGA----T
[35]	'Dpurp1K'	--TGA----T
[36]	'DpurpCRLC2'	--TGA----T
[37]	'DcitrCU2A4'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[38]	'DcitrDdCR'	--TAAA---T
[39]	'Ppal'	---ATGAAA-GT
[40]	'DminNWM12'	---CCCTGAC---A
[41]	'DminNWM12'	--TAAA---T
[42]	'DtenCu2G3'	--AGAA---C
[43]	'PviolATN11'	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXATNAAATGT
[44]	'Acytostelium'	--TGAA---T
[45]	'DminATN16'	---CCCTGAC---A

Table: ITS 2 sequence data (series 7: 781-910)

	Taxa	sequence 79 80 81 82 83 84 85 86 87 88 89 90 91
		1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	TTAATG-T-C-A------C-T-AAT-----AATC-GGTCCGT--CAAA-TTGG-T--T--A--G-T-TAGAGGGCAT--TAAGCGTG--CT----G----AAAGC--TATTAGAAAGGA--TCAAT-TG-
[2]	'DdiscoV12'	TTAATG-T-C-A------C-T-AAT-----AATC-GGTCCGT--CAAA-TTGG-T--T--A--G-T-TAGAGGGTAT--TAAGCGTG--CT----G----AAAGC--TATTAGAAAGGA--TCAAT-TG-
[3]	'DdATN119'	TTAATG-T-C-A------C-T-AAT-----AATC-GGTCCGT--CAAA-TTGG-T--T--A--G-T-TAGAGGGCAT--TAAGCGTG--CT----G----AAAGC--TATTAGAAAGGA--TCAAT-TG-
[4]	'DdDcitMex'	TTAATG-T-C-A------C-T-AAT-----AATC-GGTCCGT--CAAA-TTGG-T--T--A--G-T-TAGAGGGCAT--TGAGAGTN--CT----G----AAAGC--CATTAAAAAGGA--TCAGT-TG-
[5]	'DspHLM411'	TTA----T-C-A------C-T-A-------GTTA-G-AGAG---CTAT-AAC--T--T--A----A-CAGA----CC--TA----T---T-----G----CAA-C---CT------G-AC-TTTAA-T--
[6]	'DsphaSib6'	--
[7]	'PaureumN36'	--
[8]	'DsphaGMN3'	TTA----T-C-A------C-T-A-------GTTA-G-AGAG---CTAT-GAC--T--T--A----A-CAGA----CC--TA----T---C-----G----CAA-C---CT------G-AC-TTTAA-T--
[9]	'PviolacDNC1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[10]	'PviolacDNC3'	TTA----T-C-A------C-T-A-------GTTA-G-AGAG---CTAT-GAC--T--T--A----A-CAGA----CC--TA----T---C-----G----CAA-C---CT------G-AC-TTTAA-T--
[11]	'DsphaSXN2'	TTA----T-C-A------C-T-A-------GTTA-G-AGAG---CTAT-AAC--T--T--A----A-CAGA----CC--TA----T---T-----G----CAA-C---CT------G-AC-TTTAA-T--
[12]	'DrosAlab'	TTA----T-C-A------C-T-A-------GTTA-G-TGCG---CTAA-GAC--C--T--A----A-CAGA----CC--TA----T---C-----G----CAA-C---CT------G-AC-TCTAA-T--
[13]	'DrosEDF3'	TTA----T-C-A------C-T-A-------GTTA-G-TGCG---CTAA-GAC--C--T--A----A-CAGA----CC--TA----T---C-----G----CAA-C---CT------G-AC-TCTAA-T--
[14]	'DsphaHYY1'	TTA----T-C-A------C-T-A-------GTTA-G-AGCG---CTAT-GAC--T--T--A----A-CAGA----CC--TA----T---T-----G----CAA-C---CT------G-AC-TTTAA-T--
[15]	'DrosIL'	TTA----T-C-A------C-T-A-------GTTA-G-TGCG---CTAA-GAC--C--T--A----A-CAGA----CC--TA----T---C-----G----CAA-C---CT------G-AC-TCTAA-T--
[16]	'DmucBPR11'	TTA----T-C-A------C-T-A-------GTT----TTC----CAAA-GAC--T--G--A----C-CTGATAAGTA--TA----T---CT----G----GAA-C---CTA-----G-A--TTAAA-C--
[17]	'DmucBPR31'	TTA----T-C-A------C-T-A-------GTT----TTC----CAAA-GAC--T--G--A----C-CTGATAAGTA--TA----T---CT----G----GAA-C---CTA-----G-A--TTAAA-C--
[18]	'DmucDDS21'	TTA----T-C-A------C-T-A-------GTT----TTC----CAAA-GAC--T--G--A----C-CTGATAAGTA--TA----T---CT----G----GAA-C---CTA-----G-A--TTAAA-C--
[19]	'DmucPYR'	TTA----T-C-A------C-T-A-------GTT----TTC----CAAA-GAC--T--G--A----C-CTGATAAGTA--TA----T---CT----G----GAA-C---CTA-----G-A--TTAAA-C--
[20]	'DmucFLMN'	TTA----T-C-A------C-T-A-------GTT----TTC----CAAA-GAC--T--G--A----C-CTGATAAGTA--TA----T---CT----G----GAA-C---CTA-----G-A--TTAAA-C--
[21]	'DbrefTNC11'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[22]	'DmucHTL'	TTA----T-C-A------C-T-A-------GTT----TTC----CAAA-GAC--T--G--A----C-CTGATAAGTA--TA----T---CT----G----GAA-C---CTA-----G-A--TTAAA-C--
[23]	'DminGMN3'	--
[24]	'DspWLD1'	TTA----T-N-A------C-T-A-------GTT----NTC----CAAA-GAC--T--G--A----C-CTGCTNAGCC--TN----T---CT----G----GTT-C---CTA-----G-A--TNNAA-C--
[25]	'DimpBCN21'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[26]	'DspBDW112'	TTA----T-C-A------C-T-ATG-----GTT----TTC----AAAA-GAC--T--A--A----T-CTGATGAGTA--TG----T---TT----G----GAA-C---CTA-----G-A--TTAAA-C--
[27]	'DaurstBDW4'	TTA----T-C-A------C-T-A-------GTT----TGT----AGAA-TAC--T--A--A----T-CAAATGAAAT--TT----T---CA----------AA-C---CT------G-A--TT-AA-C--
[28]	'DimpEPN11'	TTA----T-C-A------C-T-A-------GTT----TGT----AGAA-TAC--T--A--A----T-CAAATGAAAT--TT----T---CA----------AA-C---CT------G-A--TT-AA-C--
