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Phylogenetic analyses of *Zostera* species based on *rbcL* and *matK* nucleotide sequences: Implications for the origin and diversification of seagrasses in Japanese waters

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Seagrasses are composed of four families belonging to angiosperms and they are thought to become adaptive to aquatic life independently. Zosteraceae is one such family and because of the relatively high species diversity around Japan and Korea coast areas, the family might have arisen therefrom. To elucidate the origin and evolution of Zosteraceae which consists of three genera, *Phyllospadix, Zostera*, and *Heterozostera,* 2.8 kb nucleotide sequences of *rbcL* and *matK* genes in the chloroplast genome were examined for various species, including cosmopolitan *Z. marina* and endemic *Z. caulescens*. The phylogenetic analysis reveals the following three features. First, based on the synonymous nucleotide substitution rate of the rice chloroplast genome, we estimated the divergence times between Zosteraceae and its closest relative, Potamogetonaceae, and between different genera, *Zostera* and *Phyllospadix,* as approximately 100 million years (myr) and 36 myr, respectively, suggesting that Zosteraceae emerged somewhere in the period from 36 myr ago to 100 myr ago. Second, two subgenera of *Zostera*, *Zostera* and *Zosterella*, exhibit their reciprocal monophyly and appear to have differentiated from each other approximately 33 myr ago. However, the third genus *Heterozostera* branched off only 5 myr ago from the stem lineage leading to *Zosterella* and this seems too recent in comparison with the ancient divergence of the two subgenera. Third, we estimated the most recent common ancestor of subgenus *Zostera* as 6 myr. In *Z. marina* four haplotypes were found in the sample and have diversified in the past 1.5 myr. One haplotype is shared by both sides of the Japan Archipelago and its closely related haplotypes occur also in eastern Pacific Ocean. Based on these phylogeographic analyses, we propose a provisional age related classification of Zosteraceae to argue the origin and evolution.

Key words: age-related classification, chloroplast DNA, molecular clock, molecular phylogeny, seagrass

INTRODUCTION

About 450 aquatic angiosperms represent 17% of the families and 1.5% of the genera of all flowering plant species (Cook 1990; Les and Philbrick 1993; Les and Schneider 1995). Among these relatively uncommon aquatic angiosperms, there are 60 marine species (seagrasses) which belong to four monocotyledon families in a single order Alismatales (Omori 2000). This taxonomic confinement to a single order and shared morphological or physiological characters necessary for survival in marine habitats circumstantially had suggested their monophyly (den Hartog 1970; Larkum and den Hartog 1989). Actually, however, morphological analyses (Dahlgren et al. 1985) and molecular phylogenetic studies (Les et al. 1993; Waycott and Les 1996; Les et al. 1997) demonstrated the polyphyletic origin of seagrasses.

Zosteraceae is a family of marine angiosperms and comprises three genera (*Phyllospadix*, *Zostera,* and *Heterozo-*

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stera). The family is distributed mainly in temperate regions of the northern and southern hemispheres. No Zosteraceae species occur in both hemispheres, although some subtropical or tropical seagrass families (Hydrocharitaceae and Cymodoceaceae) do occur in both. In genus *Zostera*, there are 11 species which are classified into two subgenera *Zostera* and *Zosterella* (Kuo and den Hartog 2001). Subgenus *Zostera* consists of four species and they are found only in the northern hemisphere. Two of these are *Z. caulescens* and *Z. caespitosa* which are endemic to northwestern Pacific Ocean (Japanese, Korean and southeastern Russian waters) and *Zostera asiatica* occurs in Pacific Ocean including North American Coast, while the other one is *Z. marina* which is cosmopolitan and occurs in both the Atlantic and Pacific (Nakaoka and Aioi 2001; Short et al. 2001). On the other hand, subgenus *Zosterella* consists of seven species. Five species in this subgenus are limited to Australia and New Zealand (Larkum and den Hartog 1989; Les et al. 2002). The other two are *Z. japonica* which occurs in northwestern Pacific Ocean and *Z. noltii* which occurs in northern Atlantic Ocean and the Mediterranean Sea. The high species diversity of Zosteraceae in the northern hemisphere is observed around the Japan Archipelago, suggesting that the family has evolved therefrom (Nakaoka and Aioi 2001).

Fossil records also support the Asian origin of Zosteraceae. According to Larkum and den Hartog (1989), the earliest fossils that are assigned to seagrasses were discovered from the Cretaceous layer. Some fossils are well-preserved and designated as genus *Archaeozostera* and *Thalassocharis*. It is thought that these genera belong to extant families of Zosteraceae and Cymodoceaceae, respectively. *Archaeozostera* specimens described from the upper Cretaceous layer were found in several locations in Japan (Koriba and Miki 1931,1958; Oishi 1931). Based on the morphological characters, Koriba and Miki (1931, 1958) regarded these specimens as a genus that is related to modern Zosteraceae, more precisely to genus *Phyllospadix*. However, Kuo et al. (1989) argued that *Archaeozostera* is unlikely to be related to Zosteraceae and instead suggested that Zosteraceae has arisen after the Tertiary (Aioi 2000). The origin of Zosteraceae remains thus controversial. Recently, Tanaka et al. (2003) analyzed *matK* sequences of all 11 species in *Zostera* in addition to *Heterozostera tasmanica* and *Phyllospadix iwatensis*. They found the monophyly of *H. tasmanica* and subgenus *Zosterella* and that Zosteraceae consists of three taxa of genus *Phyllospadix*, subgenus *Zosterella* and *Heterozostera*, and subgenus *Zostera*. Based upon these findings, they suggested some refinements in taxonomic classification of Zosteraceae. However, Tanaka et al. (2003) did not set the time frame for the origin and evolution of the family.

The purpose of this paper is to provide the tempo and mode of evolution in family Zosteraceae together with other related seagrasses. To this end, we have retrieved all *rbcL* and *matK* sequences in the chloroplast genome which are available for Alismatales. In addition, we have collected 30 seagrasses of five species from ten locations of Japan and Korea coast areas as well as from western coast areas of North America. For these samples, we determine about 2.8 kb sequences of *rbcL* and *matK* genes. Based on these nucleotide sequences, we carry out the molecular phylogenetic analysis with special reference to the geographic distribution of *Zostera* species.

Table 1. Sampling locations and sample sizes

	Species[a]				
Location[b]	Z. mar	Z. cau	Z. cae	Z. asi	Z. jap
A. Akkeshi, Hokkaido	–	–	–	2	–
B. Yamada or Otsuchi, Iwate[c]	2	3	2		–
C. Shizugawa, Miyagi	1	–	–	–	–
D. Sajima, Hayama or Minamishitaura, Kanagawa[d]	2	1	–	–	1
E. Maisaka, Shizuoka	3	–	–	–	1
F. Asamushi, Aomori	2	2	–	–	–
G. Uchiura, Ishikawa	1	1	–	–	–
H. Gabae, Korea	1	1	–	–	–
Seattle, U.S.A	2	–	–	–	–
Ensenada, Mexico	2	–	–	–	–
Total	16	8	2	2	2

[a] Abbreviations are; Z. mar: *Zostera marina*, Z. cau: *Z. caulescens*, Z. cae: *Z. caespitosa*, Z. asi: *Z. asiatica*, Z. jap: *Z. japonica*.
[b] An alphabet (A to H) represents a sampled location on the geographic map in Fig. 1.
[c] *Z. caulescens* samples are collected from Otsuchi and others are from Yamada.
[d] *Z. japonica* sample is collected from Minamishitaura and *Z. caulescens* are from Sajima.

MATERIALS AND METHODS

Collection of seagrasses. Regarding genus *Zostera*, twelve individuals of *Z. marina* were collected from seven locations around Japan and Korea and similarly eight of *Z. caulescens* from five locations (Table 1, Fig. 1). For *Z. marina*, four more were sampled from coast areas of Mexico and Seattle. In addition, two *Z. caespitosa*, two *Z. asiatica*, and two *Z. japonica* were sampled (Table 1). The habitats of these three species are restricted to coast areas of Japan and Korea (Nakaoka and Aioi 2001 for review). In general, two samples taken from a particular location were separated at least 10 m away from each other and regarded as different individuals (Inglis and Waycott 2001). There are two types of *Z. marina* in terms of their life style, annual or perennial. In the present analysis, however, we used mainly perennial *Z. marina*. Samples from Maisaka contained both annual and perennial individuals, but there were no nucleotide differences between them.

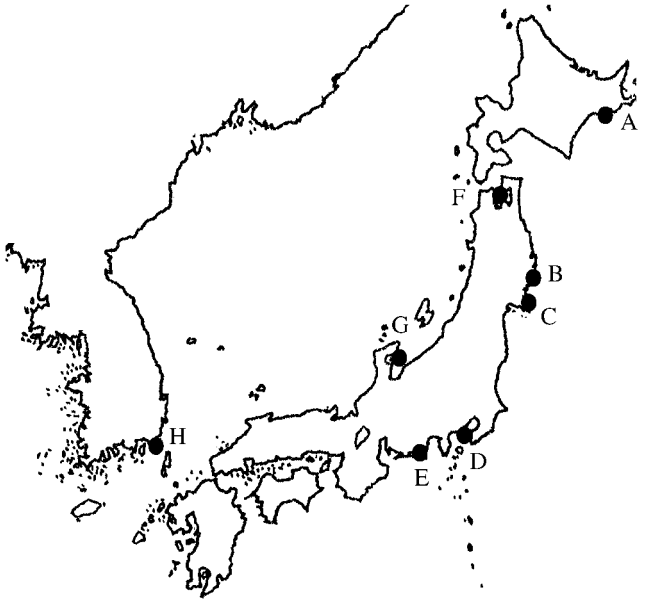

Fig. 1. Collection sites in Japan and Korea coasts. Akkeshi-bay (A), Otsuchi and Yamada-bay (B), Shizugawa-bay (C), Sagami-bay (D), Hamanako-lake (E), Mutsu-bay (F), Tukumo-bay (G), Gabae (H). These symbols are the same as those in Table 1.

DNA isolation, PCR and sequencing of chloroplast genes. Genomic DNA was extracted from 5 g of blotted wet weight of leaf materials by the Cetyltrimethylammonium bromide (CTAB) protocol (Murray and Thompson 1980; Ban 1997). The concentration of the CTAB extraction solution was 1.5% CTAB, 75 mM Tris-HCl (pH8.0), 15 mM EDTA (pH8.0), 1.05 M NaCl and that of the CTAB pellet solution was 1% CTAB, 50 mM Tris-HCl (pH8.0), 10 mM EDTA (pH8.0).

Genomic PCR was performed in 50 μl reactions containing 25 pmol of each PCR primer, 50 ng of genomic DNA, 200 μM dNTPs, and 0.6 units of ExTaq DNA polymerase (TaKaRa) in TaKaRa ExTaq buffer containing 2mM $MgCl_2$. Amplifications were carried out in a RoboCycler Gradient 96 (Stratagene) under the following standard conditions: denaturation 1 cycle of 94°C for 2 min, 50°C for 2 min, and 72°C for 3 min; 30 cycles of 94°C for 90 sec, 50°C for 2 min, and 72°C for 3 min; and an additional extension at 72°C for 15 min. The following primers were used (Tanaka et al. 1997): rbcL-Forward, ATGTCACCACAAACAGAGACTAAAGC; rbcL-Reverse, GCAGCAGCTAGTTCCGGGCTCCA; matK-Forward, TGGGTTGCCCGGGACTCGAA; and matK-Reverse, TAGAGTACTCGGCTTTTAAG. All PCR products were purified through QIAquick PCR Purification Kit (Qiagen), and were directly sequenced. Sequencing reactions were performed with ABI BigDye Terminator Kit and analyzed on an ABI 377 DNA sequencer (Applied Biosystems). To avoid sequencing errors, PCR products were sequenced two to four times in both directions. These sequences were deposited in DDBJ (DNA Data Bank of Japan). Accession numbers for these sequences are AB125348 to AB125361.

Phylogenetic analysis. In addition to 30 samples of five *Zostera* species mentioned above, 207 *rbcL* and 115 *matK* sequences (accession numbers are listed in Appendix 1 and 2 of order Alismatales were retrieved from DDBJ/Genbank/EMBL databases (as of April 2003). These sequences were aligned by CLUSTAL X (Thompson et al. 1997) and the alignment was further modified by eye. Since chloroplast DNAs have a relatively slow rate of nucleotide substitutions (Wolfe et al. 1987), there was no problem to obtain an unambiguous alignment. In the following analysis, sites or codons including any gaps were excluded. The maximum parsimony (Fitch and Farris 1974), minimum evolution (Rzhetsky and Nei 1993), and neighbor-joining (Saitou and Nei 1987) methods were used to construct the phylogeny. Programs for these tree-making methods are implemented in PHYLIP version 3.572 (Felsenstein 1995) and MEGA2 (Kumar et al. 2000). The synonymous and nonsynonymous substitutions are distinguished based on the modified Nei-Gojobori method (Nei and Kumar 2000) and multiple-hit substitutions are corrected by Jukes and Cantor (1969) method. Hereafter the nucleotide differences (p-distances) are used for studying closely related sequences and the nucleotide divergences (d-distances) after multiple-hit corrections are used for studying the time frame of the phylogeny.

