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ORIGINAL RESEARCH

Systematic Evaluation of *Isoëtes asiatica* Makino (Isoëtaceae) based on AFLP, nrITS, and Chloroplast DNA Sequences

Changkyun Kim · Hye Ryun Na · Hyunchur Shin · Hong-Keun Choi

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Abstract On the basis of amplified fragment length polymorphism (AFLP) and nucleotide sequence data from nuclear ribosomal internal transcribed spacer (nrITS) and three chloroplast DNA regions (*rbc*L, cpITS, and *trn*S-*psb*C spacer), we investigated the species delimitation and the evolutionary lineage of *Isoëtes asiatica* from Hokkaido, Japan. The neighbor-joining (NJ) dendrogram based on AFLP markers revealed the well-defined clusters (bootstrap value=100%) of *I. asiatica*. Results from the principal component analysis are largely congruent with those obtained in the NJ dendrogram. The maximum parsimony analysis, based on data from nrITS and three chloroplast DNA sequences, supported a monophyly of three species, *I. asiatica*, *Isoëtes echinospora*, and *Isoëtes maritima* from Hokkaido, Kamchatka, and Alaska regions. The distinct species status of *I. asiatica* was also well supported in the combined chloroplast DNA phylogeny. Therefore, *I. asiatica* appear to represent example of gradual speciation due to spatial isolation of ancestral populations followed by genetic divergence. Our results also suggest that *I. asiatica* is probably not the ancestral diploid of the polyploids occurring in East Asia.

Keywords AFLP · Chloroplast DNA · *Isoëtes* · *I. asiatica* · Molecular phylogeny · nrITS

C. Kim · H. R. Na · H.-K. Choi (✉)
Department of Biological Science, Ajou University,
Suwon 443-749, Republic of Korea
e-mail: hkchoi@ajou.ac.kr

H. Shin
Department of Biology, Soonchunhyang University,
Asan 336-745, Republic of Korea

Introduction

Isoëtes asiatica Makino is the only diploid (2*n*=22) species among the six Japanese taxa (Makino 1904; Takamiya et al. 1994, 1997). This species is a small submerged plant growing in higher elevation lakes and ponds from central to northern Honshu and Hokkaido, although its distribution range extends to Sakhalin and Kamchatka (Pietsch 1991; Kuvaev 1993; Takamiya et al. 1997). Because *I. asiatica* differs from the other Japanese polyploid taxa (e.g., *I. sinensis* and *Isoëtes japonica*) with regard to its echinated ornamentation of megaspore, habitats, and geographical distribution pattern, *I. asiatica* has not been regarded as an ancestral diploid for the other Japanese taxa (Watanabe et al. 1996; Takamiya et al. 1997). Furthermore, *I. asiatica* was considered to be closely related to the Alaska–Aleutian *Isoëtes echinospora* Dur. complex (Love 1962) and treated as *I. echinospora* ssp. *asiatica* (Makino) Love on the basis of the chromosome number (2*n*=22) and megaspore surface ornamentation (Love 1962). Although this broad delimitation of *I. echinospora*, with the inclusion of *I. asiatica*, is relatively well defined on the basis of spore morphology and cytological studies (Love 1962; Takamiya et al. 1994, 1996; Watanabe et al. 1996), its molecular phylogenetic relationship has not been addressed.

The aim of the present study is to evaluate the phylogenetic relationship of *I. asiatica* found in the Hokkaido and Kamchatka regions using amplified fragment length polymorphism (AFLP), sequences of nrITS, and chloroplast DNA markers. Specifically, we ask the following: (1) Should *I. asiatica* be considered a separate species? (2) Is *I. asiatica* of Hokkaido–Kamchatka closely related to the Alaska–Aleutian *I. echinospora* complex instead of *Isoëtes* species from East Asia as hypothesized in morphological and cytological literature? (Love 1962; Takamiya et al. 1997).

Materials and Methods

Plant Materials

For AFLP analysis, leaf samples from a total of 42 individual plants were collected from three species (*I. asiatica, I. echinospora*, and *Isoëtes maritima*) from Hokkaido (Japan) and Alaska (North America). All voucher specimens are deposited in the herbarium of Ajou University. Identification of each species followed Makino (1904), Watanabe et al. (1996), and Britton et al. (1999) and was based on spore wall ornamentation and chromosome numbers. Mega- and microspore ornamentation was observed with a Jeol JSM-6380 scanning electron microscope (Choi et al. 2008). Chromosome counts were determined as described by Kott and Britton (1980). Full details are provided in Table 1. We also included one individual of *I. asiatica* from Kamchatka (Russia) and six *Isoëtes* species of East Asia in the phylogenetic analysis using nrITS and three chloroplast DNA sequence data (Table 1). Additionally, sequences of 20 *Isoëtes* species taken from GenBank (http://www.ncbi.nlm.nih.gov) were included for nrITS phylogeny ("Appendix 1").

DNA Extraction and AFLP Analysis

Total DNA was extracted from 0.5 g of fresh leaves by a rapid DNA minipreparation method (Chen and Ronald 1999). Extracted DNA was dissolved in Tris–EDTA buffer (10 mM Tris–HCl, pH8.0 and 1 mM EDTA, pH8.0); the DNA concentration of each sample was determined with a spectrophotometer (Geneflow Ltd., Staffordshire, UK). AFLP analysis was performed as described by Vos et al. (1995), and the procedure is outlined in Kim et al. (2009). To illustrate the genetic relationship among *I. asiatica*, *I. echinospora*, and *I. maritima*, we analyzed the matrix of AFLP bands with neighbor joining (NJ) and principal component analysis (PCA). For details, see Kim et al. (2009).

DNA Amplification and Sequencing

The nrITS regions, including the 5.8S gene, were amplified by PCR with the use of the primer ITS1 located in the 18S region and ITS4 located in the 28S region (White et al. 1990). Twenty-five microliters containing 200 ng of genomic DNA was amplified in the PTC 200 according to the following PCR program: 4 min at 94°C, followed by

Table 1 Collection data, spore ornamentation, and chromosome number of plant material used in this study

Species	Voucher collection	Abbreviation	Megaspore[a]	Microspore[a]	Chromosome number (2n)	Number of individuals		
						AFLP	nr ITS	cp DNA
I. asiatica	Hokkaido, Japan. *J Jung 45* (AJOU)	IAH	Echinate	Laevigate	22	10	10	10
	Kamchatka, Russia. *VL Komarov sn* (K)	IAK	Echinate	Laevigