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Evolutionary history of the seagrass genus *Posidonia*

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ABSTRACT: Seagrasses are the structural species of one of the most important coastal ecosystems worldwide and support high levels of biodiversity and biomass production. *Posidonia* is one of the most ancient seagrass genera and displays a contrasting disjunct biogeographic pattern. It contains one single species in the Northern Hemisphere, *P. oceanica*, which is endemic to the Mediterranean Sea, and has up to 8 recognized taxa in the Southern Hemisphere, which in Australia are divided into 2 complexes, *P. ostenfeldii* and *P. australis*. A phylogeny based on a nuclear marker (rRNA-ITS) revealed an ancient split between the northern (i.e. Mediterranean) and southern (i.e. Australian) taxa, followed by a separation of the 2 recognized Australian complexes. However, the species belonging to the *P. ostenfeldii* complex were indistinguishable, suggesting an ecotypic origin or a recent speciation. Therefore, among the 7 morphologically described Australian species only 4 species lineages can be discriminated. The organelle markers *nad*7 intron, *trn*L–F and *mat*K/*trn*K intron were not informative for reconstructing the phylogeny of this genus, and the mitochondrial markers exhibited a strikingly slow evolutionary rate relative to other genome regions.

KEY WORDS: *Posidonia* · Phylogeny · Low evolutionary rates · Ancient diversification

INTRODUCTION

Seagrasses are clonal angiosperms that adapted to life in the sea where they form important habitat structures and enhance biodiversity and primary production in coastal ecosystems (Hemminga & Duarte 2000). Seagrasses colonized the sea about 100 million years ago (Hemminga & Duarte 2000) and presently comprise only about 60 species. They represent less than 0.02% of the angiosperm flora (Les et al. 1997) and are distributed in 5 monocotyledon families (Cronquist 1988, Cook 1990) containing 12 genera. This consistent paucity in the number of species has been attributed to several factors, including the large-scale dispersal that is facilitated by the lack of barriers (Palumbi 1994, Vermeij 1987) in the marine realm, and the uniform marine environment (Hemminga & Duarte 2000, Darling et al. 2004), the lack of co-evolution with pollinators associated with the adaptation to pollination in aquatic environments (van der Hage 1996, Ollerton & McCollin 1998, Raven 1998), lack of competition (e.g. Losos 2000) and dominance of clonal growth (Waycott et al. 2006). Yet, genetic studies in populations of *Cymodocea nodosa*, *Posidonia oceanica*, *Zostera noltii* and *Z. marina* have reported high differentiation along their distributional ranges, although species- and site-dependent (Coyer et al. 2004, Olsen et al. 2004, Arnaud-Haond et al. 2007, Alberto et al. 2008). This indicates that large-scale dispersal as a general rule does not occur for seagrasses; therefore, unusual patterns of molecular evolution may have occurred and should be explored.

Species of the genus *Posidonia* are the only members of the seagrass family Posidoniaceae and are considered to rank among the earliest marine angiosperms

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(Den Hartog 1970). Fossils of *Posidonia* found in Europe and recorded from the Cretaceous to the Miocene have remained apparently almost unchanged over this long evolutionary history (Den Hartog 1970).

Based on ecological, vegetative, reproductive and morphological characteristics, 9 species were initially described with a unique fragmented distribution. *Posidonia oceanica* is endemic to the Mediterranean Sea, whereas the other 8 described species (*P. australis*, *P. sinuosa* and *P. angustifolia* from the *P. australis* complex and *P. coriacea*, *P. denhartogii*, *P. kirkmanii*, *P. ostenfeldii* and *P. robertsoniae* from the *P. ostenfeldii* complex) are restricted to subtropical and temperate Australian waters (Cambridge & Kuo 1979, Kuo & Cambridge 1984) and are separated by about 17 000 km from their Mediterranean congeneric species. More recently, morphological information combined with allozymes revealed that *P. coriacea* and *P. robertsoniae* within the *P. ostenfeldii* complex are synonyms (Campey et al. 2000) resulting in 7 recognized species in the Australian complexes. Despite the relatively small number of *Posidonia* species, 3 groups are recognized based on the presence or absence of a primary root in the Australian and Mediterranean species respectively, on the width of their leaf (thicker but stiffer in the *P. ostenfeldii* complex) and on rhizome features (horizontal/vertical growth) as well as ecological characteristics associated with the 2 Australian groups (*P. australis* and *P. ostenfeldii* complexes) (Gobert et al. 2006).

Australian species of *Posidonia* are distributed along the southwestern, southern and southeastern coasts of Australia (Box 1). *P. oceanica* covers an area in the Mediterranean Sea between 2.5 and 5 million ha (Pasqualini et al. 1998). Previous genetic analyses (Waycott & Les 2000) and the fossil record have shown one Australian (*P. australis*) and one Mediterranean (*P. oceanica*) species to be genetically distant, which support an early evolutionary separation (Gobert et al. 2006). Based on presumed geological history and continental drift, Phillips & Menez (1988) estimated that the Australian *P. australis* and the Mediterranean *P. oceanica* diverged during the late Eocene ca. 40 million years before present (Myr BP). However, using a molecular clock based on internal transcribed spacer (ITS) and *rbc*L markers, Les et al. (2003) estimated a more recent divergence (16.7 ±12.3 Myr BP). The Tethys Sea closure during the Miocene is thought to have played an important role in the disjunction of Mediterranean and Australian taxa (Searle et al. 1987, Robertson & Comas 1998). Besides this comparison, preliminary results for *Posidonia* phylogeny on the basis of partial ITS and chloroplastidial sequences (Waycott & Les 2000, Waycott et al. 2006) showed no differences among species within the *P. ostenfeldii* complex, but had low support for Australian taxa. In the absence of details about the markers used, the lack of clear lineage sorting reported among species may be due to the low resolution of markers used or a possible synonymy of some of the morphologically defined taxa. Further analyses with various markers are therefore still needed to reconstruct a comprehensive phylogeny of the *Posidonia* genus. This would resolve species boundaries and their biogeographic history as well as clarify the pattern of molecular evolution in the genomes (organelle and nuclear) of these seagrass species.

Here we analyzed the phylogenetic relationship of all 8 morphologically recognized *Posidonia* species with a comprehensive set of markers from the 3 compartments of the genome (nuclear, mitochondrial and chloroplastidial). Our aim was to (1) help resolve the taxonomic status of the Australian species because some morphological characters used to determine species remain under debate and (2) compare the phylogenetic patterns observed with various markers from both organelle (chloroplastidial and mitochondrial) and nuclear genomes. After screening various loci for variability and phylogenetic information content to resolve the phylogeny of *Posidonia*, mitochondrial (*nad*7 intron), chloroplastidial (*trn*L–F and *mat*K/*trn*K intron) and nuclear (ITS) markers were retained.