Relative rate test. The rate heterogeneity of nucleotide substitutions within Zosteraceae was tested through the two-cluster method by Takezaki et al. (1995). In addition, the rate heterogeneity at the synonymous sites

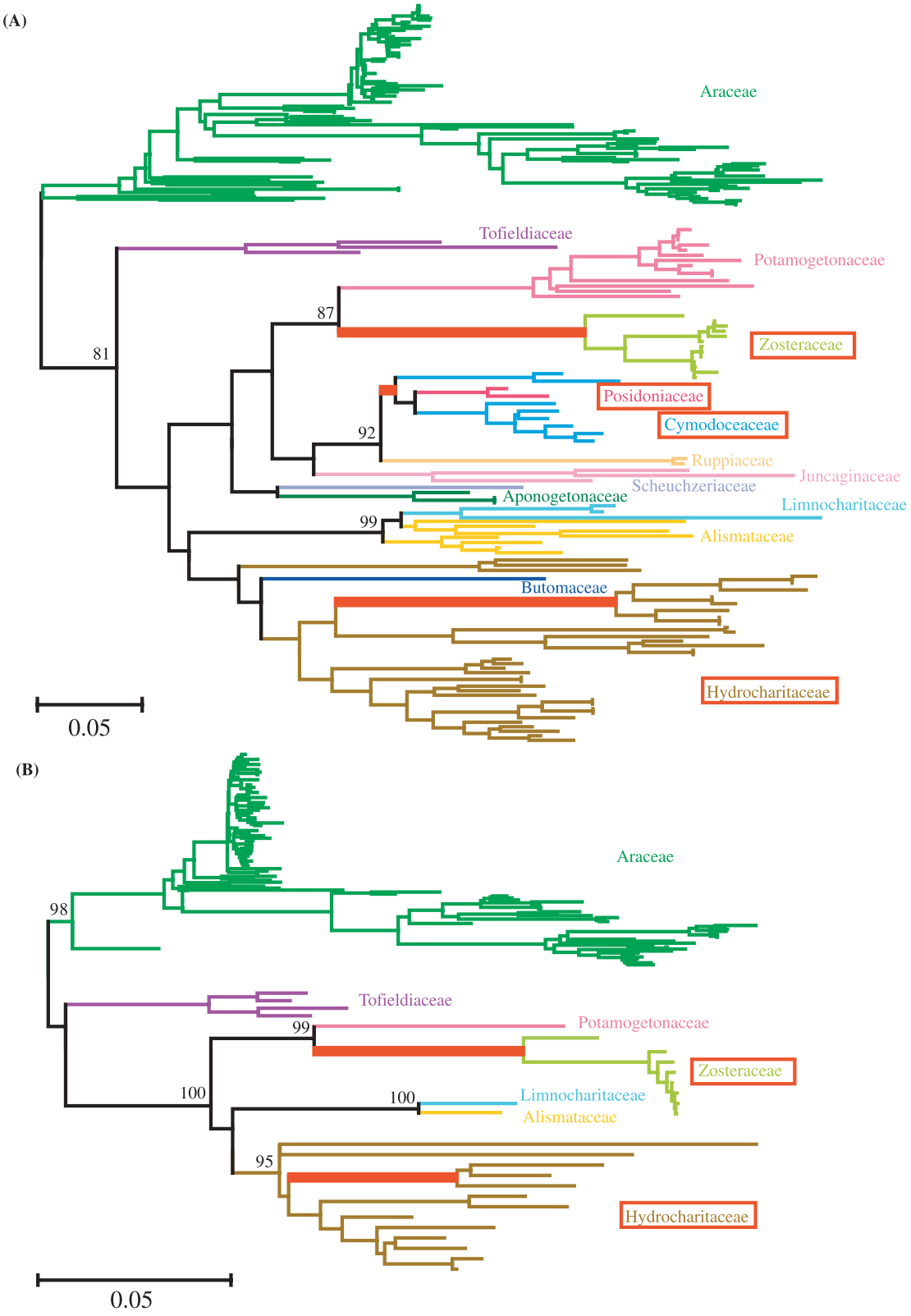

Fig. 2. NJ trees of Alismatales based on *rbcL* (A) and *matK* (B) sequences. There are 207 *rbcL* and 115 *matK* sequences available from DDBJ/Genbank/EMBL. The size of region used in the analysis is 949 bp for *rbcL* and 1157 bp for *matK*. The *d*-distance in the entire region is used for constructing the phylogenetic tree. Different colors correspond to different families. Families including seagrass species are boxed. The lineages leading to seagrasses are shown in red thick lines. The bootstrap values for each family of more than 75% are shown.

was examined by looking at the correlation in the *d*-distances between *rbcL* and *matK* genes over various species pairs. Also, the rate heterogeneity of nucleotide substitutions of Zosteraceae relative to *Oryza sativa* was tested by using *Arabidopsis thaliana* as an outgroup. This test was carried out by the Tajima's method (1993) implemented in MEGA2.

RESULTS

Phylogenetic position of Zosteraceae in order Alismatales. We determined 24 *rbcL* sequences of 1376 bp from four *Zostera* species (*Z. marina, Z. caespitosa, Z. caulescens,* and *Z. japonica*) as well as 1288 bp from *Z. asiatica* and 30 *matK* sequences of 1503 bp from the all

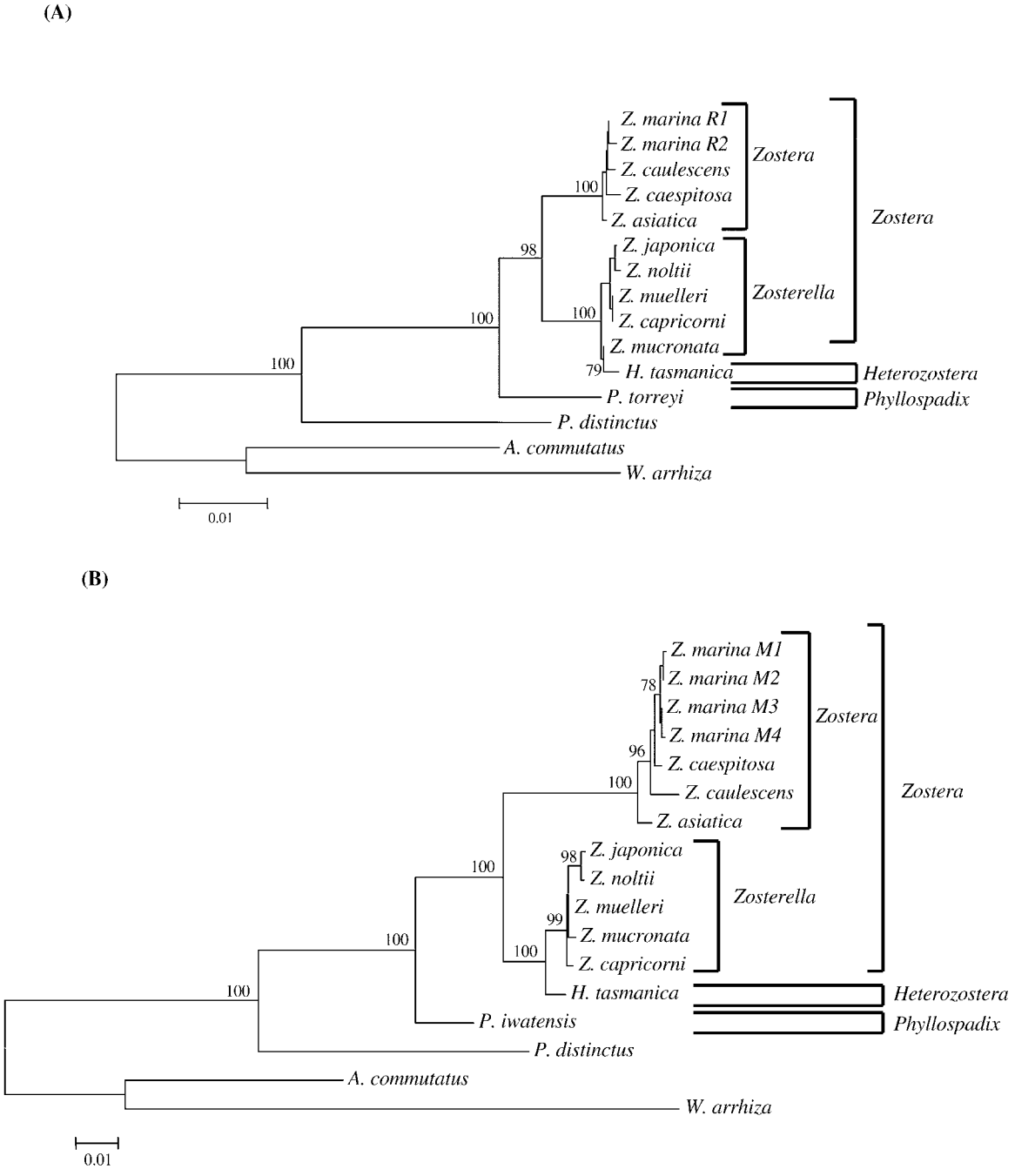

Fig. 3. NJ tress of Zosteraceae based on *rbcL* (A) and *matK* (B) sequences. The bootstrap values of more than 75% are shown. Sequences of *A. commutatus* and *W. arrhiza* from family Araceae are used as outgroups. Both trees are constructed based on *d*-distances.

five *Zostera* species. In addition, to search for the most closely related species to Zosteraceae, we used 207 *rbcL* and 115 *matK* sequences of order Alismatales to which four seagrass families (Zosteraceae, Posidoniaceae, Cymodoceaceae, and Hydrocharitaceae) and other ten families belong. The *rbcL* sequence data cover all 14 families of Alismatales while the *matK* sequence data cover seven. Using the nucleotide sequences from family Araceae as outgroups (Les et al. 1997), we first constructed the NJ trees based on the *d*-distances at *rbcL* and *matK* sequences, separately (Fig. 2). Both trees show consistently that the most closely related family to Zosteraceae is Potamogetonaceae. This conclusion does not change whether the synonymous or nonsynonymous substitutions are used. The same is true for tree-making methods. The cluster of family Zosteraceae and Potamo-

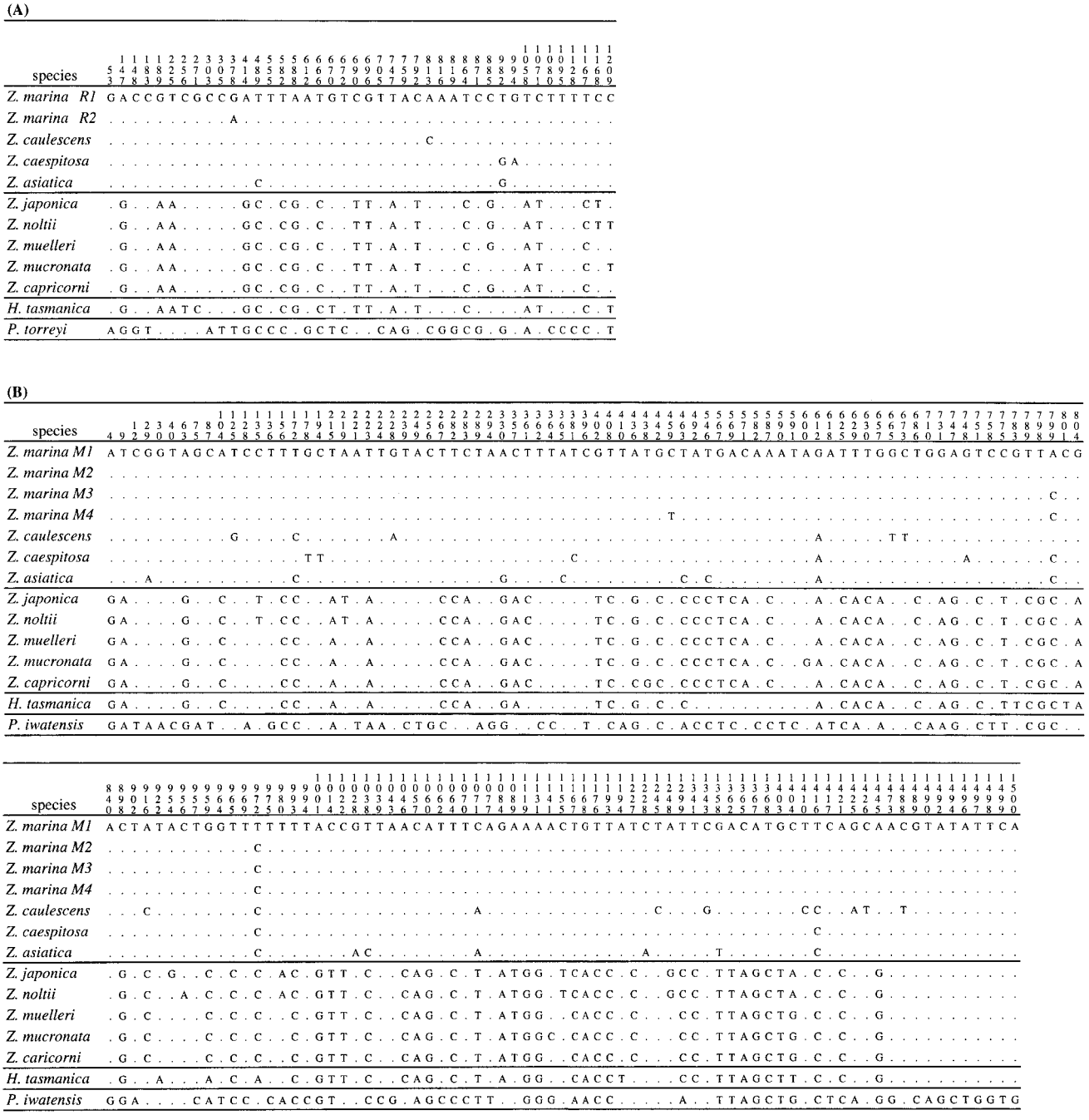

Fig. 4. DNA variation in *rbcL* (A) and *matK* (B) genes of species in Zosteraceae. Nucleotide positions of variable sites are indicated at the top. *Z. marina R1* and *R2* of *rbcL* and *M1* to *M4* of *matK* are polymorphic haplotypes. For other species, polymorphism is not detected. Dot (.) represents the same nucleotide as that in the top line.

getonaceae is always supported by more than 87% bootstrap value for *rbcL* and 99% for *matK*. It is also to be noted in Fig. 2 that the other three seagrass families form three distinct clades. Two clades of Cymodoceaceae and Posidoniaceae share a direct common ancestor and are closely related to Ruppiaceae, while seagrasses in Hydrocharitaceae form the other distantly related clade. These results support the polyphyletic origins of aquatic angiosperms (Les et al. 1997). For further investigation of Zosteraceae evolution, we hereafter focus on family Zosteraceae and Potamogetonaceae with Araceae as an outgroup (Fig. 3).

