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Molecular systematics in the genus *Dictyota* (Dictyotales, Phaeophyta): a first attempt based on restriction patterns of the Internal Transcribed Spacer 1 of the rDNA (ARDRA-ITS1)

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Abstract. *– This study represents a first attempt to reconstruct the phylogenetic relationships in the genus* Dictyota *by means of an RFLP analysis of the amplified Internal Transcribed Spacer 1 of the ribosomal region (ARDRA-ITS1). To overcome DNA extraction problems, a new protocol based on a combination of a CTAB method and a Sephaglass Bandprep Kit is presented. Seven species, mostly including more than one specimen, were included.* Padina boergesenii *was used as an outgroup. The inferred phylogenetic picture was congruent with the morphological-anatomical data. Specimens belonging to the same species invariably group together and differences among geographically isolated populations are reflected in the tree. Parsimony-based bootstrap and jackknife supports for basal nodes are generally very low and phylogenetic relationships between species are poorly resolved. This poor resolution can probably be attributed to the considerable variation in length of the ITS1 region among the species. This variation causes difficulties in sequence alignments and calls into question the use of this marker as a general tool in the genus.*

Key words: Dictyota, *Phaeophyta, marine algae, molecular systematics, ARDRA, ITS1*

1 Introduction

Systematic studies in the genus *Dictyota* (Dictyotales, Phaeophyta) have been seriously hampered by the lack of easily defined characters distinguishing species and by considerable intraspecific morphological variation. The lack of understanding of the variation of vegetative characters has resulted in developmental and other epigenetic morphological forms being described as different species (Phillips 1992). Recent efforts, which culminated in a revision of the members of the genus from the northern Atlantic Ocean (Hörnig & al. 1992a,b), have lead to a better understanding of this morphological variability. The Australian representatives of the genus, characterised by a multilayered medulla (former genus *Dilophus*), were studied by Phillips (1992) who emphasised the use of hitherto neglected characters such as the number of sporangial stalk cells and the size of mature tetrasporangia in the distinction of species. However, whereas these new morphological characters proved very useful to discriminate notoriously difficult species [e.g. *D. fastigiata* Sonder and *D. gunniana* (J.Agardh) Hörnig, Schnetter &

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Prud'homme van Reine], for a majority of tropical species such characters are non-discriminative (De Clerck & Coppejans 1999; De Clerck 1999). Most tropical representatives of *Dictyota* have nearly identical reproductive structures and thallus anatomy, and can generally be distinguished only on the basis of overall habit and sizes of interdichotomies and cells.

As DNA analysis has been shown to be a very effective tool to investigate relationships between organisms or between groups of organisms, phycologists have also begun to apply a variety of genomic techniques. Hardly any results based on molecular data exist for Dictyotales. Chloroplast DNA was partly characterised for *D. dichotoma* (Hudson) Lamouroux by Kuhsel & Kowallik (1985, 1987) and a partial sequence of the SSU rDNA (873 nucleotides, 3'- end) of *Taonia atomaria* (Woodward) J.Agardh was published by Tan & Druehl (1993). Lee & King (1996) tried to reconstruct the phylogeny of several genera of the Dictyotales, but a critical examination of the data showed that the reported sequences do not originate from brown algae. This study initiates the use of molecular markers for taxonomic purposes in *Dictyota*. Comparison of rDNA characteristics between different species, has shown to be a valuable approach for phylogenetic inferences in the algae (Zechman & al. 1990). However, the taxonomic resolution of the Small and Large Subunits is in brown algae limited to the level of orders and families and to a lesser extent to genera (Saunders & Druehl 1992, 1993; Tan & Druehl 1993; Saunders & Kraft 1995; Rousseau & al. 1997). Characteristics of the Internal Transcribed Spacers (ITS1 and ITS2) offer a much better taxonomic resolution at species and generic levels (Stache-Crain & al. 1997; Peters & al. 1997). Therefore, this study attempts to characterise the ITS of *Dictyota* and assesses its use in reconstructing phylogenetic relationships. It was our initial intention to compare both the ITS1 and ITS2, but amplification of the ITS2 region proved to be problematic and no PCR products of sufficient quality could be obtained with DNA from several specimens. Hence this study only investigates the applicability of the ITS1 region to unravel the phylogeny of *Dictyota*. Specifically, we performed an Amplified rDNA Restriction Analysis (ARDRA) (Heyndrickx & al. 1996) of this DNA region. In order to obtain the highest discrimination several restriction enzymes were used. The banding patterns were combined and the obtained composite pattern was analysed phylogenetically. It is beyond the scope of this introductory study to reconstruct a complete phylogeny of the genus, because only a limited number of species was included. Nevertheless, preliminary hypotheses on interrelationships are discussed.