MATERIALS AND METHODS

Plant material, markers amplification and sequencing. We followed the methods described by Campey et al. (2000) to investigate 7 distinct Australian species in this study. Sampling of *Posidonia* in Australia and in the Mediterranean Sea was performed in 2002; DNA was extracted by means of the 1% N-cetyl N,N,N-trimethylammonium bromide (CTAB) method (Doyle & Doyle 1987) and stored at –20°C. Sampling sites and number of individuals per species analyzed in this study are described in Table 1. Several individuals of each of the following species were collected: *P. australis*, *P. angustifolia*, *P. sinuosa*, *P. coriacea*, *P. denhartogii*, *P. kirkmanii* and *P. oceanica* (the latter from geographically distant sites within the Mediterranean, i.e. Spain, Italy, Cyprus, Greece, Malta and Tunisia). In total, 31 individuals were collected. All Australian taxa were identified in the field. An outgroup taxon, *Ruppia cirrhosa*, was selected based on phylogenetic distance from the ingroup (Les et al. 1997).

Mitochondrial regions: Mitochondrial coding sequences of *Posidonia* species available in GenBank (apocytochrome b [*cob*], accession nos. DQ8591498 and DQ859149; ATPase [*atp*1], accession nos. DQ85 9111 and DQ859112) were aligned and analyzed to

Box 1. Distribution, habitat preference and taxonomy of Australian *Posidonia* species

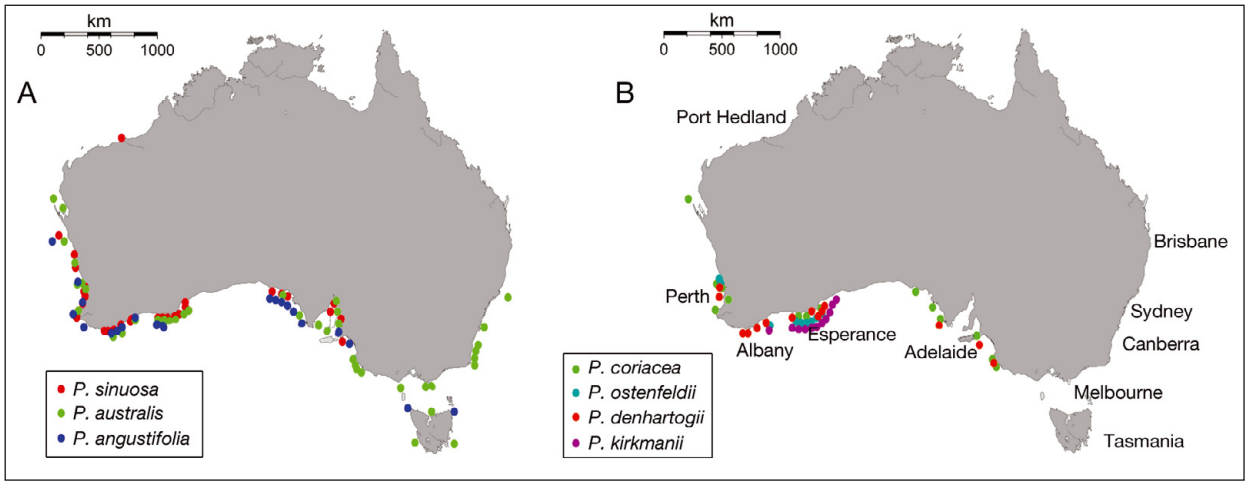

Fig. B1. Distribution of *Posidonia* species around Australia

Distribution: The complex *Posidonia australis* is composed of *P. australis*, *P. sinuosa* and *P. angustifolia* (all previously described as '*P. australis*') (Cambridge & Kuo 1979). The species that gives the name to this complex has the widest distribution among Australian *Posidonia* species and is found from western Australia (from Shark Bay), around the southern and southeastern coastlines and along the northern coast of Tasmania (Cambridge & Kuo 1979, Kuo & Cambridge 1984, Trautman & Borowitzka 1999 Fig. B1A). *P. sinuosa* has a similar geographic range in Western Australia (although its northern limit is Port Headland) but does not extend farther east than Kingston in southern Australia (Trautman & Borowitzka 1999), while *P. angustifolia* is found from the Houtman Abrolhos and along the southwest coast of Western Australia to Encounter Bay, southern Australia (Cambridge & Kuo 1979). *P. australis* and *P. sinuosa* typically inhabit relatively sheltered coastal waters where they are protected from strong currents, whereas *P. angustifolia* is found in more exposed habitats (Kuo & Cambridge 1984, Carruthers et al. 2007).

Species from the *Posidonia ostenfeldii* complex are distributed along the coast of southern Australia, and *P. coriacea*, the most widely distributed of this complex, is also found along the western coast (Fig. B1B). Individuals belonging to the *P. ostenfeldii* complex grow in open waters along the coast and in high energy coastal sublittoral habitats (Kuo & Cambridge 1984).

Taxonomy: The first species of *Posidonia* genus to be described was *P. oceanica* Linnaeus, 1735 in Systema Naturae, although the genus was then named *Zostera*. The genus itself was described by König 70 yr later and then, the former *Z. oceanica* was renamed by Delile (1813) and included here as *P. oceanica*. In 1858, another species was described and recognized as member of *Posidonia* genus; it was geographically distant from *P. oceanica* and had different characteristics such has the absence of a primary root. Joseph Hooker and his colleague Thomas Thomson (1858) named it *P. australis* because of its location. Much later, Den Hartog (1970) described a distinct species based on the morphology of the leaves and rhizome, *P. ostenfeldii*.

Within *Posidonia australis* 2 different forms were recognized, the 'broad leaved' form and the 'narrow leaved' form. The different forms occurred in sympatry and until 1979 were thought to be the same species (Cambridge & Kuo 1979). A study by Cambridge & Kuo (1979) recognized differences in the shape of the epidermal cells, the position of fibre cells in the leaf and leaf sheath and the presence around the rhizome of straw-like fibres, which distinguished 2 species that also differed in habitat, growth patterns and depth distribution. The 'broad leaved' form is now formally recognized as *P. australis* and the 'narrow leaved form' is now called *P. angustifolia*. A third 'narrow leaved' species was previously included in *P. angustifolia* or in *P. ostenfeldii* but is now known as *P. sinuosa* (Cambridge & Kuo 1979).

