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Nucleoli, rRNA genes and ITS region in *Posidonia oceanica* (L.) Delile

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The maximum number of nucleoli was counted in interphase nuclei of *Posidonia oceanica*, and a restriction pattern of nuclear rDNA was obtained after digestion with four restriction endonucleases and Southern hybridization. *P. oceanica* has only one type of ribosomal gene whose size was estimated to be 18.5 kbp long. The nucleotide sequence of the entire ITS region was also determined by direct sequencing of PCR amplified DNA fragments. The sequence of the ITS region was aligned with those of homologous regions of other monocots available in literature, and phylogenetic trees were obtained.

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The wide uniformity of morphological characters in aquatic groups of plants, such as Alismatidae, is due to an extensive phenotypic plasticity. This leads frequently to uncertain interpretation of data concerning relationships among species.

The monocot *Posidonia oceanica* (L.) Delile (family Posidoniaceae, subclass Alismatidae) is endemic to the Mediterranean sea and among other species of marine angiosperms, is one of the most interesting because of its extensive distribution and ecological role (BOUDOURESQUE et al. 1984).

Other species of the same genus live in the oceans surrounding Australia. The genus is regarded as one of the ancient marine angiosperms and has undergone little changes during its long evolutionary history (DEN HARTOG 1970). It is characterized by the prevalence of vegetative reproduction, (HUTCHINSON 1975; PHILBRICK and LES 1996), stability of chromosome number ($2n = 20$) and uniformity of chromosome morphology (KUO et al. 1990).

In populations of *P. oceanica*, analysis by means of DNA fingerprinting has indicated a very low genetic variability (PROCACCINI et al. 1996). In this species no molecular data are available on rDNA structure, now widely utilized for exploring phylogenetic relationships among taxa (ZIMMER et al. 1989; TROITSKY et al. 1991; HAMBY and ZIMMER 1992; HSIAO et al. 1994).

In eukaryotes the ribosomal rRNA genes (rDNA) contain a transcription unit and an intergenic spacer DNA (IGS), which exhibits length and sequence heterogeneity. These genes are clustered in multiple copies at one or more nucleolar organizer regions (NORs). In higher plants each transcription unit consists of coding sequences corresponding to 18S, 5.8S and 25S rRNAs. The rDNA coding sequence are highly conserved in the plant kingdom, as well as in animals and fungi. Their arrangement and the presence of different restriction sites can be useful in taxonomic and evolutionary studies, though they are not informative for exploring phylogenetic relationships among closely related taxa. In contrast, the internal transcribed spacers between 18S and 5.8S (ITS 1) and between 5.8S and 25S (ITS 2) are variable among closely related species and have been demonstrated to be phylogenetically informative (BALDWIN 1992; BALDWIN et al. 1995).

In the present work we report data on the nucleoli, the structure of rDNA and the nucleotide sequence of ITS region of *P. oceanica*.

MATERIAL AND METHODS

Plant material

Rhizomes of *P. oceanica* were collected near Tarquinia (Viterbo, Italy) on a sandy and reefy ground at a depth of 5 meters. The material was rinsed in distilled water and shoot apex meristems, excised from rhizomes, were frozen in liquid nitrogen and stored at −80°C until use.

Nucleoli preparation

Root tips were fixed in Carnoy and nucleoli were stained with silver nitrate according to MEHRA et al. (1984).

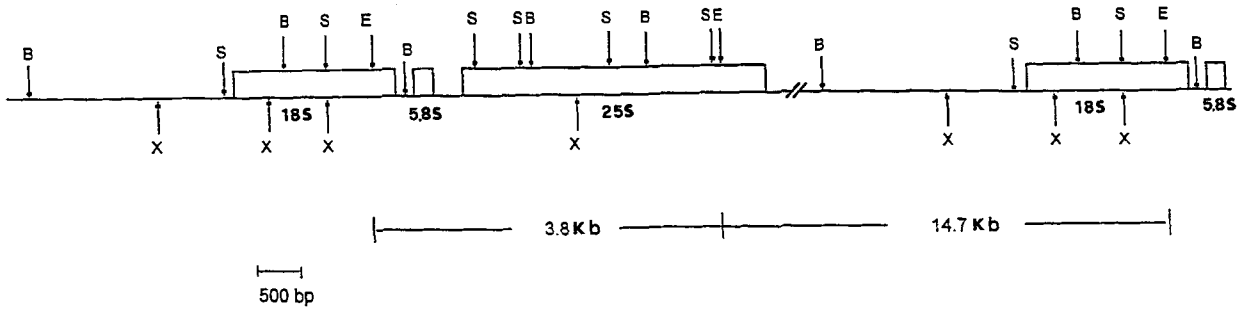

Fig. 1. Restriction map of the *P. oceanica* ribosomal DNA. B, E, S and X represent BamHI, EcoRI, SacI and XbaI restriction sites. The boxes indicate the coding regions for rRNAs. The size of EcoRI fragments are indicated.

DNA extraction, labelled probes preparation, restriction enzymes and Southern hybridization

DNA extraction was carried out following the CTAB method described by ROGERS and BENDICH (1994). The restriction pattern of DNA was obtained by single and double digestion analysis of total nuclear DNA with four endonucleases: Eco RI, Bam HI, Sac I and Xba I (Gibco BRL, Life Technology, Italy), and Southern hybridization with 18S and 25S homologous rDNA probes PCR labelled with dUTP digoxigenin (Boehringer Mannheim). Chemioluminescent signals of the restriction fragments were developed with Tropix procedure for CDP substrate (Perkin Elmer Italy) and detected by means of autoradiography.

DNA amplification and sequencing

Amplification of ITS1, ITS2 and 5.8S was performed in a Perkin Elmer (mod. 2400) thermocycler that was set to run at 95°C for 1 min for initial denaturation, followed by 30 cycles at 95°C for 30 sec, at 48°C for 30 sec, at 72°C for 90 sec and finally at 72° for 7 min. Reaction was performed in a thin wall tube in 50 µl of total volume.

"ITS 5" and "ITS 2" or "ITS 3" and "ITS 4" primers (WHITE et al. 1990) were used at a concentration of 0.5 mM. Genomic DNA (100 ng) was added to other components of Taq polymerase kit (Gibco BRL, Life Technology, Italy). Amplification products were cloned in pGEM-T vector (Promega) and entirely sequenced on both strands by the chain termination method (SANGER et al. 1977).

The sequence of the entire ITS region of *P. oceanica* is deposited in the EMBL data base (accession no. AJ225091, POA225091)

Sequences analysis

The sequences of the entire ITS region (ITS1, 5.8S and ITS2) were aligned with those of homologous regions of the following monocots available in literature: *Brachypodium distachyon* (EMBL accession number BD58SITS), *Triticum aestivum* (TA58SRDNX), *Oryza sativa* (OSRGSBHA), *Sorghum versicolor* (SV04795). The 5.8S sequence of *Potamogeton natans* was deduced from the rRNA sequence published by TROITSKY et al. (1991). The multiple alignments were performed using the Clustal W computer program (HIGGINS et al. 1992). Phylogenetic reconstruction was performed using "branch and bound" or "heuristic" options of PAUP (version 3.1.1., SWOFFORD 1991). Bootstrap values were obtained from 100,000 replicate parsimony anlaysis using "heuristic" options and closest additon of PAUP.

RESULTS

Nucleoli

The maximum number of nucleoli was counted in several interphase nuclei stained with silver nitrate and resulted to be two of similar size (not shown).

Restriction map of ribosomal RNA gene

On the basis of the restriction pattern obtained with single and double digestions with previously mentioned endonucleases, we tentatively constructed a restriction map of rDNA of *P. oceanica*, and found that, in our material, only one type of rDNA, about 18.5 kb long, is present (Fig. 1).

Two Eco RI restriction sites were located near the 3′ end of both the 18S and 25S regions. This is similar to what is found in most plant species in which these sites are highly conserved, though monocots show some exceptions. For example in some *Pooideae*, such as species of *Brachypodium*, the Eco RI restriction site near the 3′ end of the 18S region is absent (SHI et al. 1993). One Bam HI site was located in the 18S region, two sites in the 25S region, one in the intergenic spacer. This Bam HI restriction pattern is more or less similar to the one found in monocots, such as *Ornithogalum montanum* (DE DOMINICIS 1989). The occurrence is

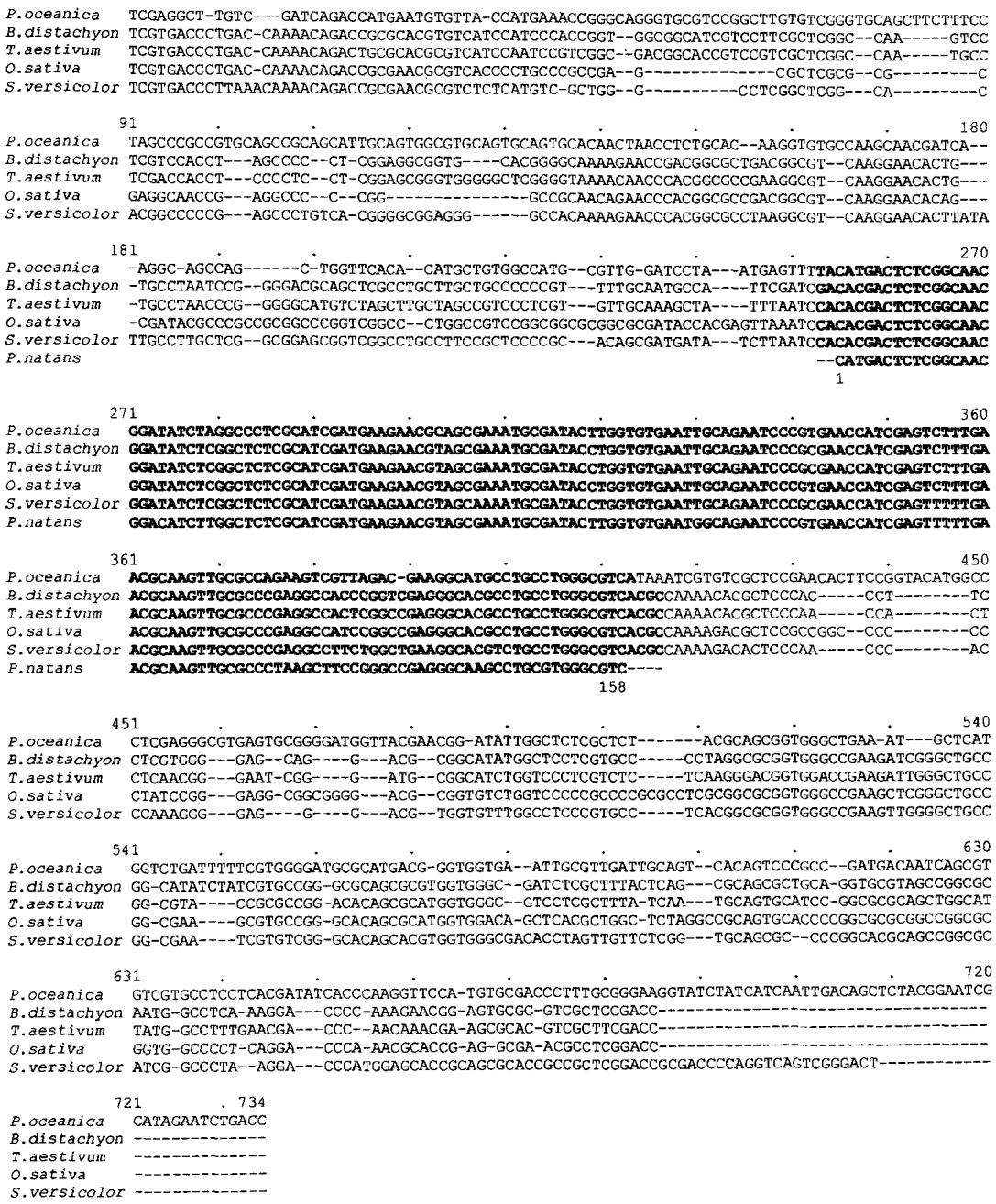

Fig. 2. Aligned DNA sequences of the internal transcribed spacer region from *P. oceanica* and other monocotyledonous plants (EMBL accession numbers are reported in Materials and Methods). The 5.8S sequences of *Potamogeton natans* (TROITSKY et al. 1991) is also reported. Numbers indicate conservative nucleotide positions of 1-734 (5′-3′) from the beginning of ITS1 region to the end of ITS2 region. Characters in bold indicate 5.8S sequences. Dashes denote gaps.

worth mentioning of an extra Bam HI site in ITS 1 of *P. oceanica*, which is quite unusual in other plant species. Sac I restriction pattern showed one cleavage site in the 18S region, four sites in the 25S and one near the 3′ end of the IGS region. Two Xba I cleavage sites were located in the 18S region, one in the 25S and one in the IGS region.

ITS region

Sequence analysis of the amplified ITS region of *P. oceanica* indicated that the length of ITS1 was 226 bp with 56 % GC, while ITS2 was 301 bp long with 54 % GC, and 5.8S was 160 bp long with 53 % GC.

The result of the multiple alignments of the sequence corresponding to the entire ITS region of *P.*

Table 1. *Pairwise divergence between ITS region sequences (a) and 5.8S sequences (b) from P. oceanica and other monocotyledonous plants. Above the diagonal are mean distances (1.0 = 100%), below the diagonal are the absolute number of nucleotide substitutions*

		1	2	3	4	5
a)						
1	*P. oceanica*	–	0.330	0.365	0.337	0.356
2	*B. distachyon*	190	–	0.158	0.199	0.207
3	*T. aestivum*	210	94	–	0.207	0.229
4	*O. sativa*	186	112	117	–	0.191
5	*S. versicolor*	206	119	131	107	–
b)						
		1	2	3	4	
1	*P. oceanica*	–	0.110	0.091	0.085	
2	*P. natans*	18	–	0.091	0.085	
3	*T. aestivum*	15	15	–	0.018	
4	*O. sativa*	14	14	3	–	

oceanica, with those of homologous regions of other monocots is reported in Fig. 2.

Many gaps had to be introduced in ITS region of *P. oceanica* when the sequence was aligned with those of *B. distachyon*, *T. aestivum*, *O. sativa* and *S. versicolor*. The sum of the gap lengths was 182 nucleotide sites out of the total of 734 sites of the entire ITS region. The total proportion of nucleotide sites included in the inferred gaps ranged from 5.9 % (44 nucleotide sites) in *P. oceanica* to 19.4 % (143 nucleotide sites) in *O. sativa*. In *P. oceanica* and *P. natans* the 5.8S sequence is 160 and 158 bp long, respectively, that is shorter than in other examined taxa in which it is 164 bp long. More exactly, in *P. oceanica*, 5.8S has an internal gap of a single nucleotide and is three nucleotides shorter at the 3′ end; in *P. natans* it is two nucleotides and four nucleotides shorter at the 5′ and 3′ ends, respectively. Comparison of ITS region sequence pairs gave divergence values ranging from 15.8 % to 36.5 % of nucleotides (Table 1a). Divergence among *P. oceanica* and the other taxa was higher than 30 %. Alignment of all ITS region positions resulted in a matrix of 734 characters (Fig. 2) of which 323 were variable. This variation is equally distributed between ITS1 (44.3 %) and ITS2 (48 %). Among variable characters, 77 were potentially informative phylogenetically (Fig. 3). ITS2 accounted for most of this variation (60 %) compared to 35 % in ITS1 and 5 % in 5.8S.

The ITS sequence data of the five monocotyledons were tested for phylogenetic signal. (Fig. 4a) The tree length distribution of 100,000 random parsimony trees was clearly skewed, with $g1 = -0.82$, suggesting that there is a strong phylogenetic signal in the data set (HUELSENBECK 1991).

Taxon	Nucleotide position (in Fig. 2)
	111111111222222222223333444444444444555555555
	233467901233458900001123444488923355588899900146678 8
	938796203445288614567965089234220234667935701264914 9
P. oceanica	AT?CGGAGAGGTCAG?TTTCTTGGTAGTTTAGGACGG?AAGTTCTCGGAGTT
B. distachyon	CTTCCCCTTTGGGCAGCCTCTCTACCGCCCGCCCCGGCAACCTTGATGGGTC
T. aestivum	CCTTCCCTTTGGCAAGTCTACTTATTACCTGCCACACCACTCTTCATAGACC
O. sativa	ACCGCGAG??CCGCGCCTCGCTGGCAATTCGGGCATCTGCTCCCCCAAGATC
S. versicolor	ACCGG?CGAGCCGAGCCTCGCCCGTTACTCAGCAAAGTGTCTCTGCAAAGCT
	555666666666666666666666
	999003333344445566667778
	138270123901354803481251
P. oceanica	GTGCTTGTCTCCCCCGTCATGACG
B. distachyon	TAGCCCAATTCAAGCAAGGTGCTC
T. aestivum	TAATTTTATTTTAC?CAGACACTC
O. sativa	GCACTCGGTCCTCGACCCG?GACG
S. versicolor	GTGTCCATCCTA?GAACCGCACCG

Fig. 3. Data matrix of potentially phylogenetically informative nucleotide positions from the ITS region of *P. oceanica* and other monocotyledonous plants. Vertical columns are nucleotide positions as given in Fig. 2. Horizontal rows are nucelotide states from individual sequences. "?" = gaps or missing nucleotides.

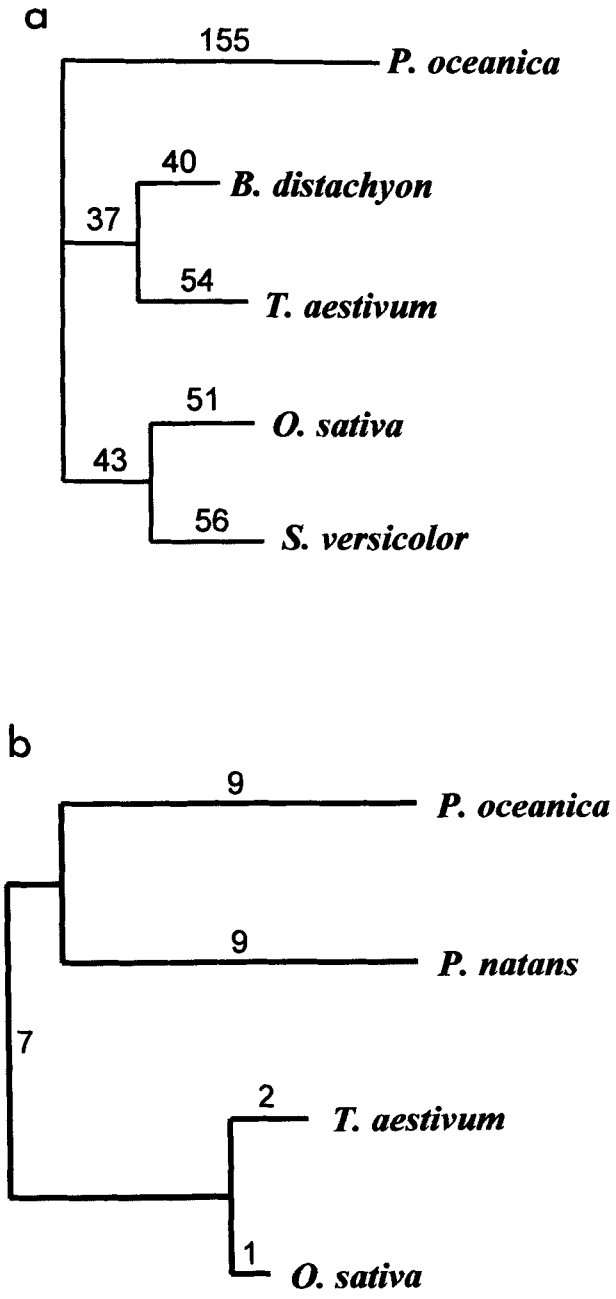

Fig. 4 a and b. Phylogenetic trees obtained from Wagner parsimony analysis using the Branch-and-Bound option of the PAUP program. **a** The phylogram of the single maximum parsimony tree generated from potentially informative ITS sequence data. Tree length = 456 steps; CI = 0.678; R = 0.37. **b** The phylogram of the maximum parsimony tree generated from 5.8S sequences of four species; Tree length = 28 steps; CI = 0.7; RI = 0.6. Gaps were treated as missing data. The numbers above the branches show the number of changes within the lineage.

Parsimony Wagner analysis using only the informative sites generated a tree of 456 steps in length, as shown in Fig. 4a. The value of the consistency index (CI) was 0.678 and the value of the retention index (RI) was 0.37. The evolutionary tree estimated by the maximum likelihood method was essentially similar in its topology to the maximally parsimonious tree (data not shown).

For 5.8S analysis, the sequence of *P. oceanica* was compared to three 5.8S sequences (*P. natans*, *T. aestivum* and *O. sativa*) of monocots examined by TROITSKY et al. (1991). In the alignment of the 5.8S region there were 25 variable sites, of which only five were informative. The estimated distance matrix is shown in Table 1b. The divergence value ranged from 1.8 % to 11 %. Separate Wagner branch-and-bound analysis of 5.8S data resulted in a parsimonious tree with CI value of 0.7 and RI value of 0.6 (Fig. 4b), and the $g1$ value of 100,000 random tree was -0.69.