[29]	'DmucorEW5'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[30]	'PviolSTR'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[31]	'PviolFCM'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[32]	'PviolATN1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[33]	'PviolATN'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[34]	'DpurpAT17'	TTA----T-C-AA-----C-T-A-------GC-----T-AG---AT-A-ACC--T--GGTT----C-CTGTTTT-TC--TA----T---A-----T----ATT-A---TTT-----A-ATGCTTGA-CG-
[35]	'Dpurp1K'	TTA----T-C-AA-----C-T-A-------GC-----T-AG---AT-A-ACC--T--GGTT----C-CTGTTTT-TC--TA----T---A-----T----ATT-A---TTT-----A-ATGCTTGA-CG-
[36]	'DpurpCRLC2'	TTA----T-C-AT-----T-TAA-------GCG----TGCG---ATAA-ACC--A--G-AA----C-CTGTTTT-G---AA----T---AG----C----CAA-A---ATT-----G-ACGTTTGAGCC-
[37]	'DcitrCU2A4'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[38]	'DcitrDdCR'	TTAATG-T-C-A------C-T-AAT-----AATC-GGTCCGT--CAAA-TTGG-T--T--A--G-T-TAGAGGGCATTATAAG-GTA--TT----G----GAAGC--CATTAAAAAGGA--TCAAT-TG-
[39]	'Ppal'	TTTAA--A-C-AT-----C---AA------ATT--GAAAAGT--CTAACTT---TCCA--A-CG-T-T-TAGAA--T--TAGTAGTTT-CT----G-----ACGCG-TCTTT-TGTGTAG-ACTAT-AG-
[40]	'DminNWM12'	TTA----TGCGAT-----CATGAAA-----GTAA-GCTCC----CTG--TTC--T--T--A----T-CTCA-ACCTC--TAG---TGAGCT----A----ATAAC---TTC-----ATAC-TAAAT-T--
[41]	'DminNWM12'	TTAATG-T-C-A------C-T-AAT-----AATC-GGTCCGT--CAAA-TTGG-T--T--A--G-T-TAGAGGGCAT--TAAGCGT---CT----G----AAAGC--TATTAGAAAGGA--TCAAT-TG-
[42]	'DtenCu2G3'	GGATTGCT-C-A------C---ATT-----AA---G--CCAT--CT---TT---T--T--T--G-C-TGGAGGG------AGG-GT---AT----G----C---C--CATTT----G----TCCAT-T--
[43]	'PviolATN11'	TGAACGCA-C-ATGATGGC---ATTGGCCCTTTGGGTTAAGTGTCATACTTGGGTGAG--AGCGATCTCTATAT--T--AAATATTAAACTTTTTATTAATACAAATTTTTT-TGTATAA-ACAAT-CGT
[44]	'Acytostelium'	T------A-C--------C-----------AT------AAG----TG--TT---T-----A-C--T-T-TA--------------T---C-----G-----ACGA--TCT------------CT------
[45]	'DminATN16'	TTA----TGCGAT-----CATGAAA-----GTAA-GCTCC----CTG--TTC--T--T--A----T-CTCA-ACCTC--TAG---TGAGCT----A----ATAAC---TTC-----ATAC-TGAAT-T–

Table: ITS 2 sequence data (series 8: 910-1040)

	Taxa	sequence 92 93 94 95 96 97 98 99 100 101 102 103 104 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	-T-GT---TG-A-T-TCGA--------C----CT----AT---CT-CTAA--T-A-TG-TTAG--T-CGG-GTA-TG-TCC----GG--CA-----G-T---T-TAGC-GGCA-GGG-GGAACG----G-
[2]	'DdiscoV12'	-T-GT---TG-A-T-TCGA--------C----CT----AT---CT-CTAA--T-A-TG-TTAG--T-CGG-GTA-TG-TCC----GG--CA-----G-T---T-TAGC-GGCA-GGG-GGAACG----G-
[3]	'DdATN119'	-T-GT---TG-A-T-TCGA--------C----CT----AT---CT-CTAA--T-A-TG-TTAG--T-CGG-GTA-TG-TCC----GG--CA-----G-T---T-TAGC-GGCA-GGG-GGAACG----G-
[4]	'DdDcitMex'	-T-GT---TG-A-T-TCAA--------C----CT----AT---AT-ATAA--T-T-TG-TTAG--T-CGG--TA-TG-TCC----GG--CA-----G-T---T-TAXX-XXXX-XXXXXXXXXX----X-
[5]	'DspHLM411'	-TCGG---A--T-T-TAAA--------C----CT----AT---AT--TGTG-TGT-TA-GT-G--T-A-G--AA-TA-ACA----AT--CT-----G-T---T-TAGT---C--GG----TTTG----A-
[6]	'DsphaSib6'	--
[7]	'PaureumN36'	--
[8]	'DsphaGMN3'	-TCGG---A--T-T-TAAA--------C----CT----AT---AT--TGTG-TGC-CA-GT-G--T-A-G--AA-TA-ACA----AT--CT-----G-T---T-TAGT---C--GG----TTTG----A-
[9]	'PviolacDNC1	-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[10]	'PviolacDNC3'	-TCGG---A--T-T-TAAA--------C----CT----AT---AT--TGTG-TGC-CA-GT-G--C-A-G--AA-TA-ACA----AT--CT-----G-T---T-TAGT---C--GG----TTTG----A-
[11]	'DsphaSXN2'	-TCGG---A--T-T-TAAA--------C----CT----AT---AT--TGTG-TGT-CG-GT-G--T-A-G--AA-TA-ACA----AT--CT-----G-T---T-TAGT---C--GG----TTTG----A-
[12]	'DrosAlab'	-TCGG---A--T-T-TAAA--------C----CT----AT---AT--TGTG-TGC-CA-GT-G--T-T-G--AA-TA-ACA----AT--CT-----G-T---T-TAGT---C--GG----TTTG----A-
[13]	'DrosEDF3'	-TCGG---A--T-T-TAAA--------C----CT----AT---AT--TGTG-TGC-CA-GT-G--T-T-G--AA-TA-ACA----AT--CT-----G-T---T-TAGT---C--GG----TTTG----A-
[14]	'DsphaHYY1'	-TCGG---A--T-T-TAAA--------C----CT----AT---AT--TGTG-TGC-CA-GT-G--T-A-G--AA-TA-ACA----AT--CT-----G-T---T-TAGT---C--GG----TTTG----A-
[15]	'DrosIL'	-TCGG---A--T-T-TAAA--------C----CT----AT---AT--TGTG-TGC-CA-GT-G--T-T-G--AA-TA-ACA----AT--CT-----G-T---T-TAGT---C--GG----TTTG----A-
