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ORIGINAL ARTICLE

Biogeography of North Pacific *Isoëtes* (Isoëtaceae) Inferred from Nuclear and Chloroplast DNA Sequence Data

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Abstract Recent advances in phylogenetics indicate that reticulate evolution has played an important role in the emergence of *Isoëtes* species in the North Pacific region. However, the biogeographical origin of the North Pacific *Isoëtes* species remains contentious. We present a fossil-calibrated phylogeny of species from the North Pacific region based on molecular data. Within this framework, we discuss their ancestral areas and biogeographical history. North Pacific *Isoëtes* are divided into two clades: clade I, consisting of East Asian, Papua New Guinean, and Australian species, and clade II, consisting of West Beringian and western North American species. Within clade I, Australian *Isoëtes* species were an early divergent group, and Papua New Guinea's species form a sister clade to the East Asian species. Biogeographical reconstructions suggest an Australasian origin for the East Asian species that arose through long-distance dispersal during the late Oligocene. Within clade II, *I. asiatica* from West Beringia forms a clade with *I. echinospora* and *I. muricata* from Alaska. Western North America was the area of origin for the dispersal of *Isoëtes* species to West Beringia via the Bering land bridge during the late Miocene. Our study identifies the biogeographic origin of the North Pacific *Isoëtes* and suggests long-distance dispersal as the most likely explanation for their intercontinental distribution.

Keywords: Biogeography, Chloroplast DNA, *Isoëtes*, molecular phylogeny, North Pacific, nrITS

Introduction

Understanding the mechanisms that generate disjunct distribution patterns of some species has long been a major focus of biogeography (Cox and Moore 2010). The biogeographical origins and evolution of the disjunct distributions of many plant groups have been examined through molecular phylogenetic analyses (Oliver et al. 2006; Havill et al. 2008; Kiefer et al. 2009; Qiu et al. 2009). Some intercontinental disjunctions, e.g., the major division in the phylogram of liverwort lineages between Laurasia and Gondwana (Vanderpoorten et al. 2010), have been explained by vicariance (Nelson and Platnick 1981). Other studies have invoked relatively recent long-distance dispersal events to explain the intercontinental disjunctions of mosses and liverworts (Perrie and Brownsey 2007; Devos and Vanderpoorten 2009; Yu et al. 2010). Among aquatic lycopsids, there is little consensus regarding historical biogeography because the local adaptations of these plants to their habitats and their singular lack of morphological variation pose challenges to phylogenetic and biogeographical characterization (Hoot et al. 2006; Kim et al. 2010b).

Isoëtes L. (quillwort) is the single genus of the family Isoëtaceae, a group of heterosporous lycopsids that includes 200 or more extant species found in lakes, wetlands, and terrestrial habitats throughout the world (Hoot et al. 2004). This genus represents a group of land plants with a long history dating back to the Devonian period (Pigg 2001). Extant species are mostly locally derived endemics with restricted geographical ranges (Hoot et al. 2006). Polyploidy has been considered a key speciation process in *Isoëtes*. Nearly 60% of known *Isoëtes* species are allopolyploids (Takamiya 1999; Troia 2001; Hoot et al. 2004; Liu et al. 2004; Tayler et al. 2004; Kim et al. 2009b). For example, *I. sinensis* Palmer is an allotetraploid resulting from the hybridization of two diploids, *I. taiwanensis* DeVol and *I. yunguiensis* Q.F. Wang & W.C. Taylor (Tayler et al. 2004). Local adaptation and allopolyploidy, which likely represents a significant speciation process in this genus, have both made it difficult to characterize the biogeographical origins of *Isoëtes* species (Hoot et al. 2004; Kim et al. 2010b).

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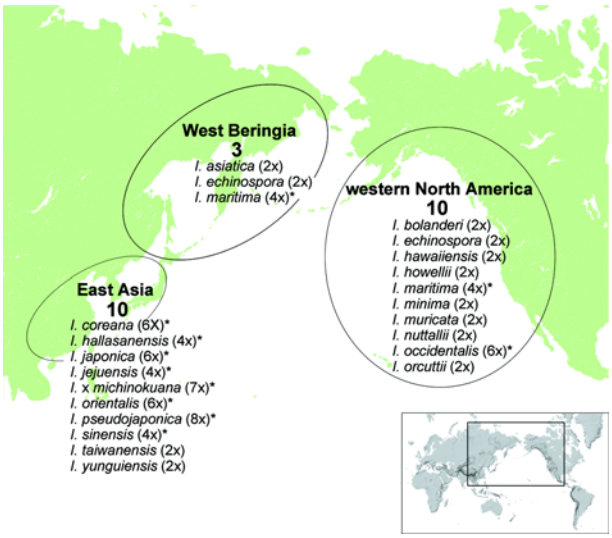

Fig. 1. Geographical distribution and number of *Isoëtes* species in the North Pacific region. An asterisk indicates polyploids that were excluded from this study.

There are presently 21 recognized species of *Isoëtes* in the North Pacific region, including East Asia, West Beringia, and western North America (Fig. 1). In East Asia, two of the currently recognized 10 species of *Isoëtes* are diploids (2n = 22). The other eight are polyploids, including three tetraploids (2n = 44), three hexaploids (2n = 66), one heptaploid (2n = 77), and one octoploid (2n = 88) (Takamiya et al. 1997; Wang et al. 2002; Liu et al. 2005; Choi et al. 2008; Jung et al. 2009). Based on molecular phylogenetic analyses, the East Asian *Isoëtes* species form a single clade (Kim et al. 2009a, 2010b); however, the exact biogeographical origin of East Asian *Isoëtes* is unclear.

Based on the distribution pattern of chromosome numbers in China, Liu et al. (2004) suggested a West China origin for the East Asian *Isoëtes* species that have dispersed through water. The diploid species (*I. hypsophila*, 2n = 22) occurs at high altitudes in West China, whereas the polyploid species (*I. sinensis*, 2n = 44) is found at low altitudes in East China. However, this study did not include species from other regions (e.g., Australia and North America) in the analysis. According to Hoot's molecular phylogenetic study (Hoot et al. 2006), the Australian and East Asian *Isoëtes* form a clade and the Australian species were derived from West China (*I. hypsophila*). However, these results were also founded on limited sampling, which resulted in a phylogram with an ambiguous basal clade due to insufficient resolution. Moreover, the complex patterns of allopolyploid evolution and the lack of resolution in East Asian and Australian species have been interpreted as indicating active speciation rather than ancient or relictual speciation (Hoot et al. 2004; Kim et al. 2010b).