2 Material and methods

2.1 DNA extraction

Algal samples were desiccated in silica gel (Chase & Hills 1991). Voucher specimens were processed as herbarium specimens and deposited in GENT (table 1). Total cellular DNA was extracted from 20-50 mg dry material. Samples were ground in liquid nitrogen in 800 µl of 2% CTAB extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 mM NaCl, 20 mM EDTA, pH 8.0, 2% w/v CTAB, 2 µl mercapto-ethanol). After 2 hours of incubation at room temperature, two choloroform-isoamylalcohol (24:1) extractions were performed. The DNA was then bound to Sephaglass beads (20 µl) with the addition of 1 ml of Gel Solubilizer (6N NaI). Following centrifugation (1 min, 15,000 g) the pellet containing the glass beads and the DNA was then washed in 160 µl of wash buffer, after which 1 ml of 50% EtOH was added. The samples were placed overnight at 4°C, centrifuged (1 min, 15.000 g) and air dried. DNA was eluted from the glass beads by addition of 100 µl of 0.1 X TE or H_2O at 55°C

2.2 Polymerase Chain Reaction

PCR was performed in 50 µl reaction volumes, containing 5 µl of template DNA, 200 µM dNTP's, 0.5 µM of each primer, 1.5 mM $MgCl_2$ standard Eurotaq reaction buffer and 1.25 units of Taq DNA polymerase (Eurogentec, Belgium). The PCR in a Perkin-Elmer thermocycler (Gene Amp PCR System 9601) proceeded as follows: an initial denaturation step at 94°C for 4 min followed by 33 cycles of 1 min at 94°C, 2 min at 55°C and 2 min at 72°C. Amplification products were checked for correct molecular weight and yield on EtBr-stained 1% agarose gels. Several primer combinations were tested, of which the TW5-5.8S1R couple was the most successful. Reaction products were also obtained with TW7-LSU115R, ITS1-LSU115R, ITS1-JO6 and TW7-JO6 (fig. 1, table 2). TW5, TW7, ITS1 and ITS4 are universal primers initially designed by White & al. (1990). JO6 was designed by Van Oppen & al. (1995). 5.8S1R, 5.8S1F and LSU115R are Phaeophyta specific primers designed by Müller & al. (1998) and Peters & Burkhardt (1998).

Table 1. Collection sites and reference numbers of the samples
ODC and HEC refer to the herbarium collections (GENT) of the first and last author respectively.

Species	Locality	Herbarium number
D. bartayresiana Lamouroux	Tanzania, Dar es Salaam, Mbudya Island	ODC 466
	Tanzania, Pemba, Tondooni, Verani	ODC 490
	Tanzania, Zanzibar, Zanzibar, Chwaka	ODC 710
	Tanzania, Zanzibar, Pongwe	ODC 715
	Papua New Guinea, Motupore Island	ODC 272
D. ceylanica Kützing	Tanzania, Dar es Salaam, Pongojo Island	ODC 688
D. cervicornis Kützing	Tanzania, Zanzibar, Chwaka	ODC 707
	Tanzania, Zanzibar, Pongwe	ODC 713
D. crispata Lamouroux	Tanzania, Zanzibar, Ras Fumba	ODC 332
D. dichotoma (Hudson) Lamouroux	France, Bretagne	ODC 176
	The Netherlands, Zeeland	ODC 114
	South Africa, Cape Town	HEC 10912
	South Africa, Cape Town	ODC 822
D. humifusa Hörnig, Schnetter & Coppejans	Tanzania, Zanzibar, Matemwe	ODC 738
D. pulchella Hörnig & Schnetter	Panama, San Blas Field Station	ODC 594
Padina boergesenii Allender & Kraft	Tanzania, Pemba, Tondooni, Verani	ODC 499