[16]	'DmucBPR11'	-T-G----A--T-TATTAA--------T----CT----GT---CA--GAT--T-C-AATAT-G--C-T-G--AA-TA-ACA----AAA-CT-----G-T---T-TAGT---T--GG----TTCA----G-
[17]	'DmucBPR31'	-T-G----A--T-TATTAA--------T----CT----GT---CA--GAT--T-C-AATAT-G--C-T-G--AA-TA-ACA----AAA-CT-----G-T---T-TAGT---T--GG----TTCA----G-
[18]	'DmucDDS21'	-T-G----A--T-TATTAA--------T----CT----GT---CA--GAT--T-C-AATAT-G--C-T-G--AA-TA-ACA----AAA-CT-----G-T---T-TAGT---T--GG----TTCA----G-
[19]	'DmucPYR'	-T-G----A--T-TATTAA--------T----CT----GT---CA--GAT--T-C-AATAT-G--C-T-G--AA-TA-ACA----AAA-CT-----G-T---T-TAGT---T--GG----TTCA----G-
[20]	'DmucFLMN'	-T-G----A--T-TATTAA--------T----CT----GT---CA--GAT--T-C-AATAT-G--C-T-G--AA-TA-ACA----AAA-CT-----G-T---T-TAGT---T--GG----TTCA----G-
[21]	'DbrefTNC11'	-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[22]	'DmucHTL'	-T-G----A--T-TATTAA--------T----CT----GT---CA--GAT--T-C-AATAT-G--C-T-G--AA-TA-ACA----AAA-CT-----G-T---T-TAGT---T--GG----TTCA----G-
[23]	'DminGMN3'	--
[24]	'DspWLD1'	-T-G----N--T-TNTTAA--------T----NT----GN---CA--NAT----CNAATAT-N--C-T-G--AA-TA-NCA----AAA-CT-----G-C---T-TANT---CC-GG----TTCA----G-
[25]	'DimpBCN21'	-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[26]	'DspBDW112'	-T-G----A--T-TATTAA--------T----CT----AT---CA--GAT--T-C-AGTGT-A--T-T-G--AA-TA-ACA----AAAACT-----G-T---T-TAGT---T--GG----TTCG----A-
[27]	'DaurstBDW4'	-TTGGGA-A--T-AATTAA--------A----TT----AT---AT--TAA--T-C-GGTTT-GACC-T----CA-TATATA----AAA-CT-----G-T---T-TAGT---T--GG----TAAA----T-
[28]	'DimpEPN11'	-TTGGGA-A--T-AATTAA--------A----TT----AT---AT--TAA--T-C-GGTTT-GACC-T----CA-TATATA----AAA-CT-----G-T---T-TAGT---T--GG----TAAA----T-
[29]	'DmucorEW5'	-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[30]	'PviolSTR'	-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[31]	'PviolFCM'	-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[32]	'PviolATN1'	-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[33]	'PviolATN'	-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[34]	'DpurpAT17'	-T-GT---T--A-TATTAT--------C----ATAATAAT---ATTATGAGTTTA-AC-CT-G--TATAG--TT-GT-TCATATTAG--CA-----GGA---AGACGTG-ATCGGG----CCTG----T-
[35]	'Dpurp1K'	-T-GT---T--A-TATTAT--------C----ATAATAAT---ATTATGAGTTTA-AC-CT-G--TATAG--TT-GT-TCATATTAG--CA-----GGA---AGACGTG-ATCGGG----CCTG----T-
[36]	'DpurpCRLC2'	-T-GT---T--T-TCT--T--------C----AT-------------TGA--T-C-ACTCT----T-T-G--AA--A-TCC----CG--CT-----G-T---T-TCGT---T--GG----CCAG----T-
[37]	'DcitrCU2A4'	-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[38]	'DcitrDdCR'	-T-GT---TG-A-T-TCGA--------C----CT----AT---TA-ATAA--A-T-TG-TTGG--T-TGGCGTA-T----C----GG--CA-----G-----T-TAGC-GGCA-GGGTAGCACG----G-
[39]	'Ppal'	CA-GA---AA-AGT-TTAA--------C---GCT----AA---CA-ATT---T-T-TGTTT----T-TGAAAAAGTA-GTA----GT--CT----AG-T---A--AGT--TCT-TT--C-TCTA----G-
[40]	'DminNWM12'	-TAGT---A--ACTTTTAA--------A----TT----TT--AAT--TGATAT-C-AA-TT-G--T-A-GTAAATTAATCA----AT--C------GTT---TGTATT---AAAGG---ATCCAT---TA
[40]	'DminNWM12'	-T-GT---TG-A-T-TCGA--------C----CT----AT---CT-CTAA--T-A-TG-TTAG--T-CGG-GTA-TG-TCC----GG--CA-----G-T---T-TAGC-GGCA-GGG-GGAACG----G-
[42]	'DtenCu2G3'	-T--T---T----T-TC-A--------C----AT----AT---CT-CGCA--T-T-AG-AT----T-CGG----------C--------CA-----------T-TAAT-TTTT-GG----TCC-------
[43]	'PviolATN11'	TT-GATATAATAGT-TTAATTTTTAGTCTCTGATTAA-AAGTTAA-ATTA--T-T-TTTATAG--T-TGATCTA-TA-GTA----GT--CTTTGCAG-TGGTA-TAATCGACT-TTGGTTTCAATTTCGA
[44]	'Acytostelium'	CA----------------A--------C---GAT----------A-A-----T------CT----T-TGCAAAA--A---A--------CT-----G--------A-T--ACA-TT----T---------
[45]	'DminATN16'	-TAGT---A--ACTTTTAA--------C----TT----TT--AAT--TGATAT-C-AA-TT-G--T-A-GTAAATTAATCA----AT--C------GTT---TGTATT---AAAGG---ATCCAT---TA

Table: ITS 2 sequence data (series 9: 1040-1170)

	Taxa	sequence 105 106 107 108 109 110 111 112 113 1140 115 116 117 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	T-GTA--C-------GC-----GCTGG-GT-T---TC---TGGC-----CA---C-----CGT-TG--CAGTA----G--C--A----GG--TAAGATC-TGCGTAATACTAGTACTTGTCCG--AAAGA
