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Research Article

Phylogeny and temporal divergence of the seagrass family Zosteraceae using one nuclear and three chloroplast loci

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Seagrasses are among the most productive habitats in the marine realm, performing several crucial physical and biological ecosystem services. One group of seagrasses is the family Zosteraceae, which includes three to four genera and >20 species inhabiting temperate waters of both the northern and southern hemisphere. Species delineation depends on the type of data used, ranging from morphological to molecular. The main goal of this study was to better understand the evolution and divergence within the family, using a broad taxon sampling (>90 individuals) representing all species across the entire biogeographical range in both hemispheres and a four-locus approach (ITS1, *mat*K, *rbc*L, *psb*A-*trn*H). The concatenated four-locus analysis supported earlier studies showing four genera in the family: *Phyllospadix*, *Zostera*, *Nanozostera* and *Heterozostera*. Four species were resolved within the genus *Zostera*, four within *Nanozostera* and two within *Heterozostera*. No distinction was revealed between *H. nigracaulis* (Australia) and *H. chiliensis* (Chile), suggesting a very recent introduction to Chile. A time-calibrated phylogeny using the *rbc*L gene revealed an early divergence of *Zostera–Nanozostera*/*Heterozostera* at 14.4 Ma, followed by a late Miocene radiation of *Nanozostera–Heterozostera* at 6.4 Ma, and the *H. polychalymas–H. nigracaulis*/*tasmanica*/*chiliensis* split at 2.3 Ma. *Zostera asiatica* diverged from other species of *Zostera* at 4.6 Ma. Phylogenetic analyses indicated that *mat*K was the most informative single locus, whereas *psb*A-*trn*H (a widely used barcoding locus) was unable to resolve any entities within the Zosteraceae. A commonly used barcoding combination for plants, *rbc*L/*mat*K, distinguished all genera, but was unable to resolve several species.

Key words: barcoding, chloroplast DNA, *Heterozostera*, ITS1, molecular clock, molecular phylogenetics, seagrass, *Zostera*, Zosteraceae

Introduction

Although the highly productive seagrass ecosystem performs numerous and valuable ecosystem services, it also is one of the most vulnerable to anthropological activities (Hemminga & Duarte, 2000; Duarte, 2002; Les *et al.*, 2002; Orth *et al.*, 2006; Ralph *et al.*, 2006; McGlathery *et al.*, 2007). Nearly 65% of the world's seagrass ecosystems have been impacted (fragmentation and/or elimination) by human activities and up to 30% have been lost since the 1980s (Duarte, 2002; Orth *et al.*, 2006). Restoration efforts have been conducted worldwide; however, success rates rarely exceed 30% (Orth *et al.*, 2006; Van Katwijk *et al.*, 2009; but see Orth *et al.*, 2012).

Despite the environmental challenges to seagrasses, many species and populations display impressive signs of resilience and persistence. Indeed, disturbance may be a critical feature of 'health' in some species/populations, in that intermediate levels of disturbance promote higher diversity (Hemminga & Duarte, 2000; Nacken & Reise, 2000; Duarte *et al.*, 2006; Reusch, 2006). With greater understanding of the role of seed banks (Zipperle *et al.*, 2009*a*, 2009*b*), rhizome dispersal/reattachment (Coyer *et al.*, 2008), the relationship between genotypic diversity and environmental changes (Hughes & Stachowicz, 2004; Reusch *et al.*, 2005; Ehlers *et al.*, 2008), and the capacity for hybridization (Coyer *et al.*, 2008), it becomes more and

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more important to clarify seagrass phylogenetic history and taxonomy.

Seagrasses are an ecological rather than a taxonomic group, with three to four angiosperm lineages each making a separate transition from terrestrial to marine habitats 77 to 17 Ma (Les *et al.*, 1997; Kuo & Den Hartog, 2000; Kato *et al.*, 2003; Janssen & Bremer, 2004). One of these lineages, the order Alismatales, comprises *c*. 60 species (13 genera and five families) (Les *et al.*, 1997; Den Hartog & Kuo, 2006; Waycott *et al.*, 2006) that have successfully colonized nearly all soft-sediment coastlines worldwide from intertidal to subtidal depths <60 m (Den Hartog, 1970; Green & Short, 2003).

Within the Alismatales, members of the monophyletic family Zosteraceae are found in temperate regions of both the northern and southern hemispheres with some species extending into tropical latitudes, but no single species occurring in both hemispheres (Den Hartog, 1970; Kato *et al.*, 2003). All genera inhabit marine and brackish soft sediments except for *Phyllospadix*, which is present on rocky intertidal substrates along open coasts in the North Pacific. Previous phylogenetic analyses and molecular clock estimates based on the chloroplast *rbc*L and *mat*K loci suggested that the family Zosteraceae emerged about 100 Ma and began to diversify into the extant clades (genera *Zostera* and *Phyllospadix*; subgenus *Zosterella*) between 33–44 Ma (Kato *et al.*, 2003).

The taxonomic history of Zosteraceae is cluttered with competing circumscriptions and little resolution. Based on comparative morphology, the genus *Zostera* first included the two sections *Alega* and *Zosterella* (Ascherson, 1868). *Heterozostera* was subsequently added as a third section (Setchell, 1933) and later, all three sections were considered to be subgenera of the genus *Zostera* (Setchell, 1935), which together with *Phyllospadix*, defined the family.

Den Hartog (1970) upgraded the subgenus *Heterozostera* to the genus level, also using morphological data. A recent study combining morphological and developmental data supported four genera (*Phyllospadix*, *Zostera*, *Nanozostera* and *Heterozostera*) with no subgenera (Tomlinson & Posluszny, 2001), whereas other studies settled on three genera (*Phyllospadix*, *Heterozostera* and *Zostera*) (Kuo & Den Hartog, 2001; Den Hartog & Kuo, 2006).

In the first molecular studies of Zosteraceae, sequences of nuclear (ITS), and chloroplast (*trn*K, *rbc*L) DNA supported two genera, *Phyllospadix* and *Zostera*, with three subgenera within *Zostera* (*Zostera*, *Zosterella* and *Heterozostera*) (Les *et al.*, 2002). Further studies included the chloroplast marker *mat*K, which led Tanaka *et al*. (2003) to propose three scenarios for genus-level resolution: (1) two genera: *Phyllospadix* and *Zostera,* the later comprising *Zostera* and *Zosterella* as subgenera including *Heterozostera*; (2) three genera: *Phyllospadix*, *Zostera* and *Nanozostera* (*Heterozostera*), the latter including the subgenus *Zosterella*; and (3) four genera: *Phyllospadix*, *Zostera*, *Nanozostera* and *Heterozostera*. At the same time, and using *rbc*L and *mat*K sequences, Kato *et al.* (2003) suggested the re-classification of Zosteraceae into three genera: *Phyllospadix*, *Zostera* (subgenus *Zostera*), and *Nanozostera* (subgenera *Zosterella* and *Heterozostera*). To complicate the matter further, the genus *Heterozostera* has nomenclatural priority over *Nanozostera* (Den Hartog & Kuo, 2006). In a recent review of the morphological and molecular data, Jacobs and Les (2009) concluded that the two genus classification for Zosteraceae proposed earlier by Setchell (1935) and Les *et al.* (1997), namely *Phyllospadix* and *Zostera*, was correct with the latter composed of subgenera *Zostera*, *Zosterella* and *Heterozostera*.

The genus *Zostera* occurs worldwide in predominantly temperate seas and is represented by 13–17 species of the subgenera *Zostera* and *Zosterella* following the classification of Den Hartog and Kuo (2006). Within the subgenus *Zostera* (found only in the North Pacific and North Atlantic), four species diverged between 3 and 6 Ma, probably in the Japanese Archipelago. In contrast, the northern and southern hemisphere's species comprising the subgenus *Zosterella* diverged *c*. 2 Ma (Kato *et al.*, 2003). The more recent radiation of the subgenus *Zosterella* most likely is one reason for the difficulties in resolving the four to seven member species. For example, geographical/morphological differences distinguish four species in the subgenus (*Zosterella mucronata*, *Z. muelleri*, *Z. novaezealandica*, *Z. capricorni*), whereas nuclear and chloroplast sequences recognize only *Z. capricorni* (Les *et al.*, 1997; Kato *et al.*, 2003; Tanaka *et al.*, 2003). Note, however, that *Z. capricorni* should be regarded as *Z. muelleri* according to nomenclatural priority (Den Hartog & Kuo, 2006).

As should be evident from previous studies, incomplete taxon sampling (including the biogeographical component) and choice of loci have greatly affected the results. In the present paper, we examined sequence divergence among all species of all genera and subgenera across the full biogeographical range using four loci (one nuclear and three chloroplast). We aimed to: (1) clarify phylogenetic relationships within the Zosteraceae, (2) estimate divergence times between genera and (3) evaluate the suitability of one or more genes for DNA barcoding and rapid identification of species.

Materials and methods

Collection and DNA extraction

Specimens were collected from worldwide locations (Table S1, see supplementary material, which is available on the Supplementary tab of the article's Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2013.821187) by the authors and numerous colleagues, but voucher specimens were not obtained in many cases, as only small samples of leaves were forwarded. Small pieces (2–3 cm)

Table 1. Primer annealing temperature and sequence for each locus.

Locus (bp)	°C	Primer	Primer sequence	Reference
ITS1 (240)	40	ITS5-F	5'-ggaagtaaaagtcgtaacaagg-3'	(White *et al.*, 1990)
		ITS2-R	5'-gctgcgttcttcatcgatgc-3'	(White *et al.*, 1990)
*mat*K (688)	50	*mat*K-F	5'-aacattccctttttggagga-3'	this study
		*mat*K-R	5'-cagaatccgataaatcagtcca-3'	this study
*rbc*L (619)	68	*rbc*L-F	5' atgtcaccacaaacagaaac-3'	this study
		*rbc*L-R	5' tcgcatgtacctgcagtagc-3'	this study
*psb*A-*trn*H (370)	60	*psb*A-*trn*H-F	5'-gttatgcatgaacgtaatgctc-3'	(Sang *et al.*, 1997)
		*psb*A-*trn*H-R	5'-cgcgcatggtggattcacaatcc-3'	(Sang *et al.*, 1997)

of freshly collected leaves were blotted dry and placed into silica crystals for dehydration and storage. DNA extraction followed Hoarau *et al.* (2007) as modified by Coyer *et al*. (2009), with the exception that samples were heated to 65°C for at least 1 h.

DNA sequencing

Polymerase chain reactions (PCRs) (10-μL volume) contained 1 μL of extracted DNA as a template, along with 1 × *Taq* polymerase buffer (HotMaster, 5Prime; GmbH, Hamburg, Germany), 0.2 μM dNTP, 1.5 μM of each primer (Table 1), and 0.015 U *Taq* polymerase (5Prime). PCR conditions (MyCycler thermocycler, Bio-Rad, Hercules, CA, USA) were: 94°C, 2 min; followed by 40 cycles of 94°C, 20 s; annealing temperature (Table S1, supplementary material), 20 s; and 65°C, 1 min; followed by a final extension of 65°C, 5 min.

For all sequences, 40 ng of the dried amplification product was purified with ExoSAP-It (USB, Cleveland, OH, USA), sequenced with forward and reverse primers using the dGTP BigDye Terminator kit and visualized with the ABI3730 Genetic Analyzer (Applied Biosystems). Forward and reverse sequences were aligned using Variant Reporter 1.0 (Applied Biosystems) and by eye in BioEdit 7.0.1 (Ibis Biosciences, Carlsbad, CA, USA). GenBank accession numbers are presented in Figures 1–5.

Data analysis

Aligned sequences with indels coded as 0 or 1 (Simmons & Ochoterena, 2000). Sequences of ITS1, *mat*K, *rbc*L and *psb*A-*trn*H were analysed (one sequence for each haplotype) using Bayesian inference with MrBayes 3 (Ronquist & Huelsenbeck, 2003). The optimal models of sequence evolution HKY+G (ITS1, *mat*K and *rbc*L) and HKY rates equal (*psb*A-*trn*H) were determined with MrModeltest 2.1 (Nylander, 2004). Along with single-locus phylogenies, a species phylogeny was inferred from a multigene (ITS1, *mat*K, *rbc*L, *psb*A-*trn*H) alignment consisting of a single individual of each species using a suitable partitioning strategy and the corresponding suitable models of sequence evolution using Bayesian information criterion (BIC). Two independent MCMC searches were run for each dataset using different random starting points (four million generations each). Convergence was examined visually by plotting likelihood vs. generation for the two runs. Burn-in was set to 20 000 trees. Phylogenetic trees were rooted with species from the two closest families Posidoniaceae (*Posidonia oceanica*) and Potamogetonaceae (*Ruppia maritima*).

All four loci were sequenced from a single individual of each species. We follow the convention of Genus (subgenus) specific epithet followed by the number of samples in parentheses: *Zostera* (*Zostera*) *marina* (26); *Z*. (*Zostera*) *asiatica* (2); Z. (*Zostera*) *caulescens* (2); *Z*. (*Zostera*) *caespitosa* (2); Z. (*Zostera*) *pacifica* (2); *Z*. (*Zosterella*) *angustifolia* (wide-leaf) (8); Z. (*Zosterella*) *noltii* (13), Z. (*Zosterella*) *japonica* (9); *Z.* (*Zosterella*) *capensis* (2); *Z*. (*Zosterella*) *muelleri* (4); *Z*. (*Zosterella*) *capricorni* (13); *Z*. (*Zosterella*) *mucronata* (2); Z. (*Zosterella*) *novazelandica* (3); *Heterozostera polychalmys* (3); *H*. *nigracaulis* (18); *H*. *tasmanica* (1); *H*. *chiliensis* (1); *Phyllospadix iwatensis* (2); *P. japonicus* (2); and *P. torreyi* (1). Outgroup genera (all four loci) included *R. maritima* (2) and *P. oceanica* (1). Sequences from GenBank submissions were not used to supplement our dataset, as our requirement of all genes being sequenced from one individual could not be guaranteed.