The Australian *Posidonia ostenfeldii*, which has thicker and stiffer leaves than its congeneric *P. australis*, was redescribed in 1984 by John Kuo and Marion Cambridge (Kuo & Cambridge 1984). As for the other Australian group, taxonomists felt the different morphologic forms could actually be different species. This group of species inhabits more exposed waters with stronger wave action (Kuo & Cambridge 1984). They considered leaf morphology and anatomy — a combination of the leaf width (that can sometimes overlap), number of vascular bundles, the appearance of epidermal cells in surface view and in cross-section and fibre bundles in the leaf sheaths — as the best characteristics for distinguishing the species (Kuo & Cambridge 1984). From this reanalysis 4 additional new species were added to the *Posidonia* genus as we know it today: *P. denhartogii*, *P. robertsoniae*, *P. coriacea* and *P. kirkmanii*. A further study by (Campey et al. 2000) aimed at re-evaluating species boundaries among this complex suggested that all these species actually are synonymous. The morphologic features analyzed did not separate the species into distinct groups, suggesting a morphological continuum. In addition, no polymorphism was detected with allozymes among all taxa. When comparing *P. robertsoniae* and *P. coriacea*, those authors suggested that differences between those 2 species are largely the result of environmental and developmental factors and there is little evidence to support 2 different species (Spalding et al. 2003). The whole *P. ostenfeldii* complex was questioned until further morphological and genetic evidence could resolve their status.

Table 1. *Posidonia* taxa included in this study with sampling location and markers used. Also shown is *Ruppia cirhosa*, used as the outgroup in the analysis

Species	Locality	Markers amplified in this study
P. kirkmanii Kuo and Cambridge	Western Australia, Wylie Bay	ITS, *nad*7, *trn*L
P. kirkmanii Kuo and Cambridge	Western Australia, Wylie Bay	ITS, *trn*L, *mat*K/*trn*K intron, *trn*L–F
P. ostenfeldii den Hartog	Western Australia, Wylie Head	ITS, *nad*7, *trn*L, *trn*L–F
P. ostenfeldii den Hartog	Western Australia, Wylie Head	ITS, *trn*L
P. ostenfeldii den Hartog	Western Australia, Wylie Head	ITS, *trn*L
P. sinuosa Cambridge and Kuo	Western Australia, Lal Bank	ITS, *nad*7, *trn*L, *trn*L–F
P. sinuosa Cambridge and Kuo	Western Australia, Two Peoples Bay	ITS, *trn*L
P. sinuosa Cambridge and Kuo	Western Australia, Wanbro Sound	ITS, *trn*L
P. sinuosa Cambridge and Kuo	Western Australia, Duke of Orleans Bay	ITS, *trn*L
P. sinuosa Cambridge and Kuo	Western Australia, Duke of Orleans Bay	ITS, *trn*L
P. australis Hook. f.	Western Australia, Lal Bank	ITS, *nad*7, *trn*L
P. australis Hook. f.	Western Australia, Duke of Orleans Bay	ITS, *trn*L
P. australis Hook. f.	Western Australia, Wanbro Sound	ITS, *trn*L
P. australis Hook. f.	Western Australia, Wanbro Sound	ITS, *trn*L
P. australis Hook. f.	Western Australia, Two Peoples Bay	ITS, *trn*L
P. australis Hook. f.	Western Australia, Duke of Orleans Bay	ITS, *trn*L
P. australis Hook. f.	Western Australia, Duke of Orleans Bay	ITS, *trn*L
P. angustifolia Cambridge and Kuo	Western Australia, Lal Bank	ITS, *nad*7, *trn*L
P. angustifolia Cambridge and Kuo	Western Australia, Lal Bank	ITS, *trn*L
P. coriacea Cambridge and Kuo	Western Australia, Taylor St Jetty	ITS, *nad*7
P. coriacea Cambridge and Kuo	Western Australia, Taylor St Jetty	ITS, *trn*L
P. coriacea Cambridge and Kuo	Western Australia, Success Bank	ITS, *trn*L, *trn*L–F
P. coriacea Cambridge and Kuo	Western Australia, Two Peoples Bay	ITS, *trn*L
P. coriacea Cambridge and Kuo	Western Australia, Two Peoples Bay	ITS, *trn*L
P. denhartogii Kuo and Cambridge	Western Australia, Wylie Head	ITS, *nad*7, *trn*L
P. oceanica (L.) Delile	Spain, Rodalquilar	ITS, *nad*7, *trn*L, *mat*K/*trn*K intron, *trn*L–F
P. oceanica (L.) Delile	North Italy (Camogli), 9° 09.00′ E, 44° 20.00′ N	ITS
P. oceanica (L.) Delile	Cyprus (Paphos), 32° 26.23′ E, 34° 43.54′ N	ITS
P. oceanica (L.) Delile	Greece (Agios Nicolaos), 23° 55.62′ E, 37° 42.97′ N	ITS
P. oceanica (L.) Delile	Malta, 14° 20.00′ E, 35° 59.00′ N	ITS
P. oceanica (L.) Delile	Tunisia (Ergla), 10° 36.00′ E, 35° 53.00′ N	ITS
Ruppia cirrhosa		GenBank (AJ012292), ITS

obtain a first estimate of their putative usefulness in discriminating species. A pairwise comparison was systematically first performed with *P. oceanica* and one of the Australian representatives (*P. australis* or *P. kirkmanii*) to assess the level of polymorphism of each marker. When polymorphism was low between these supposedly most distant taxa, the marker tested was not further used on any other species.

Another marker, the intron of *nad*7 gene, encoding the subunit 7 of NADH dehydrogenase complex I, was amplified by PCR with universal primers *nad*7/2 and *nad*7/3 (Dumolin-Lapegue et al. 1997). After an initial denaturation at 94°C for 3 min, conditions were as follows: 40 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 1 min. The final extension was at 72°C for 5 min. A 25 µl reaction mixture was used that contained 250 µM dNTPs, 0.6 µM of each primer, 1× PCR buffer, 2 mM $MgCl_2$, 5 µl of template DNA (with a final concentration of about 10 ng μl^{-1}) and 2.5 U of *Taq* polymerase (Invitrogen).

Chloroplastidial regions: Three chloroplastidial regions were amplified (*trn*L, *trn*L–F and *mat*K/*trn*K intron) (Table 2) (Taberlet et al. 1991, Johnson & Soltis 1995) and one was analyzed by aligning the sequences available in GenBank (*rbc*L, U80718 and U80719). As in the case of mtDNA, chloroplastidial markers were discarded when low levels of divergence were observed between the Mediterranean and Australian representatives. For all 4 markers we used a reaction mixture of 25 µl containing 250 µM dNTPs, 0.6 µM of each primer, 1× PCR buffer, 2 mM $MgCl_2$, 5 µl of template DNA (with a final concentration of about 10 ng μl^{-1}) and 2 U of *Taq* polymerase.

The entire fragment of *mat*K region was amplified together with a significant portion of the flanking regions (*trn*K) using *trn*K-3914F (monocot) and *trn*K-2R universal primers (Johnson & Soltis 1995). Due to unspecific amplifications, the expected bands (of approximately 2600 bp) corresponding to the *trn*K region were extracted from the gel and cloned using pGEM-T Easy

vector. Sequences were then obtained using M13 primers and new specific primers (Table 2) were designed for *Posidonia* species on adjacent regions of the *mat*K/*trn*K intron that were conserved in all species.

***ITS region* (*nuclear marker*):** The complete ITS region of the nuclear ribosomal DNA (ITS1, 5.8S rDNA and ITS2) was amplified using the primers ITS1 and ITS4 (White et al. 1990) following the same conditions described in (Kumar & Shukla 2005). The 25 µl reaction mixture contained 100 µM dNTPs, 0.5 µM of each primer, 1× PCR buffer, 1.5 mM $MgCl_2$, 5 µl of template DNA (with a final concentration of about 10 ng μl^{-1}) and 1 U of *Taq* polymerase.

All PCR amplifications were performed on a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) and all PCR products were cleaned with ExoSAP-IT Clean-Up enzyme (USB) following the manufacturer's protocol and then sequenced according to the Sanger method on a genetic analyzer (model ABI 3130xl, Applied Biosystems) using forward and reverse primers.

Alignment. For individual accessions, chromatograms were first imported and processed for quality with the PreGap4 program of the Staden Package (Staden 1996). Contiguous sequences were then assembled and consensus sequences obtained using the shotgun assembly option of the Gap4 program of the Staden Package. Finally, sequences were aligned using CLUSTAL X (Thompson et al. 1997) to detect polymorphisms and check for putative sequencing errors. One haplotype/genotype per species was chosen to reconstruct phylogenies, as the various individuals/sampling units sequenced per taxa did not show any polymorphism.

Phylogenetic data analysis. Phylogenetic analyses were performed on 3 molecular data sets: (1) ITS, (2) *trn*L and (3) *trn*L–F (concatenating sequences were amplified with primers c–d and e–f after the last one was too small to be analyzed by itself; see Table 2 for description of primers c–f) by means of the same methodology and an equal number of sequences from each taxa. All 8 taxa (7 Australian taxa plus the unique Mediterranean taxon) were included for all analyses except for the *trn*L–F sequences where only 6 species were amplified for one individual/species, due to consistent failure in the amplification of *Posidonia denhartogii* and *P. australis*. In the ITS analyses, *Ruppia cirrhosa* (AJ012292) was used as the outgroup, while *P. oceanica* was used for the *trn*L data set as the outgroup as the former was shown to decrease the phylogenetic resolution.

Maximum parsimony (MP) analyses were conducted with PAUP* v. 4.0 Beta Windows (Swofford 2002). MP searches were conducted using heuristic search methods with tree bisection reconnection (TBR) branch swapping, collapse of zero length branches and equal weighting of all characters. The analyses were repeated 1000 times with the 'random addition' option. To assess confidence in clades, bootstrap tests (Felsenstein 1985) were performed using 1000 replicates with heuristic search settings identical to those of the original search.

Maximum likelihood (ML) analyses were run on GARLI v. 0.96 (Zwickl 2006) and the best-suited nucleotide substitution model to be used on ML was determined by running Modeltest v. 3.7 (Posada & Crandall 1998) on PAUP*. One single run with 10 search replicates was performed on GARLI. The number of generations without topology improvement required for termination was set to 20 000 and the maximum score improvement over recent generations required for termination was set to 0.01; the remaining settings were kept at the default. Likelihood scores were obtained with PAUP*, as the implemented algo-

Table 2. Chloroplastic markers used in this study with respective primers and PCR parameters

Chloroplastic markers	Primer (5′–3′)	Source	PCR parameters
*trn*L–F	c: CGA AAT CGG TAG ACG CTA CG d: GGG GAT AGA GGG ACT TGA AC e: GGT TCA AGT CCC TCT ATC CC f: ATT TGA ACT GGT GAC ACG AG	Taberlet et al. (1991)	94°C, 2 min; 35 × (94°C, 1 min; 53°C (c,d), 54°C (e,f), 1 min; 72°C, 2 min); 72°C, 7 min
*mat*K/ *trn*K intron	*trn*K-3914F: ATC TGG GTT GCT AAC TCA ATG G *trn*K-2R: AAC TAG TCG GAT GGA GTA G	Johnson & Soltis (1995)	94°C, 3 min; 35 × (94°C, 1 min; 53°C, 1 min; 72°C, 2 min); 72°C, 10 min
*mat*K/ *trn*K intron2	PomatKFw: ACT GGA CTA TCT GTT TCG GTG GTA TCT PomatKR: GCT TAC TAA TGG GAT ACC CGG ATA CAT	This study	94°C, 3 min; 35 × (94°C, 1 min; 60°C, 1 min; 72°C, 2 min); 72°C, 10 min

rithm performs better for optimizing branch lengths on the final topology (Zwickl 2006). Supportive bootstrap values were calculated on GARLI and resulting trees were summarized in PAUP*.

Bayesian inference (BI) was performed in MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001). As some of the models given by Modeltest 3.7 are not accepted yet with MrBayes 3.1.2, the best nucleotide-substitution model was found by running MrModeltest 1.1b (Posada & Crandall 1998, Nylander 2004) on PAUP*. Bayesian analyses were performed with 2 parallel Metropolis-coupled Markov chain (MCMC) runs of 1 000 000 generations, sampling trees every 100 generations, and 4 chains with the default heating. A Dirichlet distribution was used for the base frequency parameters. Burn-in was determined by inspection of the log-likelihoods of sampled trees and sample points collected prior to stationarity were eliminated from the sample. Convergence was confirmed by using AWTY (are we there yet?) graphical analysis (Nylander et al. 2008).

Divergence time estimation. Divergence times of the main splitting events within *Posidonia* were estimated with the Multidivtime program (Thorne et al. 1998, Kishino et al. 2001). We used the ML topology that was inferred using the ITS data set as starting phylogeny because phylogenetic relationships within the genus were better resolved. The methodology assumes autocorrelation of molecular rates among lineages (Kishino et al. 2001). This Bayesian method also requires the specification of prior distributions for parameters. The prior assumption for the mean and SD of the time of the ingroup root node (rttm) was set to 0.706 time units, and the mean of prior distribution for rate at root node (rtrate) was set at 0.138, where 1 time unit in this analysis represents 100 Myr. The SD of the prior distribution was set to its maximum value (equal to the mean) to avoid violation of the definition of a prior. The only reliable fossil of this genus corresponding to an early form of *P. oceanica*, which is from the Maastricht region, The Netherlands, and dated from the Cretaceous (70.6 to 65.5 Myr BP) (Den Hartog & Kuo 2006, van der Ham et al. 2007), was used as calibration point. The MCMC method was employed to approximate both prior and posterior distributions (Kishino et al. 2001). Initial parameter values were randomly selected to initialize the Markov chain and then a burn-in period of 100 000 cycles was completed before parameters were sampled from the MCMC. Afterwards, the state of the Markov chain was sampled every 100 cycles until a total of 10 000 samples was collected.