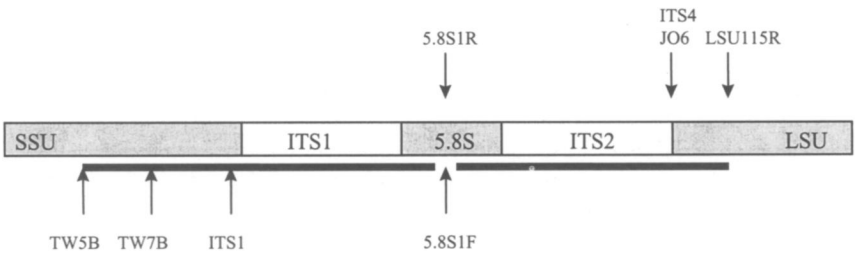

Figure 1. Position of the primers in the nuclear encoded rDNA used for amplification.

Table 2. Primers used for amplifications

Primer	Direction	Length (bp)	Position	Sequence (5'-3')
TW5B	forward	22	1541	AACTTAAAGAAATTGACGGAAG
TW7B	forward	23	1829	GGGGCAATAACAGGTCTGTGATG
ITS1	forward	19	2217	TCCGTAGGTGAACCTGCGG
5.8S1F	forward	20	2784	TGATGATTCACTGGATTCTG
5.8S1R	reverse	20	2804	CAGAATCCAGTGAATCATCA
JO6	reverse	21	3134	ATATGCTTAAGTTCAGCGGGT
ITS4	reverse	20	3148	TCCTCCGCTTATTGATATGC
LSU115R	reverse	20	3245	CTCTCCAGACTACAATTCGG

2.3 Amplified rDNA Restriction Analysis (ARDRA)

A total of 5 restriction enzymes was selected: *Dpn*II, *Hpa*II, *Mro*I, *Rsa*I and *Tsp*509I. As the restriction site of *Mro*I (a hexa-cutter), is located near the end of the SSU rDNA, it enabled us to verify the PCR product for correct identity. Indeed the smaller fragment of this digest is 490-492 bp long and represents the conservative 3'-end of the SSU. The longer fragment is variable in size and represents the ITS1 region. Digests were completed according to the manufacturers' instructions. Reaction volumes were either 20 or 30 µl, depending on the concentration of the PCR product. A 20 µl reaction volume consists of 10 µl PCR product, 7.5 µl H_2O, 2 µl reaction buffer and 5 Units enzyme. Digested DNA was size fractionated by horizontal gel electrophoresis at 90 V (3 V/cm) for 130 min in 1 X TBE-buffer (Heyndrickx & al. 1996). Samples were loaded on an agarose gel (15 X 10 cm, 20 wells) of 2% (w/v) Metaphor agarose (FMC Bioproducts, Rockland). The outer two wells were left empty and of the 18 remaining wells, 15 were used for the rDNA digests and 3 (one in the middle and one on each border of the gel) for the molecular weight marker. The marker was a combination of two separate ladders each with a different range: the Biorad 100 bp molecular (100 to 1000 bp) and Biorad 500 bp

molecular ladders (500 to 3000 bp). Banding patterns were analysed with GelCompar 4.0 (Applied Maths, Kortrijk, Belgium) in three steps: conversion, normalisation and numerical analysis. Normalisation of the patterns is of primary importance to achieve reliable databases by which an objective comparison of the banding patterns can be performed. All ARDRA patterns were normalised using the combination of the two ladders. After normalisation the patterns obtained with each of the five restriction enzymes were assembled. A similarity matrix between each pair of combined patterns was calculated with a band position tolerance of 1.0% using the Dice similarity coefficient (SD) according to the formula 2nAB/(nA+nB) with nAB, the number of bands common for pattern A and B, nA the total number of bands in pattern A, nB the total number of bands in pattern B. Dendrograms were constructed from the similarity matrices, using the UPGMA (Unweighted Pair Group Method using arithmetic Averages) clustering algorithm. In parallel with the distance method the dataset was also analysed using parsimony analysis.

Phylogenetic relationships were inferred using PAUP* 4.0b2 (Swofford 1999). *Padina boergesenii* was used as an outgroup. Most-parsimonious trees were constructed using the heuristic search option, random sequence addition (500 replicates) and TBR branch swapping. Support for individual internal branches was determined by a bootstrap (Felsenstein 1985) and parsimony jack-knifing analysis (Farris & al. 1996) as implemented in PAUP (100 replicates).

Table 3. Sizes of fragments in the digests obtained with all enzymes used

Species &	Restriction enzyme					
Herb. nr.	Tsp509I	MroI	DpnII	HpaII	RsaI	Total
D. bartayresiana ODC 466	1544/257/134	1461/490	649/427/232/145	1008/308/273/174	528/224 /200/189	1951
D. bartayresiana ODC 490	1488/260/137	1461/490	637/427/232/145	1003/308/273/174	523/221/202/190	1951
D. bartayresiana ODC 710	1488/260/137	1426/492	637/431/237/150	998/305/268/169	523/224/205/189	1918
D. bartayresiana ODC 715	1506/260/136	1470/490	637/432/237	988/304/269/174	523/222/203/188	1960
D. bartayresiana ODC 275	919/461/262/134	1384/490	654/421/232/142	507/220/199/183	523/225/212/198	1874
D. ceylanica ODC 688	1291/262/235/136	1515 /492	798/437/336/314/240/175	526/343/288/143	1525/519	2007
D. cervicornis ODC 707	449/258/131/118	776/492	433/275 /236/181/143	397/309/222/170 /109 /85	525/342/289/141	1268
D. cervicornis ODC 713	446/265/134/123	754/492	418/263/235/183/145	382/307/222/171/108/86	692/549/297/221	1246
D. crispata ODC 332	433/255/130/115	758/494	430/293/240/159/132	433/269/167/142	519/318/287/160	1252
D. dichotoma Bretagne	972/478	1376/492	1247/430 /238	512/327/284/142	1360/528	1868
D. dichotoma Zeeland	973/474/266	1376 /490	1276/431/236	310 /281/173	1368 /518	1866
D. dichotoma var. intricata Cape Town	943/467/264	1376/492	637/278/326/192	449/259/132/121	1321/526	1868
D. dichotoma var. intricata Cape Town	943/474/266	1376/492	637/278/326/192	449/261/132/121	1328 /520	1868
D. humifusa ODC 738	1798	1810/492	892/433/390/237	391/275	1908/519	2302
D. pulchella ODC 594	407/373/360/297/262/ 137	1685/492	745/438/261/240	433/269/168/143/123	1628/509	2177
P. boergesenii ODC 499	798/259/133	866/492	428/189	519/220/200/186	519/406/232/114	1358

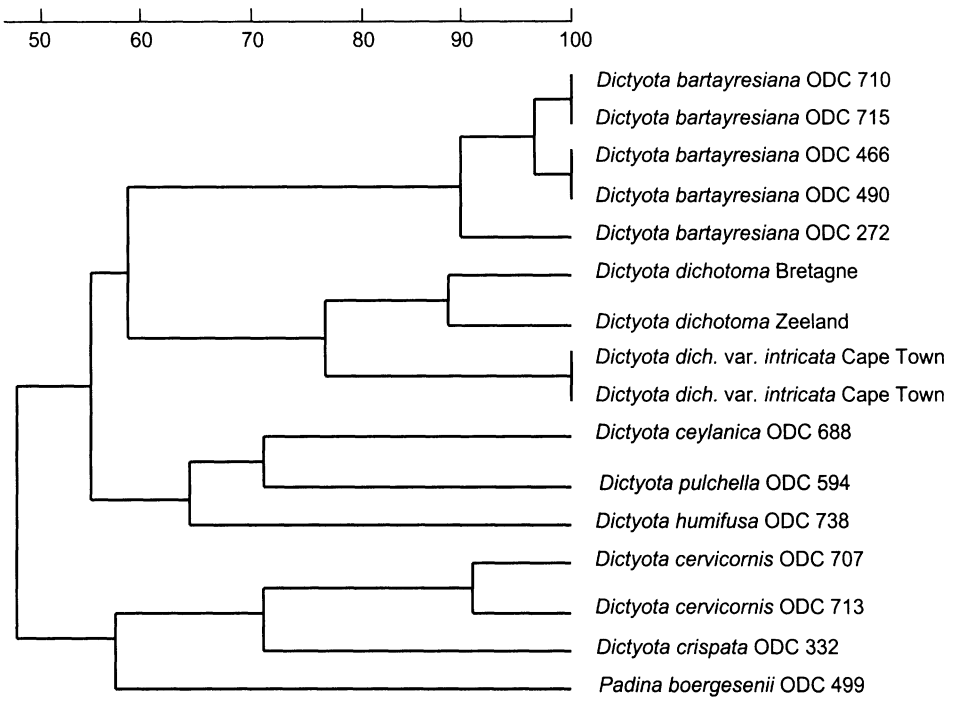

Figure 2. UPGMA tree of seven species (16 isolates) using the Dice similarity coefficient; tolerance was set at 1.0%.

3 Results

3.1 Banding patterns

The amplified ITS1 region showed much variation in length between the different species. The shortest one (*D. cervicornis*, ODC 713, 1246 bp) being 1056 bp shorter than the longest one (*D. humifusa*, ODC 738, 2302 bp). Also between isolates of a single species some variation, up to 86 bp, was observed (e.g. *D. bartayresiana*). All specimens were digested with the same set of five enzymes which resulted in 11-21 bands per specimen (table 3). Digestion with MroI always resulted in two bands of which the lower molecular weight band (490-492 bp) occurred in all specimens while the higher molecular weight band varied from 754 bp in *D. cervicornis* to 1685 bp in *D. pulchella*. Digestion with Tsp509I resulted in 3-4 bands in most specimens except for *D. humifusa* in which only a single large band was found (1798 bp). The DpnII and HpaII enzyme revealed 4 to 6 bands per digest; RsaI 2 to 4 bands per digest.

3.2 Phenetic analysis

The distance tree was constructed based on combined patterns of the individual digests (fig. 2). Below a cut-off value of 50% similarity the first lineage contains *D. cervicornis* and *D. crispata* as well as the outgroup, *P. boergesenii*. The second lineage groups the remaining *Dictyota* specimens. At about 58% similarity the latter lineage can be subdivided in a group which unites *D. bartayresiana* and *D. dichotoma* on the one hand and D. *ceylanica, D. humifusa* and *D. pulchella* on the other hand. All *D. bartayresiana* isolates are grouped with a minimum similarity of 90% between the specimen from Papua

New Guinea (ODC 272) and the Tanzanian specimens (ODC 466, 490, 710, 1713). The banding patterns of the Tanzanian *D. bartayresiana* specimens were very similar, 97-100%. The *D. dichotoma* group, can be subdivided in a South African and a northeast Atlantic lineage which show relatively low similarities (78%). Between the northeast Atlantic isolates a similarity of 90% was observed, while specimens from Cape Town, South Africa were identical in banding pattern. *D. ceylanica, D. humifusa* and *D. pulchella* group together at about 63% similarity and *D. ceylanica* appears to be a sister taxon of *D. pulchella* at 70% similarity.

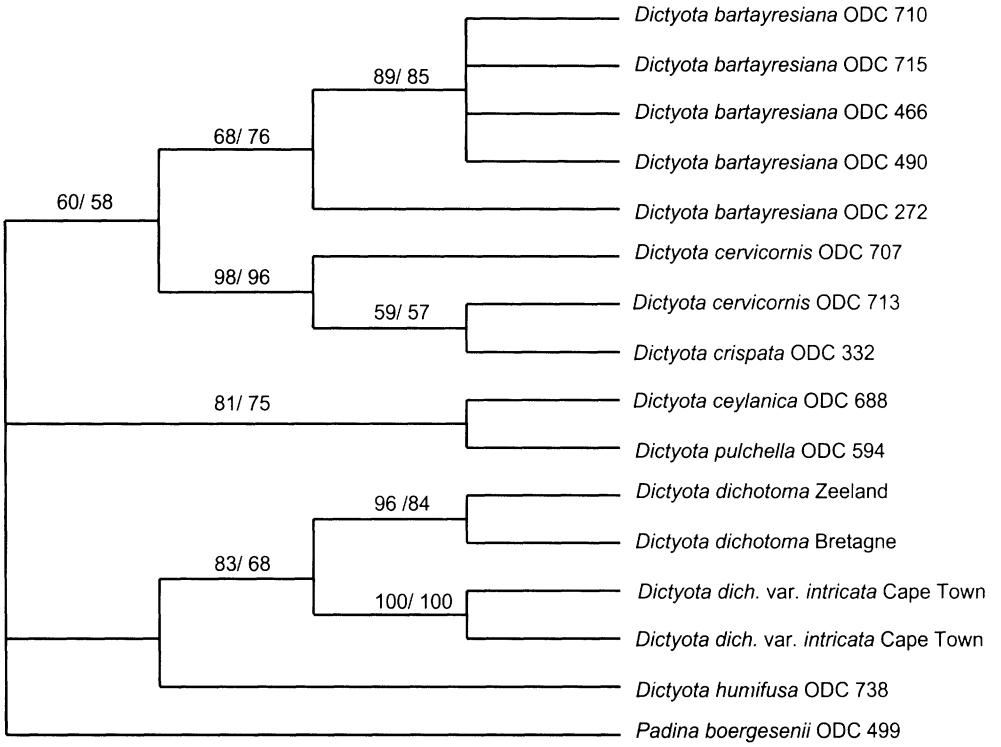

Figure 3. Strict consensus tree resulting from parsimony. Bootstrap and jackknife values (100 replicates), indicated above internodal branches, are shown for branches with support ≥ 50%.

3.3 Parsimony analysis

Maximum parsimony produced 4 most parsimonious trees of 116 steps. These differed only in the relative placement of the respective specimens of *D. bartayresiana*. The overall topology of the strict consensus tree (fig. 3) is similar to the distance tree. There is perfect agreement with the distance tree on the position of the terminal nodes. The five specimens of *D. bartayresiana* form a single clade, with the specimen from Papua New Guinea (ODC 272) being a sister to the East African specimens (ODC 466, ODC 490, ODC 710, ODC 715). *D. cervicornis* and *D. crispata* group together with the *D. bartayresiana* clade, but bootstrap (60) and jacknife (58) values are relatively low. *D. ceylanica* and *D. pulchella* form a well-supported clade but the position of this group remains unresolved. The *D. dichotoma* complex is well-supported as a monophyletic group. The position of *D. humifusa*, which groups as sister to the *D. dichotoma* clade, however, is not supported above 50%.

4 Discussion

DNA extraction in brown algae in general and in the Dictyotales in particular poses important difficulties as most methods that are successful in other groups of organisms cannot be applied with success. The method applied in this study, a combination of a CTAB method (Doyle & Doyle 1987; Rogers & al. 1989) and a Sephaglas Bandprep Kit (Pharmacia), was successful for a number of taxa, but yielded no amplifiable DNA for others. In general the method was unsuccessful when applied to thick, coarse, parenchymatous thalli (e.g. *Spatoglossum asperum* J.Agardh, *Stoechospermum polypodioides* (Lamouroux) J.Agardh and *Stypopodium zonale* (Lamouroux) Papenfuss). No DNA could be extracted from a number of medium sized *Dictyota* species either (e.g. *D. ciliolata* Sonder ex Kützing, and *D. friabilis* Setchell). Perhaps these failures can be related to the amount of contaminating polyphenols, which show considerable variation within and among species (Targett & Arnold 1998). To improve the DNA extraction method for applicability to a wider variety of Dictyotalean genera, either the present method should be modified or more time-consuming methods should be used. The latter can take up to several days and require special equipment (e.g. CsCl density gradient ultracentrifugation). Quality of the PCR products is often related to the purity of the DNA samples. If the template DNA is of high quality, PCR will yield a pure product of sufficient quantity that can be reproducibly digested with restriction enzymes. However, amplification with an impure DNA template will rarely result in good PCR products. The generated banding patterns are then frequently difficult to interpret as the bands are weak and because of the presence of indiscrete bands which may form a smear over the gel. Even in DNA of sufficient quality the yield of the PCR for the ITS1 region was always much higher than for the ITS2 region, despite several attempts to adjust the PCR conditions. No immediate explanation could be found. Perhaps secondary structures of the Internal Transcribed Spacer hinder the amplification of the locus. The existence of secondary structures in this non-coding part of the genome has been demonstrated for a number of algal groups (Coleman & Mai 1997; Mai & Coleman 1997; Peters & al. 1997), but their function still remains unclear. Secondary structures, such as stem-loops, are known to render PCR amplification and sequencing more difficult (Saunders & Druehl 1993).

The ARDRA-ITS1 technique proved successful in the genus *Dictyota*. Restriction patterns are highly reproducible and specimens that belong to a single species based on morphological observations group together in the trees obtained. Additionally, geographical differences are reflected in the restriction patterns. A single specimen of *D. bartayresiana* from Papua New Guinea showed a somewhat lower similarity to the other 4 specimens of the species from Tanzania, but still grouped in the same lineage. In a similar way the *D. dichotoma* var. *intricata* specimens from South Africa were distinct from the European representatives of the same species. The tendency to reveal geographic variation in restriction patterns could indicate that Dictyotales are a rather old lineage within the Phaeophyta. Moreover, the observation that sequence data of the ITS1 region of *D. cervicornis* (De Clerck, unpublished) could not be aligned to similar sequences of other brown algae might be indicative of an early differentiation of the order. ITS regions of other brown algae are generally remarkably conserved. Saunders & Druehl (1993) noticed a 90% similarity in the ITS1 region between two kelp species from two morphologically diverse families, *Alaria marginata* Postels & Ruprecht and *Postelsia palmaeformis* Ruprecht. Similarly, ITS 1 and ITS2 sequences of several species of the Desmarestiaceae only diverged 0.2-14.6% (Peters & al. 1997). It is most likely that the divergence patterns observed from restriction patterns in the genus *Dictyota* will be mirrored by a much higher divergence in sequence data of the same molecule. This is also reflected in the length differences of the PCR products among the different isolates. Neither Saunders & Druehl (1993), Stache-Crain & al. (1997) nor Peters & al. (1997) reported large inserts or deletions. Length differences as observed in the genus *Dictyota* have been commonly observed in Chlorophyta (Bakker & al. 1995), but have never been reported for any group of Phaeophyta. The observed high divergences of the ITS region in *Dictyota* affect its use in reconstructing phylogenies. On

species and population level the ITS region may provide a good resolution by means of the ARDRA technique, but more distant relationships (e.g. between genera) could be affected by convergences, parallelism and non-homology of bands with a less reliable tree as the direct result. Towards future sequence work the large differences in length could pose serious problems as the individual sequences may be extremely difficult to align.

The distance and parsimony analyses result in trees with similar topologies. Especially towards the terminal nodes, both analyses are highly congruent. Internal nodes show a number of incongruences. From the bootstrap and jackknife supports it becomes clear, however, that most of the internal nodes are only weakly supported, limiting the value of the ARDRA-ITS1 technique for uncovering intraspecific relationships in *Dictyota*.

D. cervicornis and *D. crispata* share a number of morphological characters not encountered in the other representatives of the genus included in this analysis such as combination of surface proliferations and involucrate sporangia. Detailed study of the paraphyses which surround the antheridial sori of *D. crispata* have shown to be of a different nature than those observed in all other Dictyota species, being multicellular and pigmented (De Clerck 1999; De Clerck & Coppejans, unpublished results). These morphological observations are reflected in the distance tree, where both species form a distinct lineage together with *P. boergesenii*, which was selected as an outgroup. In the parsimony analysis, however, *D. cervicornis* and *D. crispata* group together with *D. bartayresiana*, although the bootstrap (60) and jackknifing (58) supports are low. At present it, therefore, remains uncertain whether the genus *Dictyota* as currently defined is polyphyletic and a new genus should be described to accommodate *D. cervicornis* and *D. crispata*. The banding patterns of *P. boergesenii* and *D. cervicornis-crispata* might be superficially similar but in fact not homologous.