DISCUSSION

The subclass Alismatidae represents the largest group of aquatic angiosperms and includes all known marine angiosperms and hydrophylous monocotyledons. The phylogenetic relationship of this subclass with other angiosperms has been studied by several authors (reviewed by LES and HAYNES 1995). Comparative methods based on morphological and molecular data indicate that Alismatidae is relatively primitive within monocotyledons and argue strongly for its monophyly. Although rRNA genes have been used extensively for assessing phylogeny in plants, molecular data concerning these genes in Alismatidae are limited to the nucelotide sequences of 18S and 25S of four genera (*Echinodorus*, *Sagittaria*, *Najas* and *Potamogeton*, HAMBY and ZIMMER 1992) and the 5.8S sequence of *P. natans* (TROYTSKY 1991), while no data are available on the general structure of these genes.

The results reported in this paper, on nucleoli, rRNA genes and ITS region of *P. oceanica*, are the first data concerning Alismatidae. In literature, to our knowledge, few details are reported on chromosome morphology of *P. oceanica* (DEN HARTOG et al. 1987). Though we had some difficulties in the cytological investigations, we were able to count a maximum number of two nucleoli indicating the presence of two NORs in the somatic chromosomes of this species.

Results obtained with restriction pattern of rDNA indicate that *P. oceanica* has only one type of repeat unit, 18.5 kbp long. This size is due to the remarkable length of the IGS which makes the rDNA unit of *P. oceanica* one of the longest among ribosomal genes in monocots. The presence of a unique type of rDNA seems to be in agreement with the great genetic uniformity ascribed to this species on the basis of different approaches such as karyological studies and DNA fingerprinting (DEN HARTOG et al. 1987; PRO-

CACCINI et al. 1996). On the other hand the low level of polymorphism could be related to the fact that, in *P. oceanica*, as in other seagrasses, the vegetative reproduction is prevalent over the sexual one and so the probability of genetic recombination is rather low.

We also report the sequence of the entire ITS region of *P. oceanica* and its comparison with those of homologous regions present in other monocots.

The size of the ITS1 and 5.8S regions in *P. oceanica* is comparable to those of other angiosperms (HSIAO et al. 1994), while ITS2 is about 30 % longer. It will be interesting to verify whether the unusual size of the ITS2 is a common feature among species of Alismatidae given the putative monophyly of this subclass.

In early hypothesis on the aquatic origin of monocotyledons HENSLOW (1911) suggested that the Alismatidae subclass represents a basal lineage of monocots. A similar conclusion was drawn by TROITSKY et al. (1991) and HAMBY and ZIMMER (1992) who performed phylogenetic studies analyzing rDNA sequence data. However, the latter authors noted that such result is preliminary because of their limited sampling.

On the contrary cladograms generated from morphological data (DAHLGREN and BREMER 1985), rubisco-SSU amino acid sequence data (MARTIN and DOWD 1991) and *rbc*L sequence data (CHASE et al. 1993) indicated that the Alismatidae subclass is probably not basal but, instead, originated from a monocotyledon ancestor.

We aligned the 5.8S sequence of *P. oceanica* with the homologous ones from three monocots (*P. natans*, *O. sativa* and *T. aestivum*) examined by TROITSKY et al. (1991). The most parsimonious tree inferred from 5.8S sequence data support the monophyletic origin of the two alismatids (Fig. 4b). The phylogenetic position of *P. oceanica* is also supported by the tree obtained using the entire ITS sequence data (Fig. 4a). This analysis was made comparing the ITS of *P. oceanica* with those of 4 (*S. versicolor* was used rather than *S. bicolor*) out of 10 species which HSIAO et al. (1994) studied to estimate phylogenetic relationships in monocots. Our ITS tree shows that *P. oceanica* occupies a lineage which is paraphyletic to both the Pooideae group and the one comprising *S. versicolor* and *O. sativa*. Moreover both our cladograms indicate that the Alismatidae subclass, as suggested by other authors (DAHLGREN and BREMER 1985; MARTIN and DOWD 1991; CHASE et al. 1993), is not basal within monocotyledons but, instead, originated from a primitive ancestor common to other monocots.

Studies designed to characterize the ITS region of species closely related to *P. oceanica* are under way in our laboratory. We believe that analysis of ITS sequence data might be a useful tool for phylogenetic studies of Alismatidae, as shown for other plant groups (BALDWIN 1992).

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