A cautious time-frame of diversification in the family Zosteraceae was estimated by inferring a chronogram based on the *rbc*L gene using Beast v1.7.3 (Drummond *et al.*, 2012). The *rbc*L alignment consisted of 12 haplotypes, 10 belonging to Zosteraceae species (*Phyllospadix*, *Heterozostera* and *Zostera*), and the outgroup species *P. oceanica* and *R. maritima.* The root of the tree was constrained in geological time based on the earliest fossil records assigned to seagrasses, which were discovered from the Cretaceous layer (Larkum & Den Hartog, 1989). The root was, therefore, constrained at 66–145 Ma (Walker *et al.*, 2012) with a uniform prior. We applied the synonymous nucleotide substitution rate of 0.9–1.2 × 10^9 per site per year for *rbc*L from the *Oryza sativa* chloroplast genome (Li, 1997), which did not differ from the rate estimate for *rbc*L for

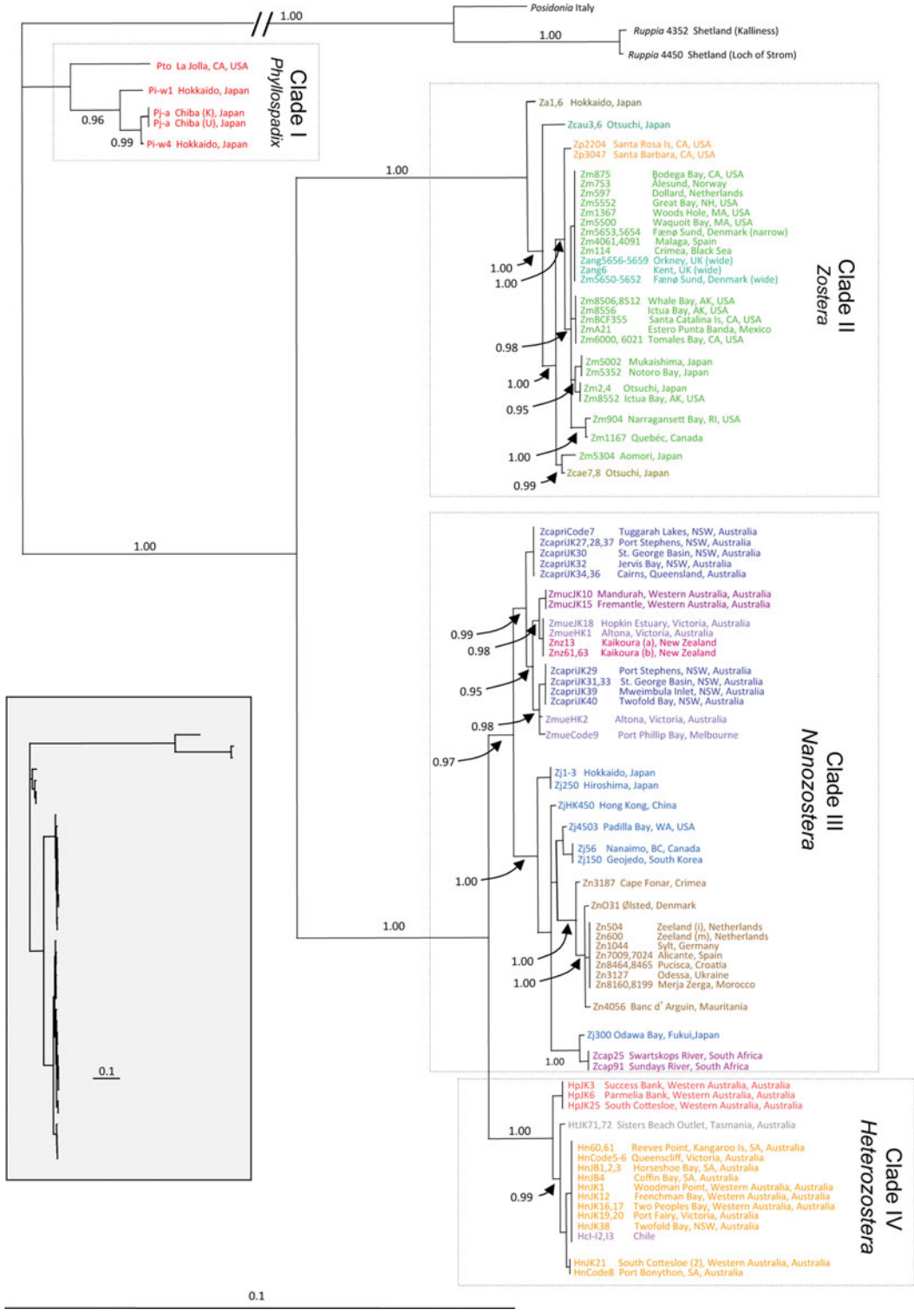

Fig. 1. Bayesian phylogenetic tree based on concatenated ITS1 (nuclear), and *mat*K, *rbc*L and *psb*A-*trn*H (chloroplast) sequences (1917 bp, 43 indels) reveals four clades. Clade genera after Tomlinson & Posluszny (2001) and Tanaka *et al.* (2003). Numbers represent Bayesian posterior probability values, where values ≥ 0.95 represent statistical significance ($P \leq 0.05$) and values <0.90 were not presented. See text for details and models of evolution. We use the three-genus classification (Den Hartog, 1970; Kuo & Den Hartog, 2001; Den Hartog & Kuo, 2006) in the figure for comparison with previous work. In the main figure, the outgroup branch was truncated to reveal clade detail; insert shows intact branch. Abbreviations: Pto, *Phyllospadix torreyi*; Pi, *P. iwatensis*, Pj, *P. japonicus*; Za, *Zostera asiatica*; Zcau, *Z. caulescens*; Zp, *Z. pacifica*; Zm, *Z. marina*; Zang, *Z. angustifolia*; Zcae, *Z. caespitosa*; Zcapri, *Zosterella capricorni*; Zmuc, *Z. mucronata*; Zmue, *Z. muelleri*; Znz, Z. *novaezealandicaa*; Zj, *Z. japonica*; Zn, *Z. noltii*; Zcap, *Z. capensis*; Hp, *Heterozostera polychalmys*; Ht, *H. tasmanica*; Hn, *H. nigracaulis*; Hc, *H. chiliensis*.

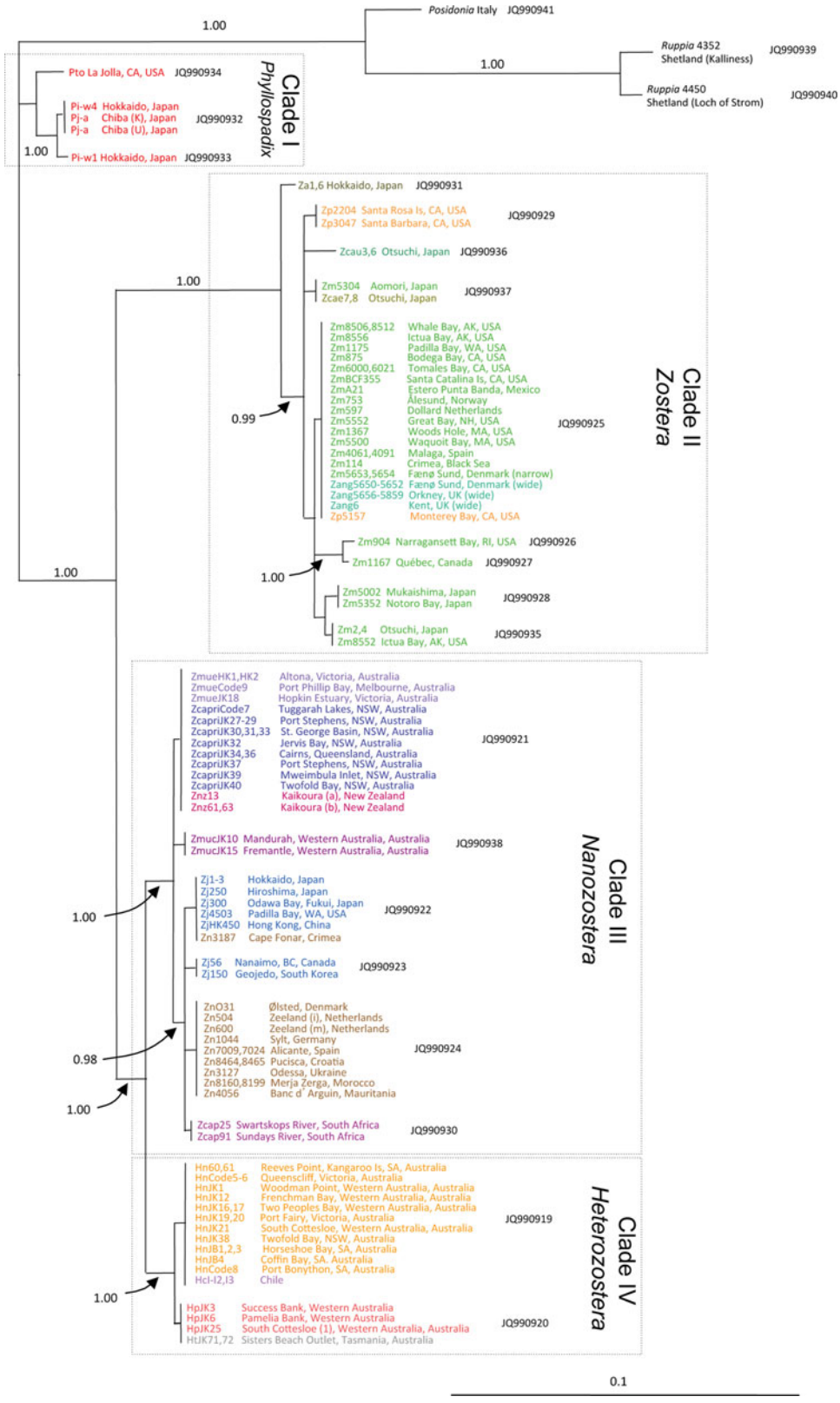

Fig. 2. Bayesian phylogenetic tree based on ITS1 (nuclear) sequences (240 bp, 21 indels). Numbers represent Bayesian posterior probability values; model of evolution = HKY + G; GenBank Accession numbers are listed on the right side of each sequence. See legend of Fig. 1 for key to abbreviations.

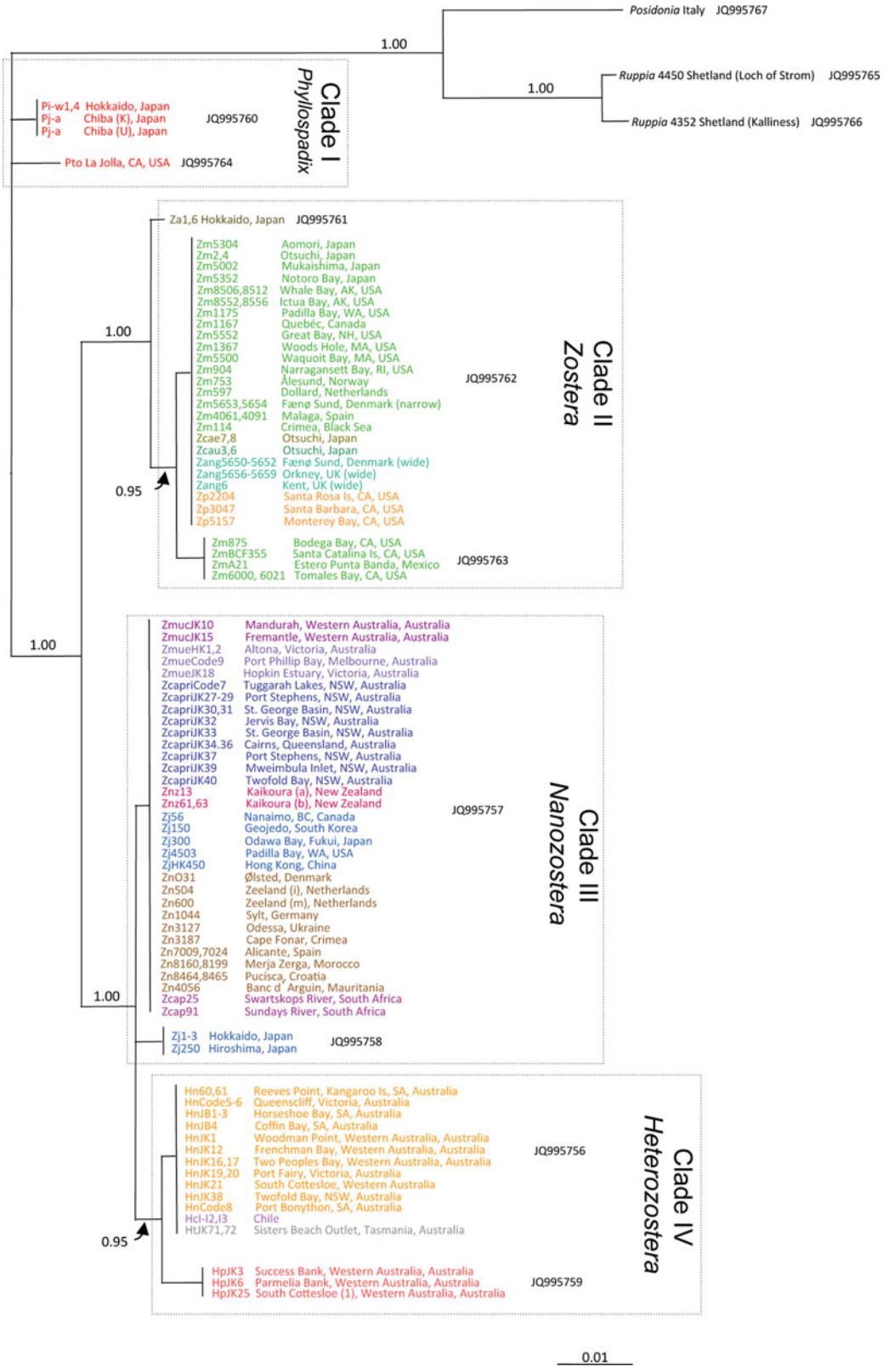

Fig. 3. Bayesian phylogenetic tree based on *mat*K (chloroplast) sequences (688 bp). Numbers represent Bayesian posterior probability values; model of evolution = HKY + G; GenBank Accession numbers are listed on the right side of each sequence. See legend of Fig. 1 for key to abbreviations.