Diversification rates through time. We investigated rates of diversification using the constant rate (CR) test with the γ statistic (Pybus & Harvey 2000, Pybus & Rambaut 2002) as implemented in the R package LASER v.2.3 (Rabosky 2006a,b). Because we have complete species sampling for the genus, the CR test is appropriate without having to perform a Monte Carlo simulation to account for missing lineages. Under a pure birth process (constant rates) γ-values follow a standard normal distribution with a mean of 0 (Pybus & Harvey 2000). Negative γ values suggest a slowdown in the rate of diversification through time whereas positive values indicate an increase in diversification rates towards the present. Therefore, the null hypothesis of constant rates is rejected at the 5% level in a 1-tailed test if $\gamma < -1.645$ (Barraclough & Vogler 2002). We also analyzed the semilogarithmic lineage through time (LTT) plot to evaluate whether the number of lineages was consistent with a constant rate of diversification through time. The relative cladogenesis statistics implemented in End-Epi 1.0 (Rambaut et al. 1997) allowed examining rates of diversification across the Bayesian-inferred linearized tree to identify branches in *Posidonia* phylogeny that show higher rates than expected under a constant model.

RESULTS

Phylogenetic analysis

Mitochondrial markers

Percentages of divergence for the 3 markers tested were between 0 and 0.2% among all species including the Mediterranean–Australian comparison (Table 3, including GenBank information). Due to this lack of polymorphism, those markers were not used to reconstruct phylogeny of *Posidonia* species.

Chloroplastidial markers

For the *trn*L marker the highest divergence was observed comparing *Posidonia oceanica* with species from the *P. australis* complex. The other chloroplastidial markers showed less than 1% of maximum divergence between these species, which were geographically separated by tens of thousands of kilometers; thus, they were not used for phylogenetic analyses. The only exception was *trn*L–F, which showed a cleavage due to a 200 bp deletion in *P. oceanica* compared with the remaining congeneric species. Yet, concatenated sequences of both *trn*L and *trn*L–F did not provide additional information compared with the *trn*L marker alone, and phylogenetic analysis will therefore only be reported for *trn*L sequences (Table 4). Summary statistics for the parsimony, maximum likelihood and Bayesian analyses of nuclear and chloroplastidial marker (*trn*L) are summarized in Table 4.

The alignment, as well as the values of maximum divergence level (Table 3), suggests enough resolution to separate at least 3 different groups of species based on the *trn*L region, corresponding to the clusters determined on the basis of morphology (both Australian complexes and *Posidonia oceanica*). Yet, limited or nonexistent resolution was observed when building the trees using the different methods (data not shown).

Table 3. Summary of statistics for each marker analyzed. The aligned sequence length is the size of the sequences after aligning and counting with the intersections; for *trn*F *Posidonia oceanica* is almost half of the remaining sequences with 225 bp

Marker type	GenBank accession number	Aligned sequence length (bp)	% of maximum divergence level between complexes or species
Mitochondrial			
*nad*7 intron	GQ927731 GQ927732 GQ927733 GQ927734 GQ927735 GQ927736 GQ927737 GQ927738 GQ92773	745	Between all species, 0%
cob	DQ859149 DQ859148	1020	*P. oceanica* vs. *P. australis*, 0.1%
*Atp*1	DQ859111 DQ859112	1053	*P. oceanica* vs. *P. australis*, 0.2%
Chloroplastidial			
*rbc*L	U80719 U80718	1182	*P. oceanica* vs. *P. australis*, 0.8%
*mat*K/*trn*K intron2	GQ927728 GQ927729	1150	*P. oceanica* vs. *P. kirkmanii*, 0.7%
*trn*L	GQ927746 GQ927747 GQ927748 GQ927749 GQ927750 GQ927751 GQ927752 GQ927753 GQ927754	619	*P. oceanica* vs. *P. australis* complex, 6.5%[a]
*trn*L–F	GQ927740 GQ927741 GQ927742 GQ927743 GQ927744 GQ927745	436	*P. oceanica* vs. *P. sinuosa*, 14.3%
Nuclear			
ITS	GQ927719 GQ927720 GQ927721 GQ927722 GQ927723 GQ927724 GQ927725 GQ927726 GQ927727	715	*P. oceanica* vs. *P. ostenfeldii* complex, 21.3%[a]

[a]For *trn*L, *trn*F and ITS markers only the maximum value, when comparing species and complexes, is shown

Table 4. (a) Summary of maximum parsimony analyses. CI: consistency index; RI: retention index. (b) Summary of maximum likelihood analyses. For Bayesian analyses the models chosen were GTR / F81 (Felsenstein 1981) for ITS and *trn*L, respectively. GTR: general time reversible; L: likelihood

(a) Maximum parsimony	
No. of variable characters	214/53
No. of parsimony-informative characters	63/2
CI (all characters)	0.9742/1.0000
CI (excluding invariant characters)	0.9012/1.0000
RI	0.9238/1.0000
(b) Maximum likelihood	
Model of sequence evolution	TrN (Tamura & Nei 1993) / F81 (Felsenstein 1981)
–Ln(L)	2196.71008 / 810.85175
Base frequencies	A = 0.249317, C = 0.283401, G = 0.237653, T = 0.229629 / A = 0.320819, C = 0.143566, G = 0.130724, T = 0.404891
Rate matrix	(A–C) = 1.000000, (A–G) = 3.43098, (A–T) = 1.000000, (C–G) = 1.000000, (C–T) = 1.79173, (G–T) = 1.000000 / (A–C) = 1.000000, (A–G) = 11.50114, (A–T) = 1.000000, (C–G) = 1.000000, (C–T) = 0.011513, (G–T) = 1.000000

Most differences found on alignment of sequences were indeed linked to differences in sequence structure (indels), which are considered as single events and do not increase the level of resolution much.

ITS region

ITS trees obtained from the 3 different methods were in strong agreement (Fig. 1, parsimony tree not shown) regarding not only the topology but also the branch lengths and bootstrap/posterior probability values. The divergence of Australian species from the Mediterranean species is readily apparent with additionally strong values validating the separation between the 2 Australian complexes recognized taxonomically (Fig. 1). In ML analysis, however, the species belonging to the *Posidonia ostenfeldii* complex have no statistical support to be considered as distinct species (Fig. 1). These results are also corroborated by Bayesian inference. Within the *P. australis* complex, whatever the method used, there is a clear divergence of *P. australis* and *P. angustifolia* from *P. sinuosa*.