Based on chromosome number (diploid, $2n$ = 22) and spore ornamentation (Taylor et al. 1993b), it has also been suggested that *I. hawaiiensis* W.C. Taylor & W.H. Wagner from Hawaii is closely related to *I. taiwanensis*, which was recognized as the parental species for the East Asian polyploids (Kim et al. 2010b). Therefore, to better understand the biogeographic origin of the East Asian *Isoëtes* species, we need to evaluate their phylogenetic relationships and estimate divergence times with a dataset that includes comprehensive sampling from East Asia, Australia, and North America.

A similar uncertainty in biogeographic origin is exhibited by the Beringian and western North American *Isoëtes* species. Three *Isoëtes* species occur in West Beringia, including populations in Hokkaido, Sakhalin, and Kamchatka (Taylor et al. 1993a; Takamiya et al. 1997; Fig. 1). The distribution ranges of the *Isoëtes* species in this region overlap with those of western North American species (*I. echinospora* Durieu and *I. maritima* Underw.). Ten *Isoëtes* species have been reported for western North America (Taylor et al. 1993a; Fig. 1). According to previous molecular phylogenies (Hoot et al. 2004, 2006), *Isoëtes* from western North America are divided into two clades: (1) an American species complex (*I. bolanderi* Engelm., *I. echinospora*, *I. hawaiiensis*, *I. howellii* Engelm., *I. martima* Underw., *I. muricata* Durieu, *I. occidentalis* L.F. Hend), and (2) a western North America group (*I. orcuttii* A.A. Eaton, *I. nuttalii* A. Brown, *I. minima* A.A. Eaton), the latter formed a clade with *Isoëtes* species of India, Africa, and Mediterranean. As it was in East Asia, allopolyploidy is probably a significant speciation mechanism, hindering the understanding of the biogeographic history of the region (Hoot et al. 2004). *Isoëtes asiatica* from Hokkaido and Northeastern Russia forms a monophyletic group with *I. echinospora* from Alaska, which excludes *Isoëtes* species from East Asia (Kim et al. 2009a). However, this study did not examine the origin of the *I. asiatica–I. echinospora* complex, which requires further characterization before these species may be used in phylogenetic analyses.

The present study examined the evolutionary origins of North Pacific *Isoëtes* based on phylogenetic analyses of the nuclear ribosomal ITS (nrITS) and three chloroplast DNA (cpDNA) regions (*atpB-rbcL*, *trnL*, and *trnS-psbC*). Furthermore, we conducted biogeographical analyses by using a fossil-calibrated chronogram. This study represents the broadest taxon sampling of North Pacific *Isoëtes* to date and attempts to answer important questions about their biogeographical origin and the dispersal- or vicariance-based events that led to the divergence of this genus.

Results

Analysis of nrITS Sequence Data

The nrITS alignment, comprising 34 sequences from 21 ingroup and 3 outgroup species, contained 745 characters.

Table 1. Tree statistics of the nrITS, *atpB-rbcL*, *trnL*, and *trnS-psbC*, and combined datasets from maximum parsimony (MP) analysis

Parameters	nrITS	*atpB-rbcL*	*trnL*	*trnS-psbC*	Combined		
					3 cpDNA	nrITS + 3 cpDNA	nrITS + *atpB-rbcL*
No. of sequences	34	34	31	30	34	32	51
Length of aligned matrix (bp)	745	790	421	872	2083	2828	1754
Variable characters (%)	224 (30.1)	69 (8.7)	16 (3.8)	17 (1.9)	102 (4.9)	311 (11.0)	899 (51.3)
Parsimony informative characters (%)	186 (25.0)	60 (7.6)	13 (3.1)	15 (1.7)	88 (4.2)	262 (9.3)	627 (35.7)
No. of trees (MP)	936	39	6	18	63	88	>100,000
MP tree length	289	81	17	3	120	396	1429
Consistency index (CI)[a]	0.868	0.875	0.929	0.938	0.859	0.844	0.748
Retention index (RI)	0.955	0.963	0.985	0.986	0.961	0.946	0.906
Model selected (AIC)[b]	GTR + G				GTR + I	GTR + I + G	GTR + I + G

[a]CI is calculated by excluding uninformative characters.
[b]AIC, Akaike information criterion.

There were 224 (30.1%) variable sites, of which 186 (25.0%) were parsimony informative (Table 1). Parsimony analysis of the nrITS dataset resulted in 936 equally parsimonious trees (tree length = 289; consistency index [CI] = 0.87; retention index [RI] = 0.96). The BI phylogram was identical in topology to the strict consensus of the trees sampled by MP analysis (BI tree not shown), which resolved two well-defined clades (labeled I and II; Fig. S1). Clade I included 16 species from East Asia, Papua New Guinea, West China, the Philippines, and Australia (bootstrap percentage [BP] = 89, PP = 0.98). Within clade I, *I. stevensii* J.R. Croft and *I. habbemensis* from Papua New Guinea formed a clade with East Asian *Isoëtes* species (BP = 71, PP = 0.96). Two accessions of the western Chinese species *I. hypsophila* did not group with *Isoëtes* species from East Asia. Instead, these two accessions formed a clade with *I. philippinensis* Merrill & Perry from the Philippines and two *Isoëtes* species (*I. muelleri* A. Braun and *I. inflata* E.R.L. Johnson) from Australia, but with only weak support (BP = 53, PP = 0.85). The remaining Australian *Isoëtes* species formed an unresolved group in clade I (Fig. S1). Within clade II, two well-supported subclades were recovered. *Isoëtes asiatica* from Hokkaido, Sakhalin, and Northeastern Russia formed a clade with *I. echinospora*_UJ and *I. muricata* from western North America with high support (BP = 100, PP = 1.00). *Isoëtes hawaiiensis* from Hawaii formed a clade with *I. echinospora*_RC, from the Commander Island, Kamchatka (BP = 82, PP = 1.00).

Analysis of cpDNA Sequence Data

The cpDNA *atpB-rbcL*, *trnL*, and *trnS-psbC* regions contained 69 (8.7%), 16 (3.8%), and 17 (1.9%) variable sites, and 60 (7.6%), 13 (3.1%), and 15 (1.7%) were parsimony informative sites, respectively (Table 1). Parsimony analysis of the *atpB-rbcL*, *trnL*, and *trnS-psbC* datasets resulted in 39, 6, and 18 equally parsimonious trees, respectively. Each individual cpDNA region yielded topologies with very low resolution, with most branches unresolved in the three separate MP strict consensus trees (data not shown). Despite this, individual analyses of the three cpDNA regions showed that East Asian *Isoëtes* species, except for *I. australis* and *I. alpina*, were sister species of *Isoëtes* from Papua New Guinea, the Philippines, West China, and Australia. MP analysis on the combined cpDNA data matrix yielded a strict consensus tree from 63 equally parsimonious trees (tree length = 120; CI = 0.86; RI = 0.96; Fig. S2). The strict consensus MP tree was congruent with the BI inferred topology (BI tree not shown). The strict consensus MP tree clearly indicated that *Isoëtes* species were divided into two main clades: (I) East Asia (*I. taiwanensis* and *I. yunguiensis*)–Papua New Guinea (*I. stevensii* and *I. habbemensis*)–the Philippines (*I. philippinensis*)–West China (*I. hypsophila*)–Australia (except for *I. australis* and *I. alpina*); and (II) Hokkaido, Japan–Northeastern Russia–western North America–Hawaii (*I. hawaiiensis*; Fig. S2). Consistent with the result inferred from nrITS dataset, *I. stevensii* and *I. habbemensis* from Papua New Guinea formed a clade with the East Asian *Isoëtes* species.

Analysis of Combined Sequence Data

Among the 2828 characters in the combined datasets, 262 (9.3%) were parsimony informative (Table 1). Phylogenetic analysis of the combined dataset resulted in 88 equally parsimonious trees, each with 396 steps (CI = 0.84, RI = 0.95). The BI phylogram tree (not shown) was identical in topology to the sampled 50% majority-rule consensus tree. Consistent with the results from the individual analyses, two main clades (I and II) were resolved in the consensus tree (Fig. 2). Within clade I, Australian *Isoëtes* species formed an

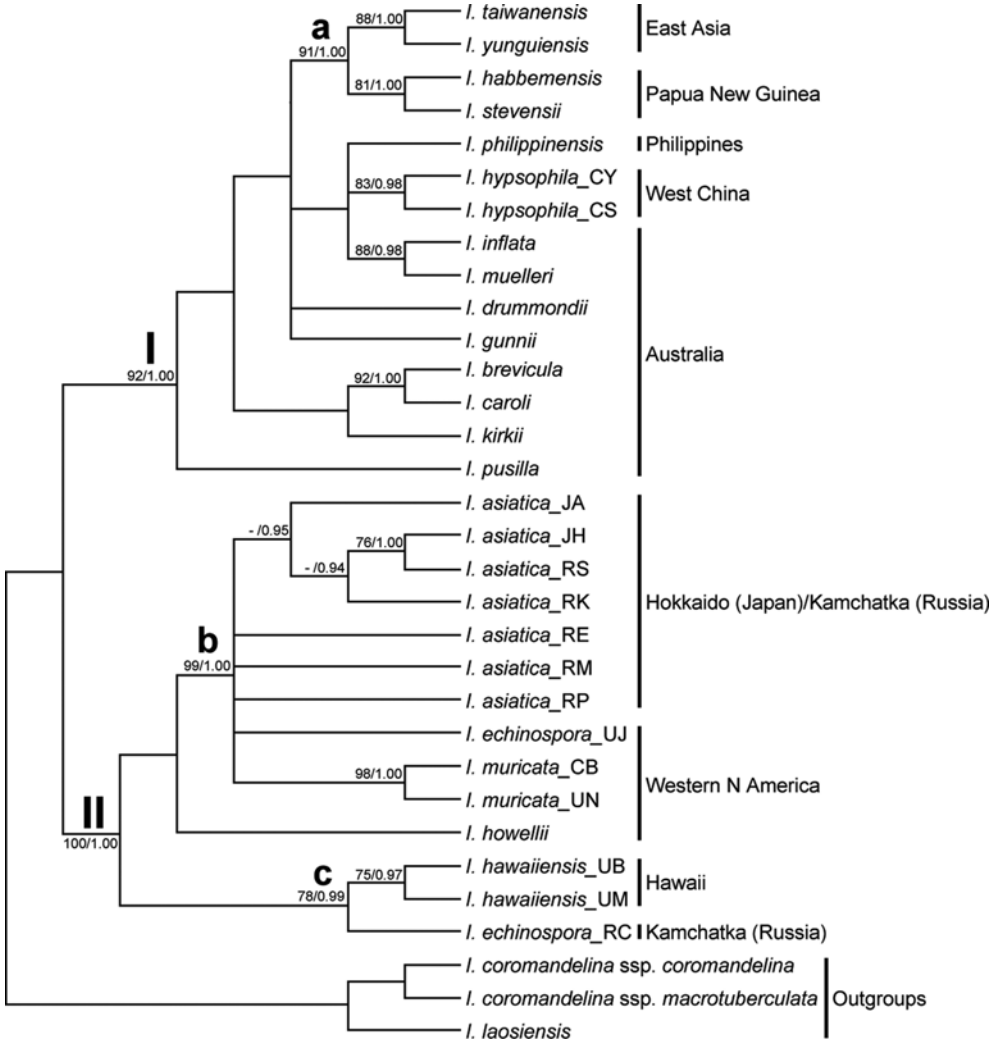

Fig. 2. A 50% majority-rule consensus tree of 88 most parsimonious trees (tree length = 396, consistency index [CI] = 0.84, retention index [RI] = 0.95) inferred from combined nrITS and three cpDNA (*atpB-rbcL*, *trnL*, and *trnS-psbC*) sequence data. Numbers above the branches indicate support values (maximum parsimony bootstrap [BP]/Bayesian posterior probability [PP]); a dash (–) indicates BP 75%. Geographical origin of *Isoëtes* species is indicated using the following codes: CB, British Columbia, Canada; CS, Sichuan, China; CY, Yunnan, China; JA, Aomori, Japan; JH, Hokkaido, Japan; RC, Commander Island, Kamchatka, Russia; RE, Elizovsky, Kamchatka, Russia; RK, Kurile Island, Russia; RM, Magadan, Russia; RN, Narichevo, Kamchatka, Russia; RP, Paratunka, Kamchatka, Russia; RS, Sakhalin, Russia; UB, Big Island, Hawaii, USA; UC, Cohoe Lake, Alaska, USA; UJ, Jewel Lake, Alaska, USA; UM, West Maui, Hawaii, USA; and UN, New Hampshire, USA.

early divergent group, and Papua New Guinea's two species (*I. stevensii* and *I. habbemensis*) formed a sister clade to the East Asian *Isoëtes* species (subclade a; BP = 91, PP = 1.00). Within clade II, *I. asiatica* from Hokkaido (Japan) and the Russian Far East formed a clade with *I. echinospora*_UJ and *I. muricata* from western North America (subclade b; BP = 99, PP = 1.00). Two accessions of *I. hawaiiensis* formed a sister clade to *I. echinospora*_RC, from the Commander Island, Kamchatka (subclade c; BP = 78, PP = 0.99).