It is currently accepted that there are three genera within family Zosteraceae, *Phyllospadix, Zostera*, and *Heterozostera*, and two subgenera *Zostera* and *Zosterella* within genus *Zostera* (den Hartog 1970). To date the origin and subsequent diversification of Zosteraceae, we used *rbcL* and *matK* sequences of *Z. noltii* (U80733, AB096170), *Z. capricorni* (AY077963, AB096167), *Z. mulleri* (AY077962, AB096169), *Z. mucronata* (U80732, AB096168), *H. tasmanica* (U80730, AB096171), *P. torreyi* and *P. iwatensis* (U80731, AB096172) together with the *Zostera* sequences we determined (Table 1). Fig. 3A (*rbcL*) and 3B (*matK*) clearly show that two subgenera *Zostera* and *Zosterella* are reciprocally monophyletic and each clade is supported by 100% bootstrap value for both *rbcL* and *matK*. The monophyly of genus *Zostera* with respect to genus *Phyllospadix* (represented by *P. torreyi* for *rbcL* or *P. iwatensis* for *matK*) is also supported by 100% bootstrap value. However, the phylogenetic position of *Heterozostera* which consists of a single species *H. tasmanica* is problematic. As Tanaka et al. (2003) and Les et al. (2002) noted, our result also shows that *Heterozostera* is more closely related to subgenus *Zosterella* than to subgenus *Zostera* (79% and 100% bootstrap value for *rbcL* and *matK*, respectively) despite its current taxonomic classification as a genus (Kuo and den Hartog 2001). As discussed later, *Heterozostera* can be ranked as the same taxonomic level as *Zosterella*.

Nucleotide differences between and within *Zostera* species. Fig. 4 shows variable sites observed among 12 Zosteraceae species, and Table 2 shows the average number of nucleotide differences (the *p*-distances) between and within these species.

The *p*-distances among the *matK* sequences reveal that *Z. marina* is more closely related to *Z. caespitosa* than to *Z. caulescens* (Table 2). There are two phylogenetically informative sites which support the clustering of *Z. marina* and *Z. caespitosa* to the exclusion of *Z. caulescens* (Fig. 4). As for the *rbcL* sequences, however, the *p*-distances between *Z. marina* and *Z. caulescens* are smaller than those between *Z. marina* and *Z. caespitosa* or between *Z. caulescens* and *Z. caespitosa* (Table 2). In fact, there is one informative site which can support the clustering between *Z. marina* and *Z. caulescens* (Fig. 4). Thus, both *matK* and *rbcL* support mutually incompatible phylogenetic relationships among these three species. This incompatibility is most easily explained by one parallel synonymous substitution in *rbcL*, so that we inferred that *Z. marina* is more closely related to *Z. caespitosa* than to *Z. caulescens*.

Among five *Zostera* species, *Z. marina* exhibits the highest nucleotide diversity (Nei and Li 1979), although it is as low as 0.07% for *matK* and 0.03% for *rbcL* (Table 2). In 16 *matK* sequences of *Z. marina*, there are four haplotypes (*M1* to *M4*) which are different at one to three segregating sites. Haplotype *M1* is common (12/16 =

Table 2. The number of nucleotide differences per locus and the per–site % nucleotide differences (in parenthesis) within and between *Zostera* species. *rbcL* (1288 bp; above the diagonal) and *matK* (1503 bp; below the diagonal)

Subgenus	*Zostera*				*Zosterella*
Species [a]	*Z. marina*[b] (16)	*Z. caulescens* (8)	*Z. caespitosa* (2)	*Z. asiatica* (2)	*Z. japonica* (2)
Z. marina	0.36 (0.03)	1.2 (0.09)	2.2 (0.17)	2.2 (0.17)	19.2 (1.5)
	0.99 (0.07)				
Z. caulescens	11.9 (0.79)	0 (0)	3 (0.23)	3 (0.23)	20 (1.6)
		0 (0)			
Z. caespitosa	5.7 (0.38)	18 (1.2)	0 (0)	2 (0.16)	21 (1.6)
			0 (0)		
Z. asiatica	12.7 (0.84)	21 (1.4)	15 (1.0)	0 (0)	19 (1.5)
				0 (0)	
Z. japonica	71.7 (4.8)	86 (5.7)	81 (5.4)	75 (5.0)	0 (0)
					0 (0)

[a] The sample sizes are given in parenthesis.
[b] The number of *Z. marina* sequences of *rbcL* is 10 and that of *matK* is 16.

0.75) and found in both northwestern and eastern Pacific Ocean, while the other three haplotypes are rare and restricted to particular sampling locations. One *M2* is represented in each of Ishikawa and Shizuoka. Likewise, one *M3* and one *M4* are represented in Mexico. In *rbcL* sequences, there are two haplotypes, designated as *R1* and *R2*, which differ by only one segregating site. Haplotype *R1* occurs in almost all samples while rare haplotype *R2* is found only in Mexico. However, *Z. caulescens*, *Z. caespitosa*, *Z. asiatica*, and *Z. japonica* are monomorphic for both *rbcL* and *matK* (Table 2).

Molecular clock at the synonymous sites of Zosteraceae *rbcL* and *matK* sequences. Phylogenetic analysis based on RFLP or DNA sequences revealed that the nucleotide substitution rate at the chloroplast genome greatly varies among different lineages (Wilson et al. 1990; Bousquet et al. 1992; Gaut et al. 1992; Bremer 2000). In order to date the origin and subsequent diversification of Zosteraceae, it is necessary to test the rate constancy and to examine whether the rate does not differ from that in other species such as *Oryza sativa* of which the synonymous substitution rate is well studied (Li 1997).

First, we tested the rate heterogeneity of nucleotide

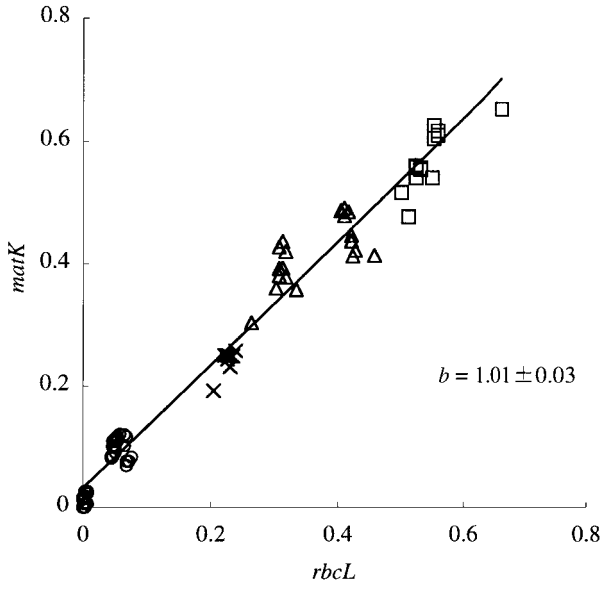

Fig. 5. Correlation in the synonymous divergences (*d*-distances) between *rbcL* and *matK* genes. Open circles represent comparison within a family of Zosteraceae, and crosses stand for comparisons between Potamogetonaceae and Zosteraceae. Open triangles represent comparisons between Araceae and Zosteraceae or Potamogetonaceae. Open squares show comparisons between *Oryza sativa* and Zosteraceae or Potamogetonaceae or Araceae. The coefficient of the regression line is $b = 1.01 \pm 0.03$.