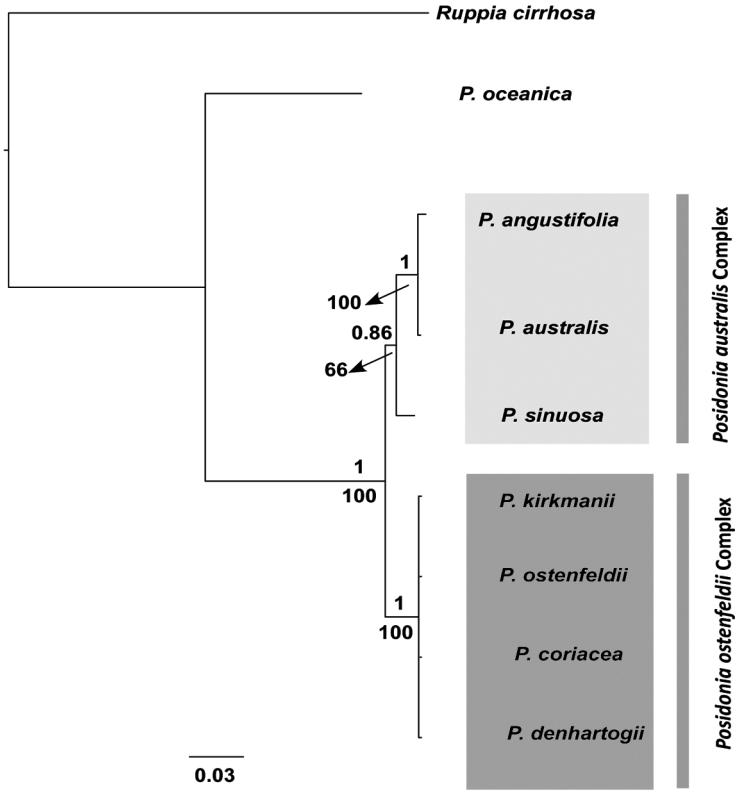

Fig. 1. Maximum likelihood [–ln(L) = 2196.71008/2527.51495] and Bayesian tree of Posidoniaceae from the ITS data set. Numbers above branches represent posterior probabilities and numbers below branches represent nonparametric bootstrap proportion (greater than 50%)

Divergence time estimation

The divergence time obtained with Multidivtime (Thorne et al. 1998, Kishino et al. 2001) between the 2 major clades (Mediterranean and Australian) was estimated to be 68.0 (65.6; 70.5) Myr BP (Fig. 2). The estimated time of the most recent common ancestor of the 2 Australian complexes was estimated at 12.8 (6.9; 23.7) Myr BP, followed by a likely latter diversification of the *Posidonia australis* complex that establishes the separation of *P. sinuosa* from the 2 other *Posidonia* lineages at about 8.4 (3.8,16.6) Myr BP.

Diversification rates through time

The LTT plot (not shown) showed a slight increase of the number of lineages towards the present, while the CR test indicated a nonsignificant γ value (γ = 1.99). The relative cladogenesis statistic did not identify any lineages with significantly higher diversification rates than expected.

Alignment of ITS fragments of *Posidonia oceanica* from different Mediterranean regions

Despite high polymorphism and genetic structure at microsatellites in *Posidonia oceanica* across the Mediterranean Sea (Arnaud-Haond et al. 2007), none of the 40 ITS sequences from samples taken from Spain to Cyprus showed any difference for any of the 708 bp that were sequenced and aligned.

DISCUSSION

Phylogeny of *Posidonia*: ancient or recent species?

The initial separation of the genus of *Posidonia* into Northern and Southern Hemisphere clusters, i.e. Mediterranean and Australian, is well supported by the ITS phylogeny. The first clade comprises a unique taxon, *P. oceanica*, that has been identified as the elder descendent of the Tethys Sea dweller *P. cretacea* (Larkum & Den Hartog 1989). The novelty of our results is that our divergence time estimates suggest an early separation (>60 Myr BP) (Fig. 2), which would have occurred in the ancient Tethys Sea and at a relatively early stage during the origin of marine angio-

sperms. This estimation is much older than the 16.7 Myr BP proposed by Les et al. (2003) using fixed substitution rates averaged from estimates obtained from distinct (nonseagrass) species and the 40 Myr BP suggested by Phillips & Menez (1988) on the basis of geological and tectonic history. According to Les et al. (2003) the fixed substitution rate of 0.27% per Myr used in that study did not reflect the rate of ITS evolution in most taxa. Divergence time estimates based on biogeographic events might be not accurate if the selected vicariance event separated previously contiguous areas under a variety of processes (Zakharov et al. 2004) as occurred in the Tethys Sea. Those processes might include multiple events of separation and mixture of the considered area or gradual vicariances that occurred over a long time period. The Bayesian approach used in the present study is expected to yield more reliable estimates by incorporating a more consistent source of calibration (fossil record) and by accommodating possible rate variation across the tree.

The deep divergence between the single Mediterranean *Posidonia oceanica* and the several Australian *Posidonia* lineages may result from either (1) an acceleration of diversification rates in some of the branches of the tree usually associated with recent adaptive radiations (Bromham & Woolfit 2004) or (2) the existence of high background extinction rates. According to the relative cladogenesis statistics test, no ancestral branches exceeding the expected rates of lineage accumulation were identified in the *Posidonia* ultrametric tree. The CR test indicated a nonsignificant positive γ value and did not support the hypothesis of a recent burst of cladogenesis. Therefore, the existence of high background extinction rates may more probably explain the deep divergence between *P. oceanica* and the Australian lineages. The estimate for the time when the the 2-antipode groups split matches the boundary for the mass extinction Cretaceous–Tertiary (K–T boundary) event. This catastrophic episode might have been responsible for a high number of extinctions of *Posidonia* ancestors, which explains the long branches separating Mediterranean and Australian clades in the reconstructed phylogenies. Stanley (2009) states that geographic range plays a role in survival from extinction at the genus level. Groups that have narrow geographic ranges or are ecologically