[2]	'DdiscoV12'	T-GTA--C-------GC-----GCTGG-GT-T---TC---TGGC-----CA---C-----CGT-TG--CAGTA----G--C--A----GG--TAAGATC-TGCGTAATACTAGTACTTGTCCG--AAAGA
[3]	'DdATN119'	T-GTA--C-------GC-----GCTGG-GT-T---TC---TGGC-----CA---C-----CGT-TG--CAGTA----G--C--A----GG--TAAGATC-TGCGTAATACTAGTACTTGTCCG--AAAGA
[4]	'DdDcitMex'	X-XXX--X-------XX-----XXXXX-XX-X---XX---XXXX-----XX---X-----XXX-XX--XXXXX----X--X--X----XX--XXXXXXX-XXXXXXXXXXXXXXXXXXXXXXX--XXXXX
[5]	'DspHLM411'	T-AAG--C-------AT-----GGAG-----T---GC---T--A-----CT---C-----CTA-T---TAGT--------C-----------T--TTT--TAT-TAAACC-CAT----GAC----T----
[6]	'DsphaSib6'	--CC-CAT----GAC----T----
[7]	'PaureumN36'	--
[8]	'DsphaGMN3'	T-AAG--C-------AT-----GGAG-----T---GC---T--A-----CT---C-----CTA-T---TAGT--------C-----------T--TTT--TAT-TAAACC-CAT----GAC----T----
[9]	'PviolacDNC1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[10]	'PviolacDNC3'	T-AAG--C-------AT-----GGAG-----T---GC---T--A-----CT---C-----CTA-T---TACT--------C-----------T--TTT--TAT-TAAACC-CAT----GAC----T----
[11]	'DsphaSXN2'	T-AAG--C-------AT-----GGAG-----T---GC---T--A-----CT---C-----CTA-T---TAGT--------C-----------T--TTT--TAT-TAAACC-CAT----GAC----T----
[12]	'DrosAlab'	T-AAG--C-------AT-----GGAG-----T---GC---T--T-----CT---C-----CTA-T---TAGT--------C-----------T--TTT--TAT-TAAACC-CAT----GAC----T----
[13]	'DrosEDF3'	T-AAG--C-------AT-----GGAG-----T----C---T--T-----CT---C-----CTA-T---TAGT--------C-----------T--TTT--TAT-TAAACC-CAT----GAC----T----
[14]	'DsphaHYY1'	T-AAG--C-------AT-----GGAG-----T---GC---T--A-----CT---C-----CTA-T---TAGT--------C-----------T--TTT--TAT-TAAACC-CAT----GAC----T----
[15]	'DrosIL'	T-AAG--C-------AT-----GGAG-----T---GC---T--T-----CT---C-----CTA-T---TAGT--------C-----------T--TTT--TAT-TAAACC-CAT----GAC----T----
[16]	'DmucBPR11'	T-TAG--C-------TT-----AGAG-----T---GC---T--A-----CT---C-----TTA-T---TAGT--------C-----------T--ATT--TAC-TGAACC-CAT----GAC----T----
[17]	'DmucBPR31'	T-TAG--C-------TT-----AGAG-----T---GC---T--A-----CT---C-----TTA-T---TAGT--------C-----------T--ATT--TAC-TGAACC-CAT----GAC----T----
[18]	'DmucDDS21'	T-TAG--C-------TT-----AGAG-----T---GC---T--A-----CT---C-----TTA-T---TAGT--------C-----------T--ATT--TAC-TGAACC-CAT----GAC----T----
[19]	'DmucPYR'	T-TAG--C-------TT-----AGAG-----T---GC---T--A-----CT---C-----TTA-T---TAGT--------C-----------T--ATT--TAC-TGAACC-CAT----GAC----T----
[20]	'DmucFLMN'	T-TAG--C-------TT-----AGAG-----T---GC---T--A-----CT---C-----TTA-T---TAGT--------C-----------T--ATT--TAC-TGAACC-CAT----GAC----T----
[21]	'DbrefTNC11'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[22]	'DmucHTL'	T-TAG--C-------TT-----AGAG-----T---GC---T--A-----CT---C-----TTA-T---TAGT--------C-----------T--ATT--TAC-TGAACC-CAT----GAC----T----
[23]	'DminGMN3'	--
[24]	'DspWLD1'	T-TCN--C-------TT-----ANAG-----T---GC---TT-T-----CTN--C-----CTN-T---TNCC--------C-----------T--ATC--CNCGTGAACC-NNT----NAC----T----
[25]	'DimpBCN21'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[26]	'DspBDW112'	T-TAG--C-------AT-----AGAG-----T---TT---TATT-----ATT--C-----TTT-T---TAGA--------C-----------T--ATT---AT-TGAACC-CAT----GAC----T----
[27]	'DaurstBDW4'	T-TAG--C-------AT-----AGAG-----T---GT---T--A-----CT---C-----TTT-T---TAGT--------C-----------T--AAA--TA--TG--CC-CAT----AAC----T----
[28]	'DimpEPN11'	T-TAG--C-------AT-----AGAG-----T---GT---T--A-----CT---C-----TTT-T---TAGT--------C-----------T--AAA--TA--TG--CC-CAT----AAC----T----
[29]	'DmucorEW5'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[30]	'PviolSTR'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[31]	'PviolFCM'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[32]	'PviolATN1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[33]	'PviolATN'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[34]	'DpurpAT17'	C-GGGGGCGTAAAA-ACCCCCGAGTGAAAAATATAGC---C--G-----CTGGTCGTT--CTACT---AAGA--------C-----------T--AAT--TT--TG-ATC-CGT----GAA----TCT-T
[35]	'Dpurp1K'	C-NGGGGCGTAAAA-ACCCCCGAGGGAAAAATATAGC---C--G-----CTGGTCGTT--CTACT---AAGA--------C-----------T--AAT--TT--TG-ATC-CGG----GAA----TCT-T
[36]	'DpurpCRLC2'	C-NAG--C-------ACA----AGGG-----TAACGC---C--C-----CT---CG-----T--T---AAGT--------C-----------T--AAA--CT---G-GTC-CGT-----AA----T----