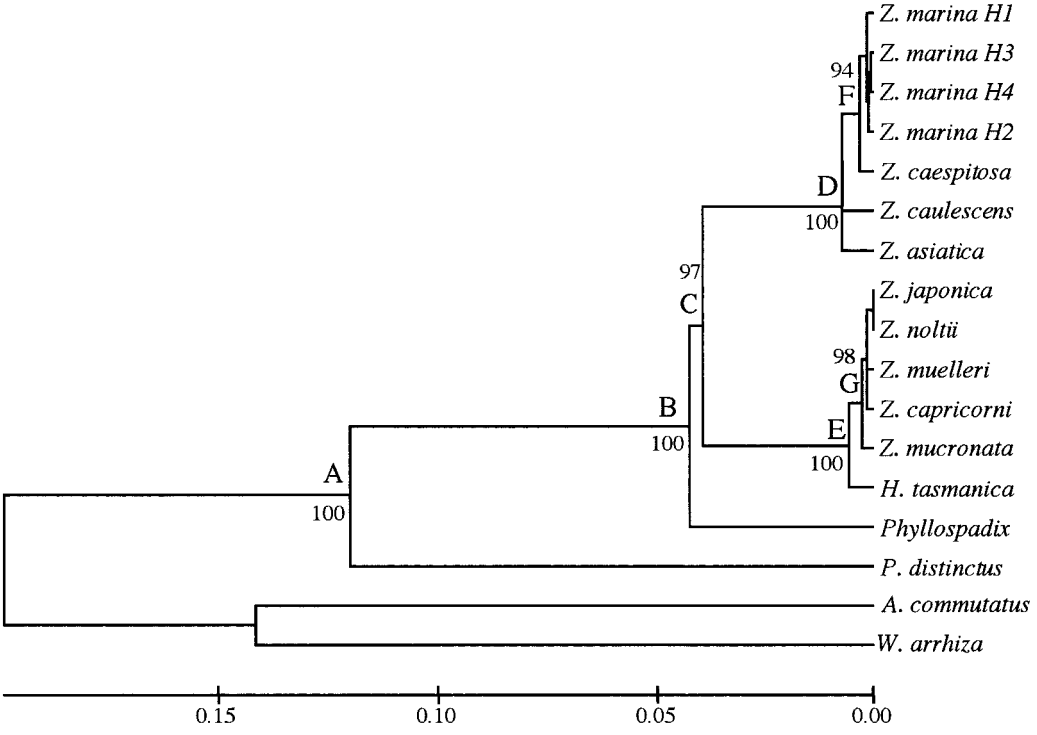

Fig. 6. NJ tree of Zosteraceae based on the synonymous divergence (*d*-distances) in the concatenated *rbcL* and *matK* sequences. The total number of synonymous sites is 809. The sequences of *A. commutatus* and *W. arrhiza* from family Araceae are used as outgroups. Symbols of A to G stand for the nodes corresponding to divergences between family Zosteraceae and Potamogetonaceae, between genus *Zostera* and *Phyllospadix,* between subgenus *Zostera* and *Zosterella*, among subgenus *Zostera* species, between *Heterozostera* and *Zosterella*, between *Z. marina* and *Z. caespitosa*, or among *Zosterella* species, respectively. Bootstrap values are shown at these nodes. Within *Zostera marina*, four haplotypes (*H1* to *H4*) are found in concatenated *rbcL* and *matK* sequences.

substitutions among Zosteraceae, Potamogetonaceae and Araceae sequences by the two-cluster test of Takezaki et al. (1995). The result shows that although there is no rate heterogeneity in *rbcL* ($\chi^2 = 10.54$, $P > 0.10$), there is significant rate heterogeneity in *matK* ($\chi^2 = 50.88$, $P < 0.01$). We then tested the rate heterogeneity at the third codon positions of *matK* and found no significance ($\chi^2 = 25.30$, $P > 0.025$). Therefore, it is likely that synonymous substitutions in *matK* have accumulated at a constant rate and that the rate heterogeneity is attributed largely to nonsynonymous substitutions. Next we tested the rate constancy of synonymous substitutions between *rbcL* and *matK*. For given species pairs, we compared the *d*-distances in *rbcL* with those in *matK* by plotting these against the X and Y axes to obtain the coefficient of the regression line. It is 1.01 ± 0.03 which is not different from 1 (Fig. 5). We therefore conclude that *rbcL* and *matK* genes have evolved at a similar rate of synonymous substitutions even when *Oryza sativa* is included. Tajima's relative rate test (1993) also shows no significant differences between rice and seagrasses in substitution rates at the third codon positions ($P > 0.122$ for *rbcL* and $P > 0.026$ for *matK*). Based on these results, we concatenate *rbcL* and *matK* sequences into one and calculate the synonymous substitutions to get more reliable estimates of species divergence times.

Fig. 6 shows the NJ tree based on the number of synonymous substitutions in the concatenated sequences. We computed the average *d*-distances (d_A, d_B, d_C, d_D, d_E, d_F, and d_G) from tips to a common node of A to G in Fig. 6. Each node corresponds to the divergence between family Zosteraceae and Potamogetonaceae, between genus *Zostera* and *Phyllospadix*, between subgenus *Zostera* and *Zosterella*, among subgenus *Zostera*, between genus *Heterozostera* and subgenus *Zosterella*, between *Z. marina* and its closely related species *Z. caespitosa*, and among subgenus *Zosterella*, respectively. We obtained $d_A = 0.120 \pm 0.012$, $d_B = 0.043 \pm 0.007$, $d_C = 0.039 \pm 0.007$, $d_D = 0.007 \pm 0.003$, $d_E = 0.006 \pm 0.003$, $d_F = 0.003 \pm 0.002$ and $d_G = 0.003 \pm 0.002$. Applying the synonymous substitution rate of $0.9 - 1.2 \times 10^{-9}$ per site per year for *rbcL* of *Oryza sativa* (Li 1997), we dated node A as 100 - 133 million years (myr), node B as 36 - 48 myr, node C as 33 - 44 myr, node D as 6 - 8 myr, node E as 5 - 7 myr, node F as 3 - 4 myr, and node G as 2 - 3 myr. We are also interested in local differentiation of subgenus *Zosterella*. There are two species confined to the northern hemisphere (*Z. japonica* and *Z. noltii*) and three are confined in the southern hemisphere (*Z. capricorni*, *Z. mulleri* and *Z. mucronata*). The synonymous divergences between these northern and southern hemisphere species range from 0.005 ± 0.002 to 0.008 ± 0.003, corresponding to the divergence time from 2 to 4 myr.