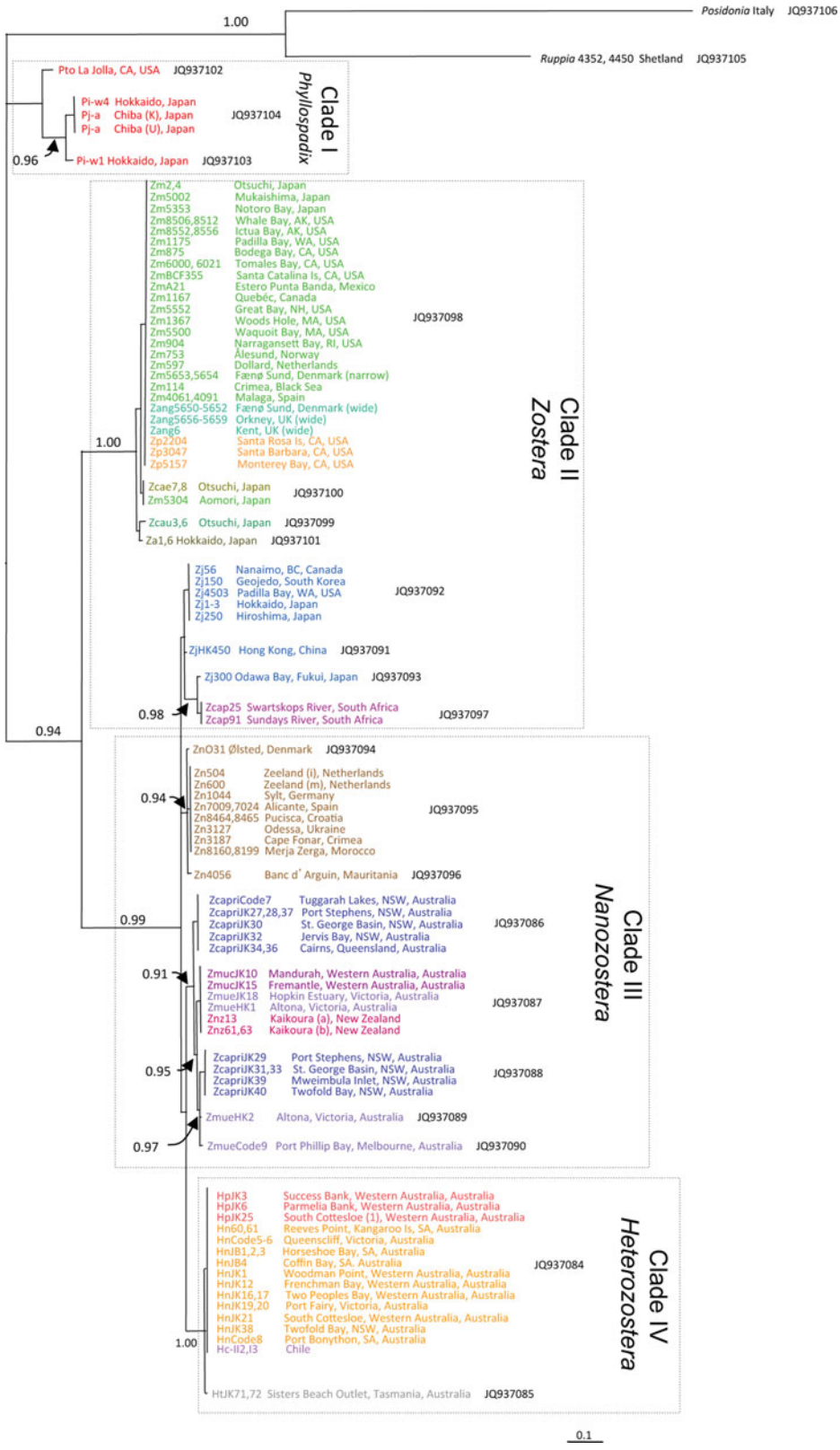

Fig. 4. Bayesian phylogenetic tree based on *rbc*L (chloroplast) sequences (619 bp). Numbers represent Bayesian posterior probability values; model of evolution = HKY + G; GenBank Accession numbers are listed on the right side of each sequence. See legend of Fig. 1 for key to abbreviations.

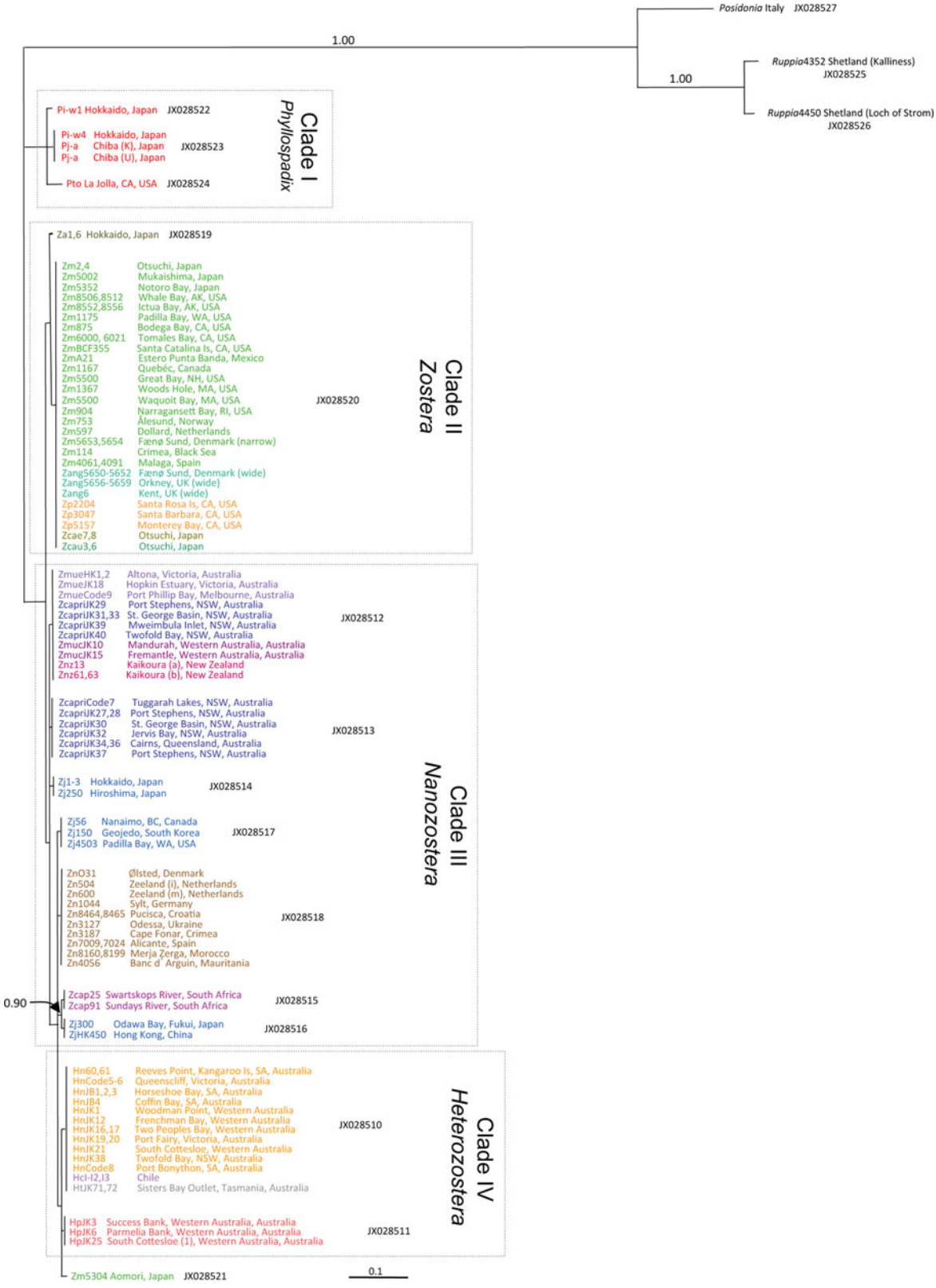

Fig. 5. Bayesian phylogenetic tree based on *psb*A-*trn*H (chloroplast) sequences (370 bp, 21 indels). Numbers represent Bayesian posterior probability values; model of evolution = HKYrates = equal; GenBank Accession numbers are listed on the right side of each sequence. See legend for Fig. 1 for key to abbreviations.