Analysis of Divergence Times

Using the combined nrITS and cpDNA *atpB-rbcL* sequence data, the crown node of the North Pacific *Isoëtes* was estimated at 96.4 Ma (95% HPD: 46.8-149.4 Ma) in the Cretaceous (Fig. 3). The BEAST dating analysis estimated the age for clade I, including *Isoëtes* species from East Asia, Papua New Guinea, West China, the Philippines, and Australia, at 54.3 Ma (95% HPD: 24.4-90.8 Ma) in the early Eocene. Within clade I, the age estimate for the node of *Isoëtes* species from East Asia and Papua New Guinea (subclade a) was 25.2 Ma (95% HPD: 5.2-51.7 Ma). The age estimate for *Isoëtes* species from West Beringia and North America (clade II) was 57.9 Ma (95% HPD: 19.6-102.3 Ma); for the Russian Far Eastern and Alaskan *Isoëtes* species (subclade b) the age was estimated at 11.2 Ma (95% HPD: 0.8-

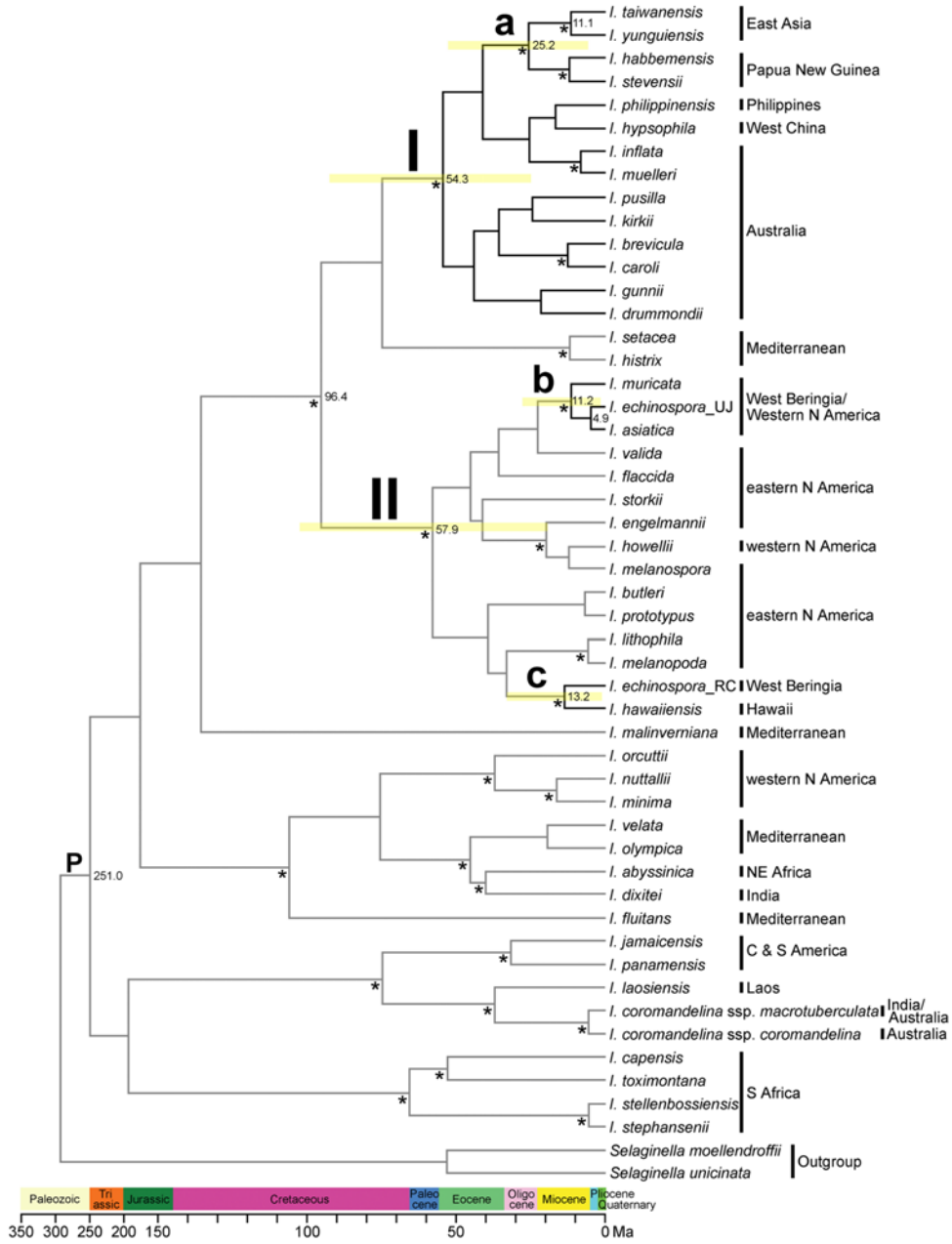

Fig. 3. Chronogram showing divergence times estimated in BEAST based on combined nrITS and cpDNA *atpB-rbcL* sequence data. Yellow bars represent 95% high posterior density for the estimated mean dates. Node labeled P is the calibration point (for more details, see Materials and Methods). An asterisk indicates branches with maximum parsimony bootstrap value >75% and posterior probability >0.90. Geological epoch is shown below the tree. The clades (I and II) and subclades (a, b, c) correspond to those in Fig. 2. Geographical origins of the *I. echinospora* accessions are indicated using the codes defined in Fig. 2.

28.5 Ma) based on the combined dataset. The divergence between Kamchatkan and Hawaiian *Isoëtes* species was estimated to be 13.2 Ma (95% HPD: 0.3-32.5 Ma) (subclade c; Fig. 3).