DISCUSSION

The origin of Zosteraceae and species divergence associated with geological events. There are two possible evolutionary hypotheses on the origin of seagrasses: they were descended from a fresh-water hydrophyte primitive stock or from a saltmarsh-type (mangrove-like) primitive stock (den Hartog 1970). Morphological and physiological evidence shows that several characters, such as salt and wave tolerance, are shared between seagrasses and saltmarsh-type plants, supporting the saltmarsh-type origin of seagrasses (Larkum and den Hartog 1989). Our molecular phylogenetic analysis has however shown that the most closely related family to Zosteraceae is Potamogetonaceae which is a family of fresh-water angiosperms (Les et al. 1997). In other seagrasses, the closest family to the clade of Posidoniaceae and Cymodoceaceae is Ruppiaceae which is also fresh-water angiosperms. Moreover, some genera within Hydrocharitaceae are marine angiosperms and these genera also appear to be derived from fresh-water angiosperms. These suggest that seagrasses have independently developed mangrove-like salt and wave tolerances in the early evolutionary phase. It is also interesting to date the origins of these seagrasses, Posidoniaceae, Cymodoceaceae, and Hydrocharitaceae. The *rbcL* synonymous divergences become 0.142 ± 0.020 between Ruppiaceae and the clade of Posidoniaceae and Cymodoceaceae as well as 0.279 ± 0.027 for seagrasses within Hydrocharitaceae. These values are comparable with the *d*-distances between Zosteraceae and Potamogetonaceae, 0.234 ± 0.026. Since there is no rate heterogeneity of synonymous substitutions at *rbcL* among these seagrasses ($P > 0.10$) and if Zosteraceae and Potamogetonaceae diverged from each other 100 myr ago, the common ancestor between Ruppiaceae and the clade of Posidoniaceae and Cymodoceaceae is dated as 69 ± 8 myr ago and the origin of seagrasses within Hydrocharitaceae is dated as 119 ± 11 myr ago. These divergence times agree well with the preposition that primitive seagrass might have occurred on shores of the Tethys Sea (Larkum and den Hartog 1989).

Since the rather ancient emergence of Zosteraceae about 100 myr ago, the family only began to diversify into two extant clades 36 myr ago. This does not contradict fossil records of *Archaeozostera* found in the Cretaceous layer in Japan (Koriba and Miki, 1931, 1958; Oishi 1931). However, Koriba and Miki (1931, 1958) concluded that these fossils belong to a genus that might be related to extant *Phyllospadix*. This conclusion is inconsistent with our estimate of the emergence time of *Phyllospadix* and supports the relatively recent origin hypothesis of Zosteraceae by Kuo et al. (1989) and Aioi (2000).

Similarly, we estimated the divergence time between *Zostera* and *Zosterella* as 33 – 44 myr. This geological

time coincides with the completion of significant continental drift. It is thought that this completion led great temperature changes; polar seas got colder and the tropical seas became warmer (Galloway and Kemp 1981; Valentine 1984; Frakes 1979). This temperature change might limit the distribution of temperate *Zostera* and *Zosterella* into either northern or southern hemisphere. Within subgenus *Zostera*, four species diverged around 3 to 6 myr ago. At that time, the Japan Archipelago had been shaped into the present form. Prior to this formation, the Japan Archipelago had been divided into a large number of small islands. If sea currents in Japanese waters changed by formation of the Japan Archipelago, it is possible that this change altered gene flow between the northern and southern part of Japanese waters. Such alternation might facilitate the diversification of *Zostera* species in Japanese waters. The geographic distribution of species in subgenus *Zosterella* between the northern and southern hemisphere was probably initiated 2 myr ago. However, unlike speciation among subgenus *Zostera* around the Japan Archipelago 6 – 8 myr ago, there is no obvious geological event that can account for the separation of *Zosterella* species between the northern and southern hemisphere and the cause remains enigmatic (Tanaka et al. 2003).

Genetic variability within *Zostera* species. The extent of nucleotide differences observed among samples sequenced is generally low. This is mainly due to the low nucleotide substitution rate ($0.9 - 1.2 \times 10^{-9}$ per site per year) of chloroplast genes compared with the rate at nuclear encoded genes (Wolfe et al. 1987; Li 1997), a relatively short generation time in flowering plants, and a relatively small effective population size of chloroplast genes owing to the cytoplasmic inheritance and haploid nature.

A pair of most diverged sequences (*M1* and *M4*) found in *Z. marina matK* sequences differs by only three substitutions. Therefore, the time back to the most recent common ancestor (MRCA) of *Z. marina* sequences becomes about 1/4 of the divergence between *Z. marina* and its closely related species *Z. caulescens* or *Z. asiatica* (Table 2). If *Z. marina* and *Z. caulescens* or *Z. asiatica* diverged 6 myr ago as estimated above, the MRCA of *Z. marina matK* sequences must have occurred 1.5 myr ago. This estimate of MRCA as the origin of polymorphism appears to be old, if we take into account a relatively short generation time of the plant. One possibility for this rather high extent of polymorphism is long-lasting limited migration between northwestern and eastern Pacific populations. However, this possibility cannot easily explain the observation that a common haplotype (*M1*) occurs in both northwestern and eastern Pacific populations and *M2* ancestral to *M4* occurs also in northwestern populations. In any case, in order to address these issues, information on local genetic differentiation is needed. In this regard, it would be worth studying microsatellites or nuclear genes which are generally much more variable than chloroplast genes.

The age related re-classification of family Zosteraceae. The phylogenetic relationships of Zosteraceae species in Fig. 6 are in good agreement with those of Tanaka et al. (2003) based on *matK* sequences of *Zostera* and *Heterozostera*. The monophyletic relationships are strongly supported at each of three taxonomic levels of subgenus, genus, and family (99 - 100% bootstrap values). The position of genus *Heterozostera* in the molecular phylogeny, however, seems to contradict the rank in the taxonomy. Since *Heterozostera* shows the most primitive features among all zosteroids, it is thought that *Heterozostera* has an ancient origin and is ranked as a genus (den Hartog 1970; Larkum and den Hartog 1989). However, molecular phylogenetic analyses support the clustering of *Heterozostera* with subgenus *Zosterella* (Les et al. 1997; Tanaka et al. 2003). In fact, some recent morphological studies suggested the sister relationship between genus *Heterozostera* and subgenus *Zosterella* (Taylor 1981; Soros-Pottruff and Posluzny 1995; Tomlinson and Posluzny 2001; Tanaka et al. 2003).