[37]	'DcitrCU2A4'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[38]	'DcitrDdCR'	T-GTA--C-------GC-----GCTGG-GT-T---TC---TGGC-----CA---C-----CGT-CG--CAGT-----G--C--A----GG--TAAGATC-TGCGTAATACTAGTACTTGTCCA--AAAGA
[39]	'Ppal'	T-AAA--C-------TT-----AAAG---T-T---TCT--TAAC-G---CT---T-----CGA-G---CAGTT----A--C--A---AGA-CT--TTT--TA--AGATTC-AATT--TGACTA--TCA-A
[40]	'DminNWM12'	CATTG--T-------ATG---AGGAG---ATTTAAGAA--T--TG----AT---C-----TTA-A---AACA--------C---------AAT--TTT--TAXXXXXXXXXXXXXXXXXXXXX--XXXXX
[41]	'DminNWM12'	T-GTA--C-------GC-----GCTGG-GT-T---TC---TGGC-----CA---C-----CGT-TG--CAGTA----G--C--A----GG--TAAGATC-TGCGTAATACTAGTACTTGTCCG--AAAGA
[42]	'DtenCu2G3'	T-TTA--A-------GT-----GAAGA-AAGT---AC---TG-T-----AT---C-----CG--C---CA-T-----A--C--A----G---T--GAT--T---TAATTC--GTTC--GGCCG--TCAAA
[43]	'PviolATN11'	TCAAAC-CATACTATTT-----GATGGCGT-T---TCTTTTACCTGAAACT---TTTAATCAA-CATTCAATTGAAAAAGCGAATAATGAACTAGTTTCGTAT-AGGTTT-AATT--T-ACAAACTCTTA
[44]	'Acytostelium'	T-AAT--C--------------GATG--------------TA--------T----------GA------A-T--------C--A---TG---T--ATT--T---CGAA-C-AATT--T------------
[45]	'DminATN16'	CATTG--T-------ATG---AGGAG---ATTTAAGAA--T--TG----AT---C-----TTA-A---AACA--------C---------AAT--TTT--TAXXXXXXXXXXXXXXXXXXXXX--XXXXX

Table: ITS 2 sequence data (series 10: 1171-1300)

	Taxa	sequence
		118 119 120 121 122 123 124 125 126 127 128 129 130
		1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	T--T-ATT-CTAAT-TCC-GCCT-CACCTTTG--T-AAGAT-TACCCGCXXXXXXXXXXXXX---XXX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[2]	'DdiscoV12'	T--T-ATT-CTAAT-TCC-GCCT-CACCTTTG--T-AAGAT-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[3]	'DdATN119'	T--T-ATT-CTAAT-TCC-GCCT-CACCTTTG--T-AAGAT-TACCCGCTGAACNTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[4]	'DdDcitMex'	X--X-XXX-XXXXX-XXX-XXXXXXXXXXXXX--X-XXXXX-XXXXXXXXXXXXXXXXXXXX---XXX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[5]	'DspHLM411'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[6]	'DsphaSib6'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[7]	'PaureumN36'	---------------T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[8]	'DsphaGMN3'	T--T-ATA--TG-T-T---GCCT-NACCTTGG--T-ANGAC-TACCCGCXXXXXXXXXXXXX---XXX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[9]	'PviolacDNC1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[10]	'PviolacDNC3'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-NAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[11]	'DsphaSXN2'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[12]	'DrosAlab'	T--T-ATX--XX-X-X---XXXX-XXXXXXXX--X-XXXXX-XXXXXXXXXXXXXXXXXXXX---XXX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[13]	'DrosEDF3'	T--T-ATN--TX-X-X---XXXX-XXXXXXXX--X-XXXXX-XXXXXXXXXXXXXXXXXXXX---XXX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[14]	'DsphaHYY1'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-ANNAC-TACCCXXXXXXXXXXXXXXX---XXX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[15]	'DrosIL'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-AGCGG-AGGAXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[16]	'DmucBPR11'	N--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[17]	'DmucBPR31'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[18]	'DmucDDS21'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[19]	'DmucPYR'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[20]	'DmucFLMN'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[21]	'DbrefTNC11'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[22]	'DmucHTL'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[23]	'DminGMN3'	--
[24]	'DspWLD1'	T--TGCTA--TG-TNT---GCCT-CACCTTGGT-T-CAGAC-TNCCCGCTGCNCTTATNCTTCNTANCCNAGCCCGTTNGACCC-TCCNTNCGGC--CN-TTNNCT---NT--TTTC-NTTTNGTT-CCC