The *Dictyota dichotoma* complex has been studied in detail by Hörnig & Schnetter (1988), resulting in the description of *D. pulchella* Hörnig & Schnetter. The latter species was erected to accommodate the tropical Caribbean representatives of the *D. dichotoma* complex after hybridisation experiments showed them to be reproductively isolated from European warm-temperate *D. dichotoma* specimens. On morphological grounds, however, *D. pulchella* is particularly hard to differentiate from genuine *D. dichotoma* due to substantial amounts of morphological variation in both species (Hörnig & al. 1992b). *D. ceylanica* Kützing, originally described from Sri Lanka and widely represented in the Indian Ocean, is morphologically indistinguishable from the Caribbean *D. pulchella* (De Clerck 1999). Our analysis shows both species to be sister taxa, which could then have diverged from a Tethyan ancestor. It is surprising that both taxa are only distantly related to the warm temperate *D. dichotoma*, to which they are morphologically very similar. The position of *D. humifusa* remains unresolved. In the distance tree *D. humifusa* groups together with *D. ceylanica* and *D. pulchella*; the parsimony analysis places *D. humifusa* in a basal polytomy.

5 Conclusion

The use of restriction data to infer phylogenies has been subject to considerable debate. The main criticism involves homology of bands and treatment of insertions and deletions (for more complete discussions, see Templeton 1983; DeBry & Slade 1985; Albert & al. 1992). Generally gene sequences provide a better resolution and the resulting phylogeny are less subject to criticism. Despite these criticisms, trees based on restriction data are in general congruent to trees based on gene sequences in bacteria (Heyndrickx & al. 1996). The phylogeny presented in this study should, however, be interpreted with care. The distance and parsimony analysis are largely congruent at the terminal nodes, but differ in the placement of the *D. cervicornis-crispata* clade and in the position of *D. humifusa*. Terminal nodes seem to be well supported as indicated by bootstrap and jackknife values, but the reliability of the trees decreases towards the base. Other factors, such as the limited number of taxa included, may also affect

the value of the present phylogenetic picture. Most of the species used can be regarded as common, tropical representatives of the genus that are differentiated morphologically only by sizes of inter-dichotomies and cortical and medullary cells (except for *D. cervicornis* and *D. crispata*). Warm temperate species, (not included in the analysis) are generally better characterised by well-defined differences in their reproductive morphology (arrangement of sporangia in sori, number of sporangial stalk cells, involucra) or cortical and medullary structure (De Clerck & Coppejans 1999). Inclusion of these species could affect the topology of the tree considerably. Therefore, the position of *D. cervicornis* and *D. crispata*, which render the genus *Dictyota* paraphyletic in the UPGMA analysis, should be considered preliminary. Although, morphological investigations have revealed that the presence of multicellular, pigmented paraphyses is unique within the genus (De Clerck & Coppejans, unpublished results), a better hypothesis for the true relationships of *D. cervicornis* and *D. crispata* to the rest of the genus can only be obtained by inclusion of a series of related genera. Only then will it become clear whether the genus is truly para- or polyphyletic and the description of new genera is justified. Inclusion of species of related genera could also bring new insights in the relationship between *Dictyota* and *Pachydictyon*. The latter genus was created to accommodate species characterised by a multilayered cortex. Future research should also examine the relationships between *Dictyota* species with a multilayered medulla (former genus *Dilophus*) and those with a unilayered medulla in order to provide supplementary evidence either for or against the merging of *Dilophus* with *Dictyota* by Hörnig & al. (1992a) or in opposition to this idea.

The observed wide divergences of the ITS region of *Dictyota* species suggests that a more conserved genetic locus might be more appropriate for unravelling the phylogenetic relationships of this group of organisms. The length variations and insertions and deletions in particular render the ITS1 region less suitable for phylogenetic research at lower taxonomic ranks. In Rhodophyta the large subunit of the RuBisCo enzyme (rbcL) proved very appropriate for inferring phylogenies at the ordinal and family level (Freshwater & al. 1995; Fredericq & al. 1996, Hommersand & al. 1999). In Phaeophyta the only published data come from a study of *Ectocarpus* and *Kuckuckia* (Stache-Crain & al. 1997), where the RuBisCo spacer was used to compare sequence divergence of geographically isolated populations. In this respect it is difficult to predict the suitability of the rbcL region in the genus *Dictyota*.

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