Biogeographical Analysis

The summary of the ancestral ranges at the nodes of major clades and subclades inferred by BBM and S-DIVA are presented in Fig. 4. Both analyses indicated that the ancestors of the species from East Asia, Papua New Guinea, the Philippines, and West China originated in Australasia (E; optimal ancestral area reconstruction for clade I in Fig. 4). The BBM reconstruction suggested Australia, followed by East Asia, as the most probable ancestral area for subclade a including species from East Asia and Papua New Guinea, whereas the

S-DIVA reconstruction for this node suggested East Asia + Australasia (CE). BBM and S-DIVA reconstructions indicated eastern North America (F) as the ancestral area for clade II. S-DIVA suggested western North America (B) or West Beringia + western North America (AB) as the most probable ancestral area for subclade b that included members of the *I. echinospora* complex from Hokkaido, Kamchatka, and western North America, whereas BBM indicated western North America (B) with 83% marginal probability (Fig. 4).

Discussion

The objective of this study was to assess the biogeographical origins of North Pacific *Isoëtes* species using nrITS and cpDNA sequence data. The individual and combined molecular analyses consistently resolved two clades: (I) the East Asian, Papua New Guinean, and Australian species, and (II) the Hokkaido (Japan), Northeastern Russian, and western North American species (Fig. 2). The phylogenetic relationships and biogeographical origins of these well-defined clades within the North Pacific *Isoëtes* species are discussed below.

The East Asian Clade

Our molecular phylogeny suggests that two diploid *Isoëtes* species (*I. taiwanensis* and *I. yunguiensis*) from East Asia form a sister group to *I. stevensii* and *I. habbemensis* from Papua New Guinea rather than to the North American or West Chinese species in the combined dataset phylogeny (subclade a in Fig. 2). The Australian species appeared as an early diverging group in clade I. The present results point to Australia and not West China as the center of origin of the East Asian species. This is reinforced by the biogeographical analysis, which identifies Australia as the ancestral region of the East Asian *Isoëtes* species in clade I with high marginal probability (Fig. 4). Moreover, two separate dispersal events from Australia to East Asia via Papua New Guinea must be inferred. These results do not support the previous hypotheses of Hoot et al. (2006) and Liu et al. (2004) who suggested that East Asian and Australian *Isoëtes* species originated in high altitudes of West China.

Two competing hypotheses have been proposed to explain the transoceanic distribution of aquatic plants, the first attributing it to dispersal and the second to vicariance (continental drift). Les et al. (2003) proposed that dispersal is the major factor accounting for the disjunctive distribution of aquatic plants. This is contrary to the viewpoint which considered vicariance as the major cause of the disjunctive distribution in aquatic taxa (e.g., Hydrocharitaceae; Chen et al. 2012). We found a transoceanic disjunction between East Asian and Australian *Isoëtes* species. Our age estimate for the node of East Asian and Papua New Guinean species is 25.2 Ma (late Oligocene epoch; subclade a in Fig. 3), followed by diversification at 11.1 Ma (late Miocene epoch) and the subsequent emergence of the monophyletic East Asian *Isoëtes* species. Thus, continental drift does not appear to have played a role in the disjunct distribution between East Asia and Australia since the great southern landmass, Gondwanaland, is thought to have broken up in the early Cretaceous (Sclater and Fisher 1974). The availability of biotic interchange between East Asia and Australia beginning at the Oligocene is too recent to support a vicariance explanation based on continental drift. Instead, relatively recent long-distance dispersal is a possible explanation for the intercontinental distribution of East Asian and Australian *Isoëtes* in clade I (Fig. 4). Intercontinental disjunctions by long-distance dispersal depend on the distance that spores can be carried (Tryon 1986). Long distance dispersal of *Isoëtes* species could be possible by waterfowl, either by plant parts and spores carried in the gut or by spore-containing mud adhering to the feathers or legs of birds (Taylor and Hickey 1992; Hoot et al. 2006). The apparent disjunction patterns between East Asian and Australian *Isoëtes* species are likely a result of long-distance dispersal mechanisms.

West Beringian and Western North American Clade

Based on the molecular phylogenetic analyses, *I. asiatica* from Hokkaido and Northeastern Russia formed a clade with *I. echinospora*_UJ and *I. muricata* from western North America, and is not sister to the East Asian species (subclade b in Fig. 2). This close relationship corroborates the previous phylogenetic analysis of *Isoëtes* species distributed in Hokkaido and Alaska inferred from AFLP markers and DNA sequence data (Kim et al. 2009a), and it holds true even with the inclusion of *Isoëtes* species from eastern North America (Fig. 3). These results indicate the unification of species within Beringia and their isolation from the *Isoëtes* species of neighboring regions (e.g., East Asia and eastern North America). The role of Beringia as a refugium or a dispersal highway between Northeastern Russia (western Beringia) and Alaska and adjacent regions of western North America (eastern Beringia) is supported by numerous studies on the relationships of various circumpolar plants (DeChine 2008; Tkach et al. 2008; Carlsen et al. 2010).

The biogeographical analysis suggests that western North America was the center of diversification and radiation for the crown clade of *I. asiatica–I. echinospora–I. muricata* to the West Beringia region, including Hokkaido and Kamchatka (subclade b; Fig. 4). The two diploid species *I. asiatica* and *I. echinospora* were proposed to have originated from southeast Siberia/Russia via allopatric speciation based on