[25]	'DimpBCN21'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[26]	'DspBDW112'	T--T-ATA--TG-T-T---GCCT-CACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TA-GXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[27]	'DaurstBDW4'	T--T-ATA--TG-T-T---GCCT-CACGTTGG--T-AAGAC-TACCCGGTGAACTTAAGCAT---ATC--AG----TA-NXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[28]	'DimpEPN11'	T--T-ATA--TG-T-T---GCCT-GACCTTGG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATN--NN----NN-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[29]	'DmucorEW5'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[30]	'PviolSTR'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[31]	'PviolFCM'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[32]	'PviolATN1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[33]	'PviolATN'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[34]	'DpurpAT17'	T--TTTTA--TG-T-T---GCCT-CATCTAAG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----T--AG---
[35]	'Dpurp1K'	T--TTTTA--TG-T-T---GCCT-CATCTAAG--T-AAGAC-TACCCGGTGAACTTAAGCAT---ATC--AG----TNNXXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[36]	'DpurpCRLC2'	T--TTTTA--TG-N-T---GCCT-CATCTAAG--T-AAGAC-TACCCGCTGAACTTAAGCAT---ATC--AG----TAAGGGGX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[37]	'DcitrCU2A4'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[38]	'DcitrDdCR'	T--T-ATT-CTAAT-TCC-GCCT-CACCTTTG--T-AAGAT-XXXXXXXXXXXXXXXXXXXX---XXX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[39]	'Ppal'	T--T-ATGGCTA-T-TAA-G--T--AATTTTG--T-ATACC-TTCTCAAAAATCTTTGGACT---TTT--AA----AA-GTTGA-ATTGACGCTG--TT-TGTTC----AC--TCAA-TTTAACTATAAA
[40]	'DminNWM12'	X--X-XXX-XXXXX-XXX-XXXX-XXXXXXXX--X-XXXXX-XXXXXXXXXXXXXXXXXXXX---XXX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[41]	'DminNWM12'	T--T-ATT-CTANN-NCC-GCCTCCACCTT-G--T-AAGAT-TAXXXXXXXXXXXXXXXXXX---XXX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX
[42]	'DtenCu2G3'	C--C-TTT-CTGGT-T------T--AC-TATG--T-AC--T-AACCTTCT--AC--AA-CGT---TT----G-------GATTT-ATCTATTCTA--GA-GTAGCG---GC--GCTG-TTACACGG-ATT
[43]	'PviolATN11'	TAAT-ATGGCTCTT-TAATG--T--TAATTTGATTTTTAAAATTGTTACTATTCTTTTGTAT---ATTCCAA----AC-ACTGATAAAGAAGCAACATTATGAACGTTAACGATAAAGTTTATCTTTATT
[44]	'Acytostelium'	T--T--TG--T--T-CAA-G--T--A-----G----ATGC-----------------GGA-T---TT------------G--G---TTG-C-CAG---T-TG--C-----C--A-AA-TTAAAC---AAA
[45]	'DminATN16'	X--X-XXX-XXXXX-XXX-XXXX-XXXXXXXX--X-XXXXX-XXXXXXXXXXXXXXXXXXXX---XXX--XX----XX-XXXXX-XXXXXXXXXX--XX-XXXXXX---XX--XXXX-XXXXXXXX-XXX

Table: ITS 2 sequence data (series 11: 1301-1430)

	Taxa	sequence
		131 132 133 134 135 136 137 138 139 140 141 142 143
		1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
[1]	'DdiscoNC4'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[2]	'DdiscoV12'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[3]	'DdATN119'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[4]	'DdDcitMex'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[5]	'DspHLM411'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[6]	'DsphaSib6'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[7]	'PaureumN36'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[8]	'DsphaGMN3'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[9]	'PviolacDNC1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[10]	'PviolacDNC3'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[11]	'DsphaSXN2'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[12]	'DrosAlab'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[13]	'DrosEDF3'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[14]	'DsphaHYY1'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[15]	'DrosIL'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[16]	'DmucBPR11'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[17]	'DmucBPR31'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[18]	'DmucDDS21'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[19]	'DmucPYR'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[20]	'DmucFLMN'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[21]	'DbrefTNC11'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[22]	'DmucHTL'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[23]	'DminGMN3'	--