Zosteraceae used by Kato *et al*. (2003). Although Kato *et al.* (2003) indicated that the substitution rate of *mat*K and *rbc*L is equal in eelgrass; we adopted a conservative approach and used the substitution rate for *rbc*L on the *rbc*L gene, not *mat*K.

The analysis was performed under a HKY+G model with an uncorrelated lognormal (UCLN) relaxed molecular clock model, using a uniform tree prior. Four independent Markov Chain Monte Carlo (MCMC) analyses were run for 20 million generations, sampling every 10 000th generation. Convergence and stationarity of the chains were evaluated in Tracer v1.4 (Rambaut & Drummond, 2007). The majority rule consensus tree was generated with TreeAnnotator v1.7.3 (Drummond *et al*., 2012), based on 7204 trees sampled across a large part of the four runs.

Results

We determined sequences of the nuclear locus ITS1 (240 bp; 18 haplotypes), and the chloroplast loci *mat*K (688 bp; 17 haplotypes), *rbc*L (619 bp; 7 haplotypes), and *psb*A-*trn*H (370 bp; 12 haplotypes) for all genera in the Zosteraceae (Table S1, supplementary material). GenBank accession numbers are listed on the corresponding figures.

Four-locus analysis

The four concatenated loci analysis yielded better resolution than when each locus was analysed separately (Figures 1–5). Four major clades were recognized, three with high support: I, *Phyllospadix* (posterior probability, p.p. = 0.65); II: *Zostera* (*Zostera*) (p.p. = 1.00); III: *Zostera* (*Zosterella*) (p.p. = 0.97) and IV: *Heterozostera* (p.p. = 1.00) (Fig. 1). Within *Zostera*, four species were resolved from the most basal *Z. asiatica* (p.p. = 1.00) to the most derived (*Z. caulescens*, p.p. = 0.99; *Z. marina*, 0.98; *Z. pacifica*, 1.00). Two individuals of *Z. caespitosa* were indistinguishable from a *Z. marina* and seven individuals of 'wide-leaf' Z. *angustifolia* were indistinguishable from *Z. marina* throughout Europe and NE USA (Fig. 1).

Within Clade III, our analysis resolved *Z. noltii* (p.p. = 1.00) and *Z. capensis* (1.00) (Fig. 1). The remaining taxa (*Z. capricorni*, *Z. mucronata*, *Z. muelleri* and *Z. novazealandica*), which occur exclusively in Australia and New Zealand, formed an extensive paraphyletic group with no resolution. *Zostera japonica* is the sister taxon to *Z. noltii*, but remains paraphyletic with respect to both *Z. noltii* and *Z. capensis*. Clade IV comprised the monophyletic genus *Heterozostera* and two species were highly resolved (0.99): *H. polychalmys* and *H. nigracaulis/chiliensis.* The status of *H. tasmanica*, however, was unclear.

Single-locus analyses

The single-locus analyses revealed progressively less resolution. The *mat*K analysis was the most informative single locus, distinguishing the subgenera *Zostera*, *Zosterella* and genus *Heterozostera* (all p.p. = 1.00), as well as *Z. asiatica* (0.99) and a cluster comprising the *Zosterella* species *Z. noltii*, *Z. japonica* and *Z. capensis* (0.98) (Fig. 2). Results of the *rbc*L analysis were similar to *mat*K except that the genus *Heterozostera* was marginally resolved (Fig. 3). ITS analysis resolved only the three subgenera (p.p. = 0.99–1.00) (Fig. 4), whereas *psb*A-*trn*H resolved only the two outgroups (1.00) (Fig. 5).

Time-calibrated phylogeny

A chronogram analysis based on a 619 bp region of the *rbc*L gene revealed a sequential progression of diversification in the Zosteraceae ranging from a fairly resolved split between the genera *Zostera* and *Phyllospadix* (p.p. = 0.90 at 23.3 Ma (95% Highest Probability Density (HPD): 60–77) to the most recent and highly resolved (p.p. = 0.95) divergence of *Z. marina/caulescens/caespitosa/pacifica/angustifolia* from worldwide locations and *Z. marina* from California to Baja California on the west coast of USA/Mexico at 1.6 Ma (95% HPD: 0–9) (Fig. 6). The highly resolved (p.p. = 0.96) divergence of the subgenera *Zostera*, *Zosterella* and genus *Heterozostera* (collectively in both the northern and southern hemispheres) occurred at 14.4 Ma (95% HPD: 3–46). Within *Zostera*, *Z. asiatica* diverged (p.p. = 1.00) at 4.6 Ma (95% HPD: 0–21). North Pacific and North Atlantic *Z. marina/caulescens/caespitosa/angustifolia* diverged (p.p. = 0.96) 1.6 Ma (HPD: 0–9) from *Z. marina* along the west coast of California and Baja California. The highly resolved (p.p. = 1.00) divergence of *Zosterella* and *Heterozostera* occurred at 6.4 Ma (95% HPD; 1–25) and *H. polychalmys*/*tasmanica* /*chiliensis* diverged (p.p. = 0.96) from *H. nigracaulis* at 2.3 Ma (95% HPD: 0–10).

Discussion

Current taxonomic status of the Zosteraceae has centred on different interpretations at the genus/subgenus level and within and between *Z.* (*Zosterella*) and *Heterozostera* in Australia and New Zealand. Our four-locus analysis (Fig. 1) supports elimination of the subgeneric designations within the Zosteraceae, as molecular divergence among the clades is highly significant and each forms a monophyletic group. Consequently, we recognize the genera *Phyllospadix* (Clade I), *Zostera* (Clade II), *Nanozostera* (Clade III) and *Heterozostera* (Clade IV), as proposed earlier by Tomlinson & Posluszny (2001) based on morphological/developmental characteristics and Tanaka *et al*. (2003) based on *mat*K sequences ('Scenario 3'). The four-genus classification differs from the morphological interpretation (*Phyllospadix*,