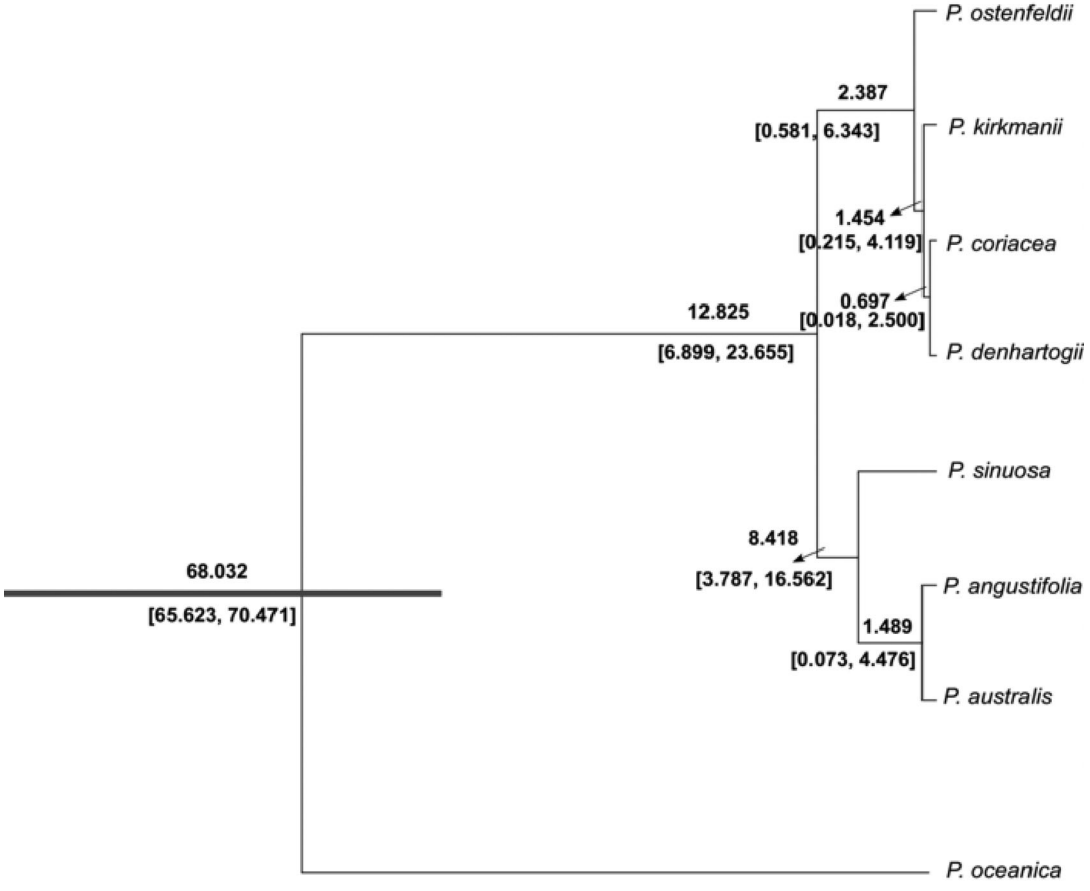

Fig. 2. Bayesian divergence dating analysis obtained with Multidivtime. Divergence dates were estimated on the ML topology derived from the ITS data set. Age estimates (in Myr, above branches) and corresponding 95% CI (bars and values in square brackets, below branches) are shown

specialized tend to experience high background extinction rates (Stanley 2009). Mediterranean and Australian *Posidonia* species share a specific Mediterranean-like climate; therefore, ecological specialization may have driven the extinction of other members of this genus between these 2 disjunct regions.

Although the Mediterranean Sea has also experienced large environmental changes during its history (Bianchi & Morri 2000), the only representative of the genus in the Northern Hemisphere is *Posidonia oceanica*. This scantiness of extant species may be due to the relatively homogeneous environment presently observed in the Mediterranean Sea in comparison to the southern Australian coasts, or to *P. oceanica* having been the only ancestral northern species that survived the recent major environmental changes in the Mediterranean Sea. However, some heterogeneity in the karyotypes of *P. oceanica* across various Mediterranean localities has been reported, with particular karyotypes being detected in one meadow of northern Africa (Algeria) (Semroud et al. 1992). Some phenological differences were also reported between eastern and western Mediterranean specimens (Bussotti et al. 1998). These observations led some investigators (Semroud et al. 1992, Bussotti et al. 1998) to question the taxonomic status of *P. oceanica* throughout the Mediterranean Sea. Biogeographic surveys of genetic diversity in *P. oceanica* (Arnaud-Haond et al. 2007, Serra et al. 2010), based on microsatellite nuclear markers, confirmed strong cleavage between populations in the eastern and western Mediterranean basins probably resulting from vicariance events during the last glaciations. Although such isolation maintained over appropriate time scales could lead to speciation, these population divergence patterns still do not support speciation (Arnaud-Haond et al. 2007, Serra et al. 2010). The results from the present phylogenetic analysis also support the existence of a single Mediterranean species, *P. oceanica*, as no polymorphism was observed for 708 bp of the ITS throughout the entire Mediterranean Sea.

Posidonia oceanica species survived the closure and partial desiccation of the Mediterranean Sea during the Messinian salinity crisis (Boudouresque 2004) in putative Mediterranean refugial areas. Despite its long history a single sequence of ITS was recorded throughout the Mediterranean Sea, which suggests either (1) a loss of ancestral polymorphism levels during a population bottleneck that would be very specific to ITS as it strikingly contrasts with high diversity and population structure previously reported with microsatellite markers or (2) the retention of ancestral polymorphism as already reported for other organisms (Presa et al. 2002, Bower et al. 2008).

The second well-supported clade is composed of the Australian species of *Posidonia*. Speciation within the Australian *Posidonia* has been suggested to have occurred during the Eocene (55.8 to 33.9 Myr BP), as a consequence of the separation of Australia from Antarctica (Kuo et al. 1990, Waycott et al. 1997). Such a hypothesis is not supported by the relaxed molecular clock analysis proposed here (Fig. 2), suggesting a much more recent estimation of speciation (no more than 23.7 Myr BP). The results obtained show remarkable differences between the ITS sequence of both geographic groups of species — Mediterranean and Australian — compared with the small differences found among the Australian species. These differences support the hypothesis of a much more recent diversification of this genus in Australia that agrees with the divergence time estimate that places it around 12 Myr ago (Fig. 2).

The morphological taxonomy of the Australian clade is only partially supported by the ITS and *trn*L marker (results not shown for the latter). The phylogenetic tree obtained differentiates with good support between the 2 complexes, *Posidonia australis* and *P. ostenfeldii*, which diverged from each other early in the history of Australian *Posidonia* taxa. This divergence is possibly correlated with the broad habitat differences experienced by both groups of species along the Australian coasts, where the *P. australis* complex is usually found associated with sheltered waters, and the *P. ostenfeldii* complex is found in open ocean environments and coastal sublittoral habitats that have high energy (Den Hartog & Kuo 2006, Carruthers et al. 2007).

More recently in the phylogenetic tree, the analysis also supports the split of the *Posidonia australis* complex into 2 clades separating *P. sinuosa* from the remaining 2 taxa, *P. australis* and *P. angustifolia*, which are more closely related. However, the distinct species within the controversial *P. ostenfeldii* complex are not statistically supported (0% divergence). We are therefore left with 2 hypotheses about these taxa: (1) either those entities are synonymous and the morphological differences might express phenotypic plasticity in response to different environmental pressures along their distinct distributional ranges, or (2) the differentiation process started recently and may be still ongoing, in which case the lineage sorting has not yet been achieved, thereby preventing us from discriminating between those protospecies with the multiple genes sequenced here. Strong phenotypic plasticity has been demonstrated by confirming, by means of ITS sequences and microsatellites, the genetic identity of distinct morphotypes of the seagrass *Zostera marina*, which previously has been considered in some studies to be a distinct species (Becheler et al. 2010). We suggest that difficult morphological identification and unresolved ITS polymorphism support the hypothesis that the *P. ostenfeldii* complex would be composed of different morphotypes or ecotypes of the same species.

Slow evolution of mitochondrial markers in *Posidonia*

Most phylogenetic studies on plants, clonal plants in particular, report only positive results, i.e. those obtained with markers with enough discriminatory power to allow phylogenetic reconstruction. This results in a paucity of information on negative results using fewer polymorphic markers thus generating a deficit of discussion on the discrepancies in the evolution of distinct genes.