To resolve this disorder, there may well be merit to an age related phylogenetic classification, at least for groups where a crude correlation exists between rank and age in existing classification (Goodman et al. 1998). In an age related phylogenetic classification, taxa at a higher rank should not only be older than taxa at a lower rank, but also taxa at the same rank should be roughly at about the same age. In this regard, it is not reasonable that genus *Heterozostera* is as old as subgenera *Zostera* and *Zosterella* (Fig. 6). It is also inappropriate that two different ranks of genera (*Zostera* and *Phyllospadix*) and subgenera (*Zostera* and *Zosterella*) have a similar age of 33 - 36 myr. Thus, our study of the time frame requires some re-classification of present subgenera *Zostera* and *Zosterella* as well as present genus *Heterozostera*.

Similar modification of ranks in Zosteraceae based on morphological characters has been proposed by Tomlinson and Posluzny (2001) or on molecular phylogenetic analysis (Tanaka et al. 2003). Tomlinson and Posluzny (2001) proposed to elevate ranks for subgenera of *Zostera* and *Zosterella* to genera, and divide family Zosteraceae into four genera, *Phyllospadix*, *Zostera*, *Nanozostera*, and *Heterozostera*. On the other hand, Tanaka et al. (2003) proposed inclusion of two subgenera *Zosterella* and *Heterozostera* into a new genus of *Nanozostera*. Our phylogenetic study supports these proposals. Although the ages of family, genus and subgenus in Zosteraceae seem to be too old as the names imply in the sense of Goodman et al. (1998), our provisional age related re-classification of the family is as follows:

Family Zosteraceae (100 myr)
- Genus Phyllospadix (36 myr)
  - Phyllospadix torreyi
  - Phyllospadix iwatensis
- Genus Zostera (33 myr)
  - Subgenus Zostera (6 myr)
    - Zostera (Zostera) marina
    - Zostera (Zostera) caespitosa
    - Zostera (Zostera) caulescens
    - Zostera (Zostera) asiatica
- Genus Nanozostera (33 myr)
  - Subgenus Zosterella (5 myr)
    - Nanozostera (Zosterella) japonica
    - Nanozostera (Zosterella) noltii
    - Nanozostera (Zosterella) capricorni
    - Nanozostera (Zosterella) mulleri
    - Nanozostera (Zosterella) mucronata
    - Nanozostera (Zosterella) novazelandica
    - Nanozostera (Zosterella) capensis
  - Subgenus Heterozostera (5 myr)
    - Nanozostera (Heterozostera) tasmanica

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Appendix 1
List of the accesion numbers in NCBI of *rbcL* sequences using Figure 2 (A)

family	accession#	family	accession#	family	accession#	family	accession#	family	accession#
Alismataceae	L08767	Araceae	AF497080	Araceae	AJ005631	Butomaceae	U80685	Hydrocharitaceae	AB004897
	L08759		AF497081		AJ005632	Cymodoceaceae	U03727		AB004898
	U80677		AF497082		AJ007543		U80686		AB004899
	U80678		AF497083		AJ007544		U80687		AF206832
	U80679		AF497084		AJ235807		U80688	Juncaginaceae	U80713
	U80680		AF497085		AY034222		U80689		U80714
	U80681		AF497086		AY034223		U80690		U80715
	U80682		AF497087		AY034224		U80691	Limnocharitaceae	U80716
Aponogetonaceae	U80683		AF497088		AY034225		U80692		U80717
	U80684		AF497089		AY034226	Hydrocharitaceae	U03726		AB004900
	AY175465		AF497090		AY034227		U03731	Posidoniaceae	U80718
Araceae	L10246		AF497091		AY034228		U80693		U80719
	L10247		AF497092		AY034229		U80694	Potamogetonaceae	L08765
	L10248		AF497093		AY034230		U80695		U03725
	L10250		AF497094		AY034231		U80696		U03730
	L10254		AF497095		AY034232		U80697		U80720
	L10255		AF497096		AY034233		U80698		U80721
	M91360		AF497097		AY034234		U80699		U80722
	M91626		AF497098		AY034235		U80700		U80723
	M96963		AF497099		AY034236		U80701		U80724
	U68092		AF497100		AY034237		U80702		U80725
	AF065474		AF497101		AY034238		U80703		U80726
	AF497060		AF497102		AY034239		U80704		U80727
	AF497061		AF497103		AY034240		U80705		U80729
	AF497062		AF497104		AY034241		U80706		AB004901
	AF497063		AF497105		AY034242		U80707	Ruppiaceae	U03729
	AF497064		AF497106		AY034243		U80708		U80728
	AF497065		AF497107		AY034244		U80709	Scheuchzeriaceae	U03728
	AF497066		AF497108		AY034245		U80710	Tofieldiaceae	AJ131774
	AF497067		AF497109		AY034246		U80711		AJ235798
	AF497068		AF497110		AY034247		U80712		AJ286562
	AF497069		AF497111		AY034248		AB004886	Zosteraceae	U80730
	AF497070		AF497112		AY034249		AB004887		U80731
	AF497071		AF497113		AY034250		AB004888		U80732
	AF497072		AJ005623		AY034251		AB004889		U80733
	AF497073		AJ005624		AY034252		AB004890		U80734
	AF497074		AJ005625		AY034253		AB004891		AY077962
	AF497075		AJ005626		AY034254		AB004892		AY077963
	AF497076		AJ005627		AY034255		AB004893		AY077964
	AF497077		AJ005628		AY034256		AB004894		
	AF497078		AJ005629		AY034257		AB004895		
	AF497079		AJ005630		AY034258		AB004896		

Appendix 2
List of the accesion numbers in NCBI of *matK* sequences using Figure 2 (B)

family	accession#	family	accession#	family	accession#
Alismataceae	AB040179	Araceae	AF78417	Araceae	AY034203
Araceae	AF78379		AF78418		AY034204
	AF78380		AF78419		AY034205
	AF78381		AF78420		AY034206
	AF78382		AF78421		AY034207
	AF78383		AF78422		AY034208
	AF78384		AF78423		AY034209
	AF78385		AF78424		AY034210
	AF78386		AF78425		AY034211
	AF78387		AF78426		AY034212
	AF78388		AF78427		AY034213
	AF78389		AF78428		AY034214
	AF78390		AF78429		AY034215
	AF78391		AF78430		AY034216
	AF78392		AF78431		AY034217
	AF78393		AF78432		AY034218
	AF78394		AB040177		AY034219
	AF78395		AB040178		AY034220
	AF78396		AY034182	Hydrocharitaceae	AB002566
	AF78397		AY034183		AB002567
	AF78398		AY034184		AB002568
	AF78399		AY034185		AB002569
	AF78400		AY034186		AB002570
	AF78401		AY034187		AB002571
	AF78402		AY034188		AB002572
	AF78403		AY034189		AB002573
	AF78404		AY034190		AB002574
	AF78405		AY034191		AB002575
	AF78406		AY034192		AB002576
	AF78407		AY034193		AB002577
	AF78408		AY034194		AB002579
	AF78409		AY034195	Limnocharitaceae	AB002580
	AF78410		AY034196	Potamogetonaceae	AB002581
	AF78411		AY034197	Tofieldiaceae	AB040157
	AF78412		AY034198		AB040158
	AF78413		AY034199		AB040159
	AF78414		AY034200		AB040160
	AF78415		AY034201		
	AF78416		AY034202		