[24]	'DspWLD1'	TTTC---C-----CGTCTTCCTC--CTNCCCTTNCTTTCNCNNTCNTCCCCNNTCNTTCCGNTCGCGNCCNCTNTGNTTTGTNCGTTTCGTTNCACTCCCTNXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[25]	'DimpBCN21'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[26]	'DspBDW112'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[27]	'DaurstBDW4'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[28]	'DimpEPN11'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[29]	'DmucorEW5'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[30]	'PviolSTR'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[31]	'PviolFCM'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[32]	'PviolATN1'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[33]	'PviolATN'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[34]	'DpurpAT17'	--
[35]	'Dpurp1K'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[36]	'DpurpCRLC2'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[37]	'DcitrCU2A4'	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
[38]	'DcitrDdCR'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[39]	'Ppal'	AAAAC--C-----TACAAAAAGCGACATCAGCCAAGCGAGACTXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[40]	'DminNWM12'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[41]	'DminNWM12'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[42]	'DtenCu2G3'	TTAA---T-----GACTTTTAGC--CATGGACAATTTATCACTAAAAGATAGGGGAACTCTAAATTTTTGGGAACCATAACATNXXXXX---
[43]	'PviolATN11'	GGTTCAACAGTTGTATATATTTCG-CCTCATCCAAGTAAGATTACCCGCTGAACTTAAGCATATCAGTAGXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[44]	'Acytostelium'	-------C-----TATAAATAGCGACATCAACTTANTGAGACTACCCGC---
[45]	'DminATN16'	XXXX---X-----XXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

Table: ITS 2 sequence data (series 12: 1530-1551)

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                                                                      sequence
                                144       145       146       147       148       149       150       151       152       153       154       155
      Taxa               1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901
 [1] 'DdiscoNC4'         XXXX-------------------------------------------------------------------------------------------------------------------------
 [2] 'DdiscoV12'         XXXX-------------------------------------------------------------------------------------------------------------------------
 [3] 'DdATN119'          XXXX-------------------------------------------------------------------------------------------------------------------------
 [4] 'DdDcitMex'         XXXXXX-----------------------------------------------------------------------------------------------------------------------
 [5] 'DspHLM411'         XXXX-------------------------------------------------------------------------------------------------------------------------
 [6] 'DsphaSib6'         XXXX-------------------------------------------------------------------------------------------------------------------------
 [7] 'PaureumN36'        XXXX-------------------------------------------------------------------------------------------------------------------------
 [8] 'DsphaGMN3'         XXXX-------------------------------------------------------------------------------------------------------------------------
 [9] 'PviolacDNC1'       XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[10] 'PviolacDNC3'       XXXX-------------------------------------------------------------------------------------------------------------------------
[11] 'DsphaSXN2'         XXXX-------------------------------------------------------------------------------------------------------------------------
[12] 'DrosAlab'          XXXX-------------------------------------------------------------------------------------------------------------------------
[13] 'DrosEDF3'          XXXX-------------------------------------------------------------------------------------------------------------------------
[14] 'DsphaHYY1'         XXXX-------------------------------------------------------------------------------------------------------------------------