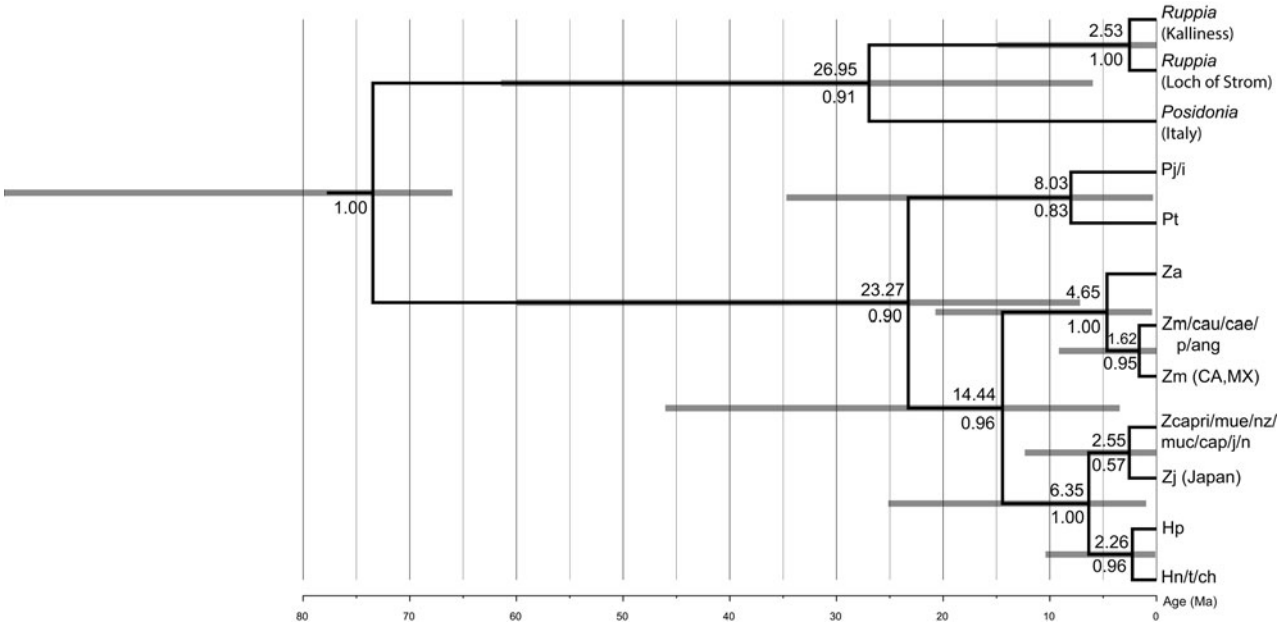

Fig. 6. Time-calibrated phylogeny. The 95% Highest Density Probability (HPD) intervals are provided for each node; upper value = node divergence time (Ma), lower value = posterior probability values. See legend of Fig. 1 for key to abbreviations.

Zostera, *Heterozostera*) of Den Hartog (1970) and Den Hartog & Kuo (2006), and the review of morphological/molecular data (*Phyllospadix*, *Zostera*) by Jacobs & Les (2009).

The four-locus analysis, as well as the *mat*K and *rbc*L analyses, strongly resolved some species within *Zostera.* For example, the analysis reinforced the designation of *Z. asiatica* (Japan, Korea, China, Russia), *Z. caulescens* (Japan, Korea, Russia), *Z. marina* ('narrow leaf', found throughout the northern hemisphere) and *Z. pacifica* ('wide leaf', found only off California) (Green & Short, 2003). The latter distinction also was supported by microsatellite data showing these species to be separate entities where they co-occur off southern California (USA) (Coyer *et al.*, 2008; Coyer, unpubl. data). However, leaf width did not correlate with molecular data in delineating the 'wide leafed' *Z. angustifolia* and 'narrow leafed' *Z. marina* where these forms co-occur in waters of the UK and Denmark (this study) and Norway (Olsen *et al.*, 2013).

Zostera caespitosa (China, Korea, Japan, Russia) was indistinguishable with both nuclear and *mat*K markers from *Z. marina* from Aomori. The mismatch most likely reflects a misidentification of the single Japanese individual.

The other area of taxonomic uncertainty in the Zosteraceae lies among the *Zostera* (*Zosterella*) taxa within Clade III (hereafter referred to as *Nanozostera*) from Australia/New Zealand, all of which reside in one partially resolved clade. Whereas previous studies using nuclear and/or chloroplast markers support only one species (*N. capricorni*) (Les *et al.*, 2002; Kato *et al.*, 2003; Tanaka *et al.*, 2003), all *Nanozostera* species are distinguished by morphological differences in leaf-tip and nervation (Den Hartog & Kuo, 2006). Our four-locus analysis revealed that *N. muelleri*, *N. capricorni* and *N. novazealandica* individuals (all from eastern Australia/New Zealand) were dispersed among three subclades, two of which were highly resolved. The inability of molecular and morphological data to converge upon a stable classification for the *N. capricorni* complex in Australia/New Zealand can be explained by several mechanisms, such as different ecotypes of a single species, nascent speciation and incomplete lineage sorting, or ongoing gene flow through hybridization/introgression. Certainly, the possibility of hybridization/introgression among Australian entities in the *N. capricorni* complex must be considered (should be identified as the *N. muelleri* complex; see Den Hartog & Kuo, 2006), and is likely to occur given documented hybridization between closely related species of *Zostera* in California (Coyer *et al.*, 2008). Indeed, the probability of molecular, geographical and morphological approaches to concordantly distinguish species is likely to be positively correlated with how long the species have been isolated by gene flow (see Wiens & Penkwot, 2002).

On the other hand, all *N. mucronata* individuals clustered in one partially resolved clade. This molecular signature, in combination with geographical restriction to Western and Southern Australia (Den Hartog & Kuo, 2006) and the unique mode of dispersal via vegetative propagules (shoots) that detach from the generative shoot (Cambridge *et al.*, 1982), is consistent with nascent speciation for *N. mucronata*.

The four-locus and *mat*K analyses also revealed that two other species of *Nanozostera*, the disjunctly distributed *N. japonica* (restricted to the North Pacific) and *N. noltii* (restricted to the NE Atlantic) (Green & Short, 2003) and each with 12 chromosomes, were highly resolved from the southern hemisphere *Nanozostera* spp., all of which have 24 chromosomes (Kuo, 2001). An unexplained exception, however, was the specimen of *N. japonica* from Odawa Bay, Japan, which clustered with *N. capensis* from South Africa. The molecular analysis and difference in chromosome count suggests that a new genus could be erected for these two clades. Although *N. japonica* and *N. noltii* are distinguished by three of 31 diagnostic morphological characters (Les *et al.*, 2002), a previous analysis of *mat*K sequences was unable to resolve the two entities (Tanaka *et al.*, 2003), possibly because of limited taxon sampling and/or limited geographical range of the examined samples. The well-defined *N. noltii* clade within the more diverse *N. japonica* clade in the four-locus analysis further suggests recent divergence of *N. noltii* into the North Atlantic from a North Pacific *N. japonica* or a common ancestor, a trans-Arctic pathway and divergence pattern already revealed for several species of marine fish, invertebrates and algae (Coyer *et al.*, 2011, and references therein).