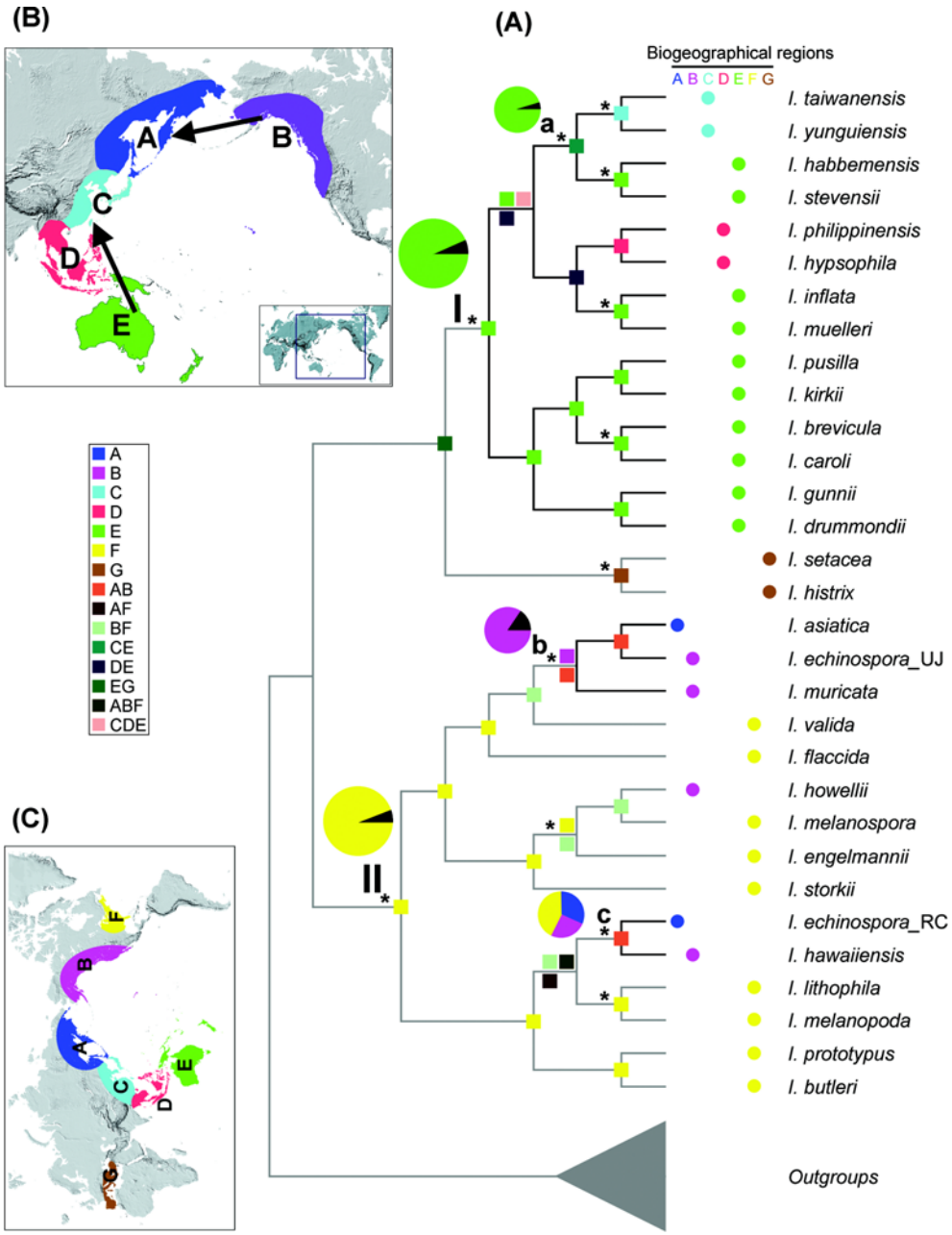

Fig. 4. (A) Bayesian Binary Method (BBM) and S-DIVA models of ancestral area reconstruction in the North Pacific *Isoëtes* species based on a reduced BEAST combined-gene chronogram. BBM ancestral area reconstructions with the highest likelihood are shown as large circles for each clade and subclade. S-DIVA ancestral area reconstructions are shown by boxes at nodes; two boxes separated by a branch indicate the ancestral ranges inherited by each of the daughter lineages arising from the node. The clades (I and II) and subclades (a, b, c) correspond to those in Fig. 2. Geographical origins of the *I. echinospora* accessions are indicated with the codes defined in Fig. 2. An asterisk indicates branches with maximum parsimony bootstrap value >75% and posterior probability >0.90. (B) Migration pathway of *Isoëtes* species in the North Pacific region based on BBM and S-DIVA. (C) Biogeographical regions used in BBM and S-DIVA: A, West Beringia; B, western North America; C, East Asia; D, South East Asia; E, Australia-Papua New Guinea; F, eastern North America; G, Mediterranean. Color key for ancestral ranges at different nodes is provided in the figure.

the distribution pattern of fossil data (Liu et al. 2004). That is, *I. asiatica* may have resulted from a dispersal event from southeast Siberia to Kuril and Hokkaido (Japan), while *I. echinospora* may have arisen from another dispersal event from southeast Siberia to western North America. However, most fossil species of *Isoëtites*, a genus related to the extant genus *Isoëtes*, are classified based on the presence of a detached sporophyll (Skog et al. 1992). Moreover, these fossil species differ considerably from the extant *Isoëtes* species. The apices of the sporophyll are spatulate and the

sporangia appear to alternate between megasporangia and microsporangia in *Isoëtites*; the megaspores of these fossils are only about four times as large as the microspores, and the corm lobes are not evident. Therefore, the biogeographical origin of the *I. echinospora* complex currently distributed in the Beringian region was likely in western North America, contrary to the proposed origin derived from fossil data.

The present results support a relatively recent origin of the crown *I. echinospora* complex, dating it to the late Miocene epoch 11.2 Ma (subclade b; Fig. 3). The lack of resolution in the molecular phylogenies between *I. asiatica* from Northeastern Russia and *I. echinospora*_UJ from Alaska indicate that they may have recently diversified (Fig. 2). The presumed recent disjunct distribution of the *I. echinospora* complex may be explained by the dispersal and migration of the complex across the Bering land bridge (BLB) and adjacent areas. The timing of the BLB in terms of opening and closing during the Tertiary has been extensively reviewed (Tiffney 1985; Wen et al. 2010). It is now known with some accuracy that the last closure of the BLB occurred 5.32 Ma (Gladenkov et al. 2002). Thus, we can infer that the BLB may have played a role in the migration of the *I. echinospora* complex from western North America to West Beringia.

The Hawaiian species, *I. hawaiiensis,* has been proposed as derived from *I. taiwanensis* or a closely related taxon based on chromosome number ($2n = 22$), megaspore ornamentation (tuberculate), and habitat (Taylor et al. 1993b). However, the results of the present study showed that *I. hawaiiensis* is a sister species of *I. echinospora*_RC from Northeastern Russia rather than of *I. taiwanensis* from Taiwan (subclade c in Fig. 2). A close relationship between *I. hawaiiensis* and *I. echinospora*, a species with a circumboreal distribution, was also supported by *LEAFY* DNA sequence data (Hoot et al. 2004). Although the biogeographical analysis does not define the ancestral region at this node, the most likely explanation for the long-distance dispersal of *I. echinospora* to Hawaii (*I. hawaiiensis*) is transport by waterfowl, as described above for clade I (Hoot et al. 2006).