[15] 'DrosIL'            XXXX-------------------------------------------------------------------------------------------------------------------------
[16] 'DmucBPR11'         XXXX-------------------------------------------------------------------------------------------------------------------------
[17] 'DmucBPR31'         XXXX-------------------------------------------------------------------------------------------------------------------------
[18] 'DmucDDS21'         XXXX-------------------------------------------------------------------------------------------------------------------------
[19] 'DmucPYR'           XXXX-------------------------------------------------------------------------------------------------------------------------
[20] 'DmucFLMN'          XXXX-------------------------------------------------------------------------------------------------------------------------
[21] 'DbrefTNC11'        XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[22] 'DmucHTL'           XXXX-------------------------------------------------------------------------------------------------------------------------
[23] 'DminGMN3'          -------------------------------------------------------------------------------------------------------------------------
[24] 'DspWLD1'           XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[25] 'DimpBCN21'         XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[26] 'DspBDW112'         XXXX-------------------------------------------------------------------------------------------------------------------------
[27] 'DaurstBDW4'        XXXX-------------------------------------------------------------------------------------------------------------------------
[28] 'DimpEPN11'         XXXX-------------------------------------------------------------------------------------------------------------------------
[29] 'DmucorEW5'         XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[30] 'PviolSTR'          XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[31] 'PviolFCM'          XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[32] 'PviolATN1'         XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[33] 'PviolATN'          XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[34] 'DpurpAT17'         -------------------------------------------------------------------------------------------------------------------------
[35] 'Dpurp1K'           XXX--------------------------------------------------------------------------------------------------------------------------
[36] 'DpurpCRLC2'        XXXXXXXXXXX--------------------------------------------------------------------------------------------------------------
[37] 'DcitrCU2A4'        XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
[38] 'DcitrDdCR'         XXXX-------------------------------------------------------------------------------------------------------------------------
[39] 'Ppal'              XXXXX------------------------------------------------------------------------------------------------------------------------
[40] 'DminNWM12'         XXXXXXXXXXX--------------------------------------------------------------------------------------------------------------
[41] 'DminNWM12'         XXX--------------------------------------------------------------------------------------------------------------------------
[42] 'DtenCu2G3'         -------------------------------------------------------------------------------------------------------------------------
[43] 'PviolATN11'        XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX------------------
[44] 'Acytostelium'      -------------------------------------------------------------------------------------------------------------------------
[45] 'DminATN16'         XXXXXXXXXXX--------------------------------------------------------------------------------------------------------------
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