Although *N. japonica* is a recent invader to the west coast of North America (first observed in 1957 in Puget Sound, WA) presumably from oyster spat imported to Puget Sound from the Akkeshi Bay region of Hokkaido, Japan beginning in the early 1900s (Harrison & Bigley, 1982), our data show a closer affinity of Nanaimo (BC, Canada) and Padilla Bay (WA, USA) individuals to a population from Geojedo, South Korea. Certainly, additional and population-level samplings, combined with microsatellite or SNP analysis, are necessary to determine the introductory origin of North American populations of *N. japonica.*

Because *Heterozostera* has the most basal morphological features within the zosteroid species, it has traditionally been accorded genus status (Den Hartog, 1970; Larkum & Den Hartog, 1989). Recent analysis based on both morphological and molecular data confirm its sister relationship to *Nanozostera* (Soros-Pottruff & Posluszny, 1995; Les *et al.*, 1997; Tomlinson & Posluszny, 2001; Kato *et al.*, 2003; Tanaka *et al.*, 2003), as does our four-locus analysis (also ITS1 and *mat*K). Furthermore, at least two of four described *Heterozostera* species (Kuo, 2005) were resolved, *H. nigracaulis* and *H. polychalmys*, which are distinguished by presence/absence of a wiry black erect stem. Status of a third species, *H. tasmanica*, was unclear: with plastid markers (maternally inherited), it grouped significantly with *H. nigracaulis* using *rbc*L and *H. polychalmys* with *mat*K; whereas the nuclear ITS1 significantly resolved *H. tasmanica* from the other two species. The pattern may indicate hybridization with the *H. nigracaulis* and *H. polychalmys* species. The identical sequences of *H. chiliensis* and *H. nigracaulis* suggest recent anthropogenic or natural introduction of *H. nigracaulis* to Chile, a hypothesis further supported by finding *mat*K sequence identity in an additional 16 individuals from four localities off the Chilean coast spanning 300 km to *H. nigracaulis* from Australia (Coyer, unpubl. data).

Time-calibrated phylogeny

A Japanese Archipelago origin of the Zosteraceae has long been assumed based on the high species diversity and occurrence of several fossil specimens of *Archeozostera*, a genus assigned to Zosteraceae, in upper Cretaceous deposits (Larkum & Den Hartog, 1989; Nakaoka & Aioi, 2001; see references in Kato *et al.*, 2003). However, other authors suggested that *Archeozostera* is not the ancestor of Zosteraceae and that the family originated later in the 'Tertiary' (now Paleocene) (Kuo *et al.*, 1989). A previous study using rates of synonymous substitution for concatenated *mat*K/*rbc*L sequences estimated the origin of Zosteraceae at 100 Ma, divergence of *Zostera* and *Phyllospadix* at 36 Ma, *Zostera–Zosterella* at 33–44 Ma and *Zosterella–Heterozostera* at 5 Ma (Kato *et al.*, 2003).

Our estimates using the synonymous nucleotide substitution rate for *rbc*L *Oryza sativa* chloroplast genome, as used by Kato *et al.* (2003), and employing a larger dataset, suggested slightly younger to comparable divergence times: *Zostera–Phyllospadix* at 23 Ma, *Zostera-Nanozostera* at 14 Ma and *Nanozostera–Heterozostera* at 6 Ma. Divergence of *Zostera* (restricted to the northern hemisphere) and *Nanozostera* (both hemispheres)/*Heterozostera* (restricted to the southern hemisphere) at the Oligocene–Miocene junction 23 Ma corresponded to the Mi-1 glaciation (Miller *et al.*, 1991) and presumably a period eliminating dispersal of marine species across the equator.

Within the genus *Zostera*, *Z. asiatica* diverged first at 4.6 Ma in the Japanese Archipelago (Green & Short, 2003; Coyer *et al.*, 2008). Until 19 Ma, Japan was part of the continental coastline of Eurasia. At this time, the continental crust split and formed a basin that subsequently spread and formed a detached island arc. Full extension of the Japanese Archipelago was completed by 15 Ma at which point Japan became a separate geographical entity (Barnes, 2003). Thus, differentiation of *Z. asiatica* occurred after a period of island formation and habitat fracturing, conditions that can promote population isolation, genetic drift and eventual speciation. Further differentiation of *Zostera* was apparent in two clades diverging at 1.6 Ma, after the first opening of the Bering Sea at 5.5 to 5.4 Ma (Gladenkov *et al.*, 2002). One clade consisted of North Pacific (Japan, Alaska and Washington State) and North Atlantic individuals (eastern Canada, eastern USA and Europe) of *Z. marina/caulescens/caespitosa*, whereas the other consisted only of *Z. marina* individuals from California and Baja California. The highly resolved distinction between Alaska/Washington and California/

Baja California *Z. marina* individuals (separated by a minimum of 1400 km) suggests nascent speciation, although individuals/populations of *Zostera* between these locations must be evaluated.

Our estimate of the *Nanozostera* and *Heterozostera* divergence (6.3 Ma) essentially agreed with the 5 Ma estimated by Kato *et al.* (2003). No significant distinction was revealed among the seven component *Nanozostera* species from both the northern and southern hemisphere. The divergence of *H. nigracaulis* from *H. polychalymas/tasmanica/chiliensis* likely occurred as the first of the Pleistocene Ice Ages began.

DNA barcoding

Candidate loci for DNA barcoding should be applicable over a wide range of taxa, with high variation among species, but conservation within species (Kress *et al.*, 2005; Hollingsworth *et al.*, 2009; Von Cräutlein *et al.*, 2011; Lucas *et al.*, 2012). The most promising DNA barcoding loci for plants are chloroplast genes and the application should employ a multi-locus approach: one robust locus to distinguish genus and family level and a more variable locus for species-level verification (e.g. *rbc*L/*mat*K, *psb*A-*trn*H) (Kress *et al.*, 2005; Hollingsworth *et al.*, 2009). Despite the worldwide importance of seagrasses as foundation species, no seagrass sequence was entered into the Consortium for the Barcoding of Life database until a recent study used the *rbc*L/*mat*K combination with *rbc*L resolving seagrass taxa up to family and genus level and *matK* for species delimitation, including some ecotypes (Lucas *et al.*, 2012).

The present study has shown that *mat*K resolved some, but certainly not all species/species complexes of the genera *Zostera* (only *Z. asiatica* was distinguished) and two lineages within *Heterozostera* (*H. polychalmys/chiliensis* and *H. nigracaulis/tasmanica*). Within the genus *Nanozostera*, the *mat*K analysis distinguished the *N. noltii/japonica/capensis* complex from *N. mucronata*, *N. muelleri* and *N. novazealandica* (e.g. *N. capricorni*) in Australia/New Zealand (see Den Hartog & Kuo, 2006). Furthermore, while *rbc*L was able to resolve genera in the Zosteraceae (agreeing with its wide use to distinguish higher taxonomic levels), the *psb*A-*trn*H locus commonly used in plants to resolve species was unable to distinguish any genera within the Zosteraceae. Consequently, our study supplements that of Lucas *et al.* (2012), in that (with respect to a barcoding function) the *rbc*L/*mat*K combination can distinguish all genera/subgenera within the Zosteraceae, but is unable to conclusively resolve several species. Inclusion of the nuclear ITS1 locus provides little additional resolution. Clearly, the choice of barcoding loci depends upon the level of resolution required and is likely to be less useful for taxa experiencing rapid diversification, especially those lineages within the genus *Nanozostera* in Australia/New Zealand.

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