The monophyly of *I. echinospora* is not supported with our sampling (Fig. 2). Similar result also presented in the previous study (Hoot et al. 2004), which did not support the monophyly of *I. echinospora*. Although we could not recognize any differences in morphological characteristics between the populations of two regions (Kamchatka and western North America), the fact that this species is genetically heterogeneous lends credence to the suggestion that it should be reevaluated with more extensive sampling of this species. Future systematic and phylogeographical studies with a broader sampling scheme at the population level are also planned to further determine the taxonomic status of *I. echinospora*_RC from the Commander Island, Kamchatka and to analyze the dispersal history and diversification of *I. echinospora*_RC and *I. hawaiiensis*.

Conclusions

This study provides a framework for testing specific biogeographical hypotheses for the North Pacific *Isoëtes* species, many of which are endemic. The molecular phylogenetic data support the hypothesis that North Pacific *Isoëtes* species can be divided into two clades: an East Asian–Papua New Guinean–Australian and a Northeast Russian–Alaskan clade. Biogeographical analyses suggest that the East Asian *Isoëtes* species most likely originated in Australia. The western North American region is the source area for the radiation of the *I. echinospora* complex toward Hokkaido, Sakhalin, and Kamchatka during recent dispersals. Our molecular phylogeny represents the most detailed molecular phylogenetic information to date relating to *I. echinospora* and illustrates the need to investigate this species more thoroughly. A comprehensive evaluation of the phylogeny and phylogeography of *I. echinospora* using molecular markers is required. *Isoëtes* species have been newly reported in South East Asia (Kim et al. 2010a; Jung et al. 2013), and the current study links these species to Gondwanaland. Future work will include additional sampling to establish the biogeographical histories of *Isoëtes* species from South East Asia, India, South America, North Australia, and Africa.

Materials and Methods

Taxon Sampling and Data Analyses

Two datasets were designed for this study. In the first phylogenetic analysis of the North Pacific *Isoëtes* species, DNA sequences of 34 accessions from 18 *Isoëtes* species from East Asia, West China, the Philippines, Northeastern Russia, Papua New Guinea, Australia, and western North America were included (Table S1). Nuclear ribosomal ITS and *atpB-rbcL* sequences were downloaded for *I. habbemensis* Alston which is distributed in Papua New Guinea, and *I. pusilla* C.R. Marsden & R.J. Chinnock and *I. kirkii* A. Braun from Australia. The western North American diploid *I. bolanderi*, which shows a strong affinity to *I. howellii* of western North America (Hoot et al. 2004), was not collected because it is listed as threatened species in Canada (COSEWIC 2006). Eight East Asian *Isoëtes* species (*I. coreana* Y.H. Chung & H.-K. Choi, *I. hallasanensis* H.-K. Choi et al., *I. jejuensis* H.-K. Choi et al., *I. japonica*, *I. sinensis*, *I.* × *michinokuana* M. Takamiya et al., *I. orientalis* H. Liu & Q.F. Wang, and *I. pseudojaponica* M. Takamiya et al.) and two western North American species (*I. maritima* and *I. occidentalis*) were excluded because they are known to be interspecific hybrids (Kim et al. 2009a, 2010b). Moreover, our preliminary study of the combined nrITS and three cpDNA (*atpB-rbcL*, *trnL*, and *trnS-psbC*) datasets indicated that East Asian polyploids formed a clade with two diploids (*I. taiwanensis* and *I. yunguiensis*). Three taxa, *I. coromandelina* L. f. ssp. *coromandelina*, *I. coromandelina* L. f. ssp. *macrotuberculata* Marsden, and *I.*

laosiensis C. Kim & H.-K. Choi, distributed in India, Australia, and South East Asia, respectively, were chosen as outgroup taxa (Marsden 1976, 1979; Kim et al. 2010a; Jung et al. 2013).

For our divergence time estimation and biogeography we analyzed North Pacific *Isoëtes* species within a broad phylogenetic framework using the second dataset. A total of 48 taxa (49 accessions) from East Asia, Australia, Africa, North America, the Mediterranean, and Central and South America were included in the molecular dating analysis using the combined nrITS and cpDNA *atpB-rbcL* data matrices. Sequences of 29 species were obtained from GenBank. Two *Selaginella* species [*S. moellendorffii* Hieron. and *S. uncinata* (Desv.) Spring] served as outgroups. The sampled taxa, localities, voucher information, and GenBank accession numbers for the nrITS and cpDNA datasets are listed in Table S1.

DNA Extraction, PCR Amplification, and Sequencing

Total genomic DNA was extracted from silica-dried plant material and herbarium specimens using a rapid DNA miniprep method (Chen and Ronald 1999). The entire nrITS region (ITS1-5.8S-ITS2) was amplified using primers ITS1 and ITS4 (White et al. 1999). The three cpDNA regions were as follows: (1) the *atpB-rbcL* intergenic spacer region, amplified using primers atpF (5'-GCTGATTTTACTGA-TAAAGATCC-3') and rbcLR (5'-TGGGTCCCTCCCTACAATTCA-3') designed for this study; (2) *trnL* (including the *trnL* gene and *trnL* intron), amplified using primers trnL2F (5'-TAGCTTTCAGATTCAG-GGAAACC-3') and trnL2R (5'-CTCCTTACTACAATTCACATTG-3') designed for this study; and (3) the *trnS-psbC* spacer region, amplified using primers trnS-psbCF22 and trnS-psbCR1106 (Kim et al. 2009a).

Genomic DNA (200 ng per 25 μL reaction) was amplified using a PTC-200 Thermal Cycler (MJ Research, Waltham, USA). The steps of the PCR program were as follows: initial denaturation for 4 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 53°C (for *atpB-rbcL* and *trnL*) or 55°C (for nrITS and *trnS-psbC*), and 1 min at 72°C; and terminal extension for 10 min at 72°C. The PCR products were purified for sequencing using a PCR Purification Kit (Qiagen, Valencia, USA) following the manufacturer's specifications. All PCR products were sequenced on an ABI3730 automated sequencer (Applied Biosystems, Foster City, USA) in both directions using corresponding PCR primer sets.