Relative to the nuclear and chloroplast genomes, mitochondrial variation was strikingly low given how long ago the divergence occurred as revealed by the nuclear genome. A complete lack of mitochondrial variation across 745 bp of a noncoding sequence and extremely low variation across 2073 bp of a coding region, even when comparing the Mediterranean *Posidonia oceanica* and the remaining Australian species, which diverged tens of millions of years ago, is perplexing.

The 3 different genomes of plants (nuclear, mitochondrial and chloroplastidial) evolve at remarkably different rates in most plant species (Wolfe et al. 1987, Palmer & Herbon 1988). When looking at synonymous substitution rates, which should not be subject to selective forces, chloroplastidial DNA (cpDNA) evolves at only half the rate of nuclear DNA (nDNA), but about 3 times faster than mtDNA (results not shown). These relative rates are maintained across the 3 genomes in most plants studied (Muse 2000). Accordingly, mtDNA markers have typically been considered not variable enough to resolve small-scale phylogenetic patterns in plants.

Despite the well-documented slow evolution of mtDNA, an almost total absence of polymorphism between species that had separated during the closure of the Tethys Sea is puzzling, and even more so given the few mitochondrial mutations (*nad*7 marker) found between the distinct genera *Posidonia* and *Cymodocea* (GenBank accession no. GQ927730) showing divergence of only 4.1% (results not shown). The mitochondrial region is remarkably less divergent than the 2 other genomes, even when comparing the ratios for other plant species already known to have slow evolving mitochondrial genomes (Wolfe et al. 1987).

Plant mtDNA is much larger than animal mtDNA (15 to 18 kb); even the smallest plant mitochondrial genome has more than 200 kb, with maximum sizes up to 2400 kbp (Palmer & Herbon 1988). In contrast with animal mitochondrial genomes, most of the plant mitochondrial genome appears to be noncoding (Lynch et al. 2006) and therefore may be expected to evolve faster than in animals, yet this has only been observed in rare exceptions (Cho et al. 2004, Parkinson et al. 2005). Several explanations for the apparent slow rate of evolution of plant mitochondria include (1) an efficient system of DNA repair and (2) a consequence of the long generation time of plants (Palmer & Herbon 1988, Hellberg 2006, Nabholz et al. 2008), which is only expected to apply if generation time is inversely correlated with germ-line cell divisions as in most animals (Whittle & Johnston 2003) but not necessarily in plants. Any demographic effects on mitochondrial mutation rate are expected to affect chloroplast mutation rates in the same way; therefore, the extreme difference between mitochondrial mutation and chloroplast rates in *Posidonia* suggest the occurrence of a mechanism specific to the mitochondrial genome, such as an efficient DNA repair system. A variety of different eukaryotes have developed highly efficient repair systems (France et al. 1996, van Oppen et al. 1999, Chen et al. 2000, Shearer et al. 2002, Duran et al. 2004, Tseng et al. 2005, Worheide 2006), whereas rare cases of plants with a high rate of nucleotide substitution in mitochondria (Cho et al. 2004, Parkinson et al. 2005) have been interpreted as the result of the loss of repair systems, e.g. key changes in the production and/or detoxification of oxygen free-radicals (Cho et al. 2004).

Considering that the divergence occurred 40 Myr BP as proposed by Phillips & Menez (1988), which lies in the lower interval estimates obtained in our analysis, the estimated ITS mutation rates (0.002 substitutions site^{-1} Myr^{-1}) fall into the values reported for ITS in woody plants (Kay et al. 2006) but are much lower than the other herbaceous species reported in this review. Higher values were also estimated for other marine macrophytes (e.g. *Fucus vesiculosus*) with a Northern/Southern Hemisphere disjunct distribution, where ITS divergence between taxa from both hemispheres was of 37 to 45% (compared with the 20.8 to 21.3% obtained here for *Posidonia*) for a later separation time estimated in the Oligocene/Miocene, ca. 38 to 7 Myr BP (Serrao et al. 1999). This indicates that either (1) besides specific mechanisms for mtDNA, some general factors cause slow DNA evolutionary rates in this genus and possibly in more seagrass species or (2) the separation between the Northern and Southern Hemisphere species may have occurred later in history as suggested by Les et al. (2003). This latter hypothesis would agree with the paleo-oceanographic history and the closure of Tethys Sea during the Miocene (20 to 10 Myr BP) that left those 2 zones isolated (Searle et al. 1987, Robertson & Comas 1998). Yet, *P. oceanica*, being a temperate taxon, may have been less affected by the closure of the Tethys Sea than were tropical species. Our results indicate that the separation of these 2 major groups was much more ancient, which therefore supports a slow evolutionary rate in the *Posidonia* genera.

The clonality and the extreme generation time of seagrass species (e.g. Reusch et al. 1999, Alberto et al. 2001) may contribute to the slow nuclear genome evolution and the lack of mitochondrial variation reported here, although other mechanism such as DNA repair may also play a role in mtDNA evolution. Seagrasses originated in the Cretaceous ~100 Myr BP (Larkum & Den Hartog 1989, Klap et al. 2000) and comprise only a few dozen species throughout the world, demonstrating exceedingly low speciation rates. The most common explanations for such low number of taxa are (1) the absence of animal pollinators, which has removed coevolutionary relationships with pollinators as drivers of speciation, as typically observed in terrestrial systems (van der Hage 1996, Ollerton & McCollin 1998, Raven 1998), and (2) the lack of barriers to gene flow in the marine environment (Hemminga & Duarte 2000, Darling et al. 2004). This last hypothesis has been contradicted by evidence of common genetic differentiation among seagrass meadows even across short distances (Coyer et al. 2004, Olsen et al. 2004, Arnaud-Haond et al. 2007, Alberto et al. 2008, Becheler et al. 2010). Although the absence of coevolution with pollinators cannot be discarded as an influencing factor, the results derived here also suggest that slow evolutionary rates may account for the poor speciation in seagrasses. Longer generation time in plants is associated with slower evolutionary rates (Charlesworth & Wright 2001, Nabholz et al. 2008) and clonal growth may extend the generation time by seriously lowering the frequency of meiotic events.

In summary, the results presented here, using a range of markers, confirm the differentiation between Australian and Mediterranean *Posidonia* as well as the existence of 2 clades of *Posidonia* species in the Australian flora. However, only 4 lineages can be discriminated out of the 7 morphotypes sampled in Australia, suggesting either a high phenotypic plasticity as reported for other seagrass species or an extremely recent diversification. The DNA evolutionary rates found also appear to be among the slowest reported for herbaceous plants, in particular for mtDNA. Those differences in evolutionary rates could possibly be due to the clonal nature, slow growth and extended generation times in the genus *Posidonia*, which agrees with reported slow speciation rates among seagrass species and a possible specific mtDNA repair mechanism similar to the one described in some clonal cnidarians (Tseng et al. 2005).

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