Phylogenetic Analysis

DNA Baser v.3 (http://www.DnaBaser.com) was used to evaluate chromatograms for base confirmation and to edit contiguous sequences. Multiple-sequence alignment was performed using MAFFT v.6.864 (http://www.genome.jp/tools/mafft; Kotoh and Toh 2008) with default alignment parameters. Gaps were treated as missing data. Maximum parsimony (MP) analyses of the individual and combined datasets were performed in PAUP* v.4.0b10 (Swofford 2002). All characters and character states were unordered and weighted equally. The aligned datasets used in this study are available from the online database TreeBASE (http://www.treebase.org/; study accession number, SN17717). The incongruence length difference (ILD) test was performed in PAUP* using 1000 heuristic search replications to assess congruence between the nrITS and cpDNA datasets (Farris et al. 1995). The ILD value indicated that the nrITS and cpDNA sequence data partitions were significantly incongruent ($P = 0.001$). However, after removing potentially rogue species (*I. australis* S. Williams and *I. alpina* Kirk), the data partitions were not significantly incongruent ($P = 0.068$). Furthermore, few strongly supported (BP >90) incongruent clades were found upon comparison of bootstrap consensus trees generated from the MP analyses of the two datasets. Thus, we chose to combine nrDNA and cpDNA datasets with their weakly incongruent trees, as suggested by Sheahan and Chase (2000) and Nie et al. (2008).

The MP analyses were run using a heuristic search with the following settings: tree bisection-reconnection (TBR) branch swapping; random additions with 1000 replicates; hold = 1; multrees = yes; steepest = yes; and collapse = yes. The maxtrees limit was not restricted in analyses of individual and combined cpDNA datasets, but it was set to 100,000 trees in the analyses of nrITS + cpDNA *atpB-rbcL* datasets. Bootstrap analyses (1000 pseudoreplicates) were conducted to examine the relative level of support for individual clades on the cladograms of each search (Felsenstein 1985).

Bayesian Markov Chain Monte Carlo (MCMC) inference (BI; Yang and Rannala 1997) of phylogeny was conducted with nrITS, cpDNA, and combined nrITS and cpDNA datasets using MrBayes v.3.12 (Ronquist and Huelsenbeck 2003). The selection of models of nucleotide substitution was based on the Akaike information criterion (AIC; Akaike 1974) as implemented in MODELTEST v.3.1 (Posada and Crandall 1998). The models were as follows: GTR + G (nrITS), GTR + I (cpDNA), and GTR + I + G (nrITS and cpDNA combined dataset). Four chains of the MCMC inference were run simultaneously, with sampling every 100 generations for a total of 6 million generations. The first 600,000 generations (i.e., 6000 trees) were discarded as the "burn-in" period of the chains. The log-likelihood scores of sample points were plotted against generation time using Tracer v.1.5 (Rambaut and Drummond 2003) to ensure that stationarity was achieved after the first 600,000 generations and the log-likelihood values of the sample points reached a stable equilibrium. In addition, AWTY (Nylander et al. 2008) was used to compare split frequencies from different runs and plot cumulative split frequencies to ensure that stationarity was reached. After removing the burn-in trees, the remaining trees were used to construct a 50% majority-rule consensus tree, with the proportion of bifurcations in this consensus tree given as posterior probabilities (PP) for robustness estimation of the BI trees.

Molecular Dating

BEAST v.1.5.4 (Drummond and Rambaut 2007) was used to estimate the divergence time of the clades and subclades of *Isoëtes* species. The BEAUti interface was used to generate input files for BEAST, in which a GTR + I + G model for the combined dataset was applied with a Yule speciation tree prior and an uncorrelated lognormal molecular clock model. We constrained the stem age of the genus using a lognormal prior with an offset of 245.5 Ma, a mean of 1.5, and a standard deviation of 0.5 based on the early Triassic *Isoëtes* species *I. beestonii* Retallack, which is the most ancient known species of the genus (Retallack 1997).

Posterior distributions of parameters were approximated using two independent MCMC analyses of 10,000,000 generations (sampling once every 1000 generations). Samples from the two chains, which yielded similar results, were combined after a 10% burn-in for each. The results were verified using Tracer v.1.5 (Rambaut and Drummond 2003) to ensure that plots from the two analyses converged on the same area and then combined. The samples from the posterior analysis were summarized on a maximum clade credibility tree, which had the maximum sum of posterior probabilities on its internal nodes, using TreeAnnotator v.1.5.4 (Drummond and Rambaut 2007) with the posterior probability limit set to 0.5, summarizing mean node heights. Means and 95% higher posterior densities (HPD) of age estimates were obtained from the combined outputs using Tracer.

Biogeographical Analysis

Biogeographic data for the *Isoëtes* species were compiled from herbarium specimens and the literature (Taylor et al. 1993a; Takamiya et al. 1997; Jung et al. 2009). The complete distribution range of *Isoëtes* was divided into 10 areas: (A) West Beringia, (B) western

North America, (C) East Asia, (D) South East Asia, (E) Australia–Papua New Guinea, (F) eastern North America, (G) Mediterranean, (H) Central and South America, (I) Africa, and (J) India (Table S1).

Ancestral areas reconstruction (AAR) and estimation of the spatial patterns of geographic diversification within *Isoëtes* were inferred using the Bayesian Binary Method (BBM) and Statistical Dispersal-Variance Analysis (S-DIVA) implemented in RASP v.3.1 (Reconstruct Ancestral State in Phylogenies, formerly S-DIVA; http://mnh.scu.edu.cn/soft/blog/RASP; Yu et al. 2015). The BBM was run with the fixed state frequencies model (Jukes-Cantor) with equal among-site rate variation for two million generations, 10 chains each, and two parallel runs. In S-DIVA, the frequencies of an ancestral range at a node in ancestral reconstructions are averaged over all trees (Yu et al. 2015). For these analyses, we used trees (the last 9000 of 10,000 trees, excluding burn-in) obtained from the BEAST MCMC output from the combined nrITS and cpDNA *atpB-rbcL* dataset. The consensus tree that is used for mapping of the ancestral distribution on each node was obtained with the Compute Condense option in RASP from the 9000 stored trees. The number of maximum areas was constrained to four because no species currently occurs in more than four areas.

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Author's Contributions

H-KC conceived and designed the experiments. CK and H-KC performed the experiments and analyzed the data. CK wrote the draft and H-KC revised the draft. All authors agreed on the contents of the paper and declared that no competing interests exist.

Supporting Information

Fig. S1. Strict consensus of 936 most parsimonious trees from parsimony analysis of the nrITS sequence data.
Fig. S2. Strict consensus of 63 most parsimonious trees from parsimony analysis of the combined cpDNA sequence data.
Table S1. Species, voucher with collection and locality data, and GenBank accession number for taxa included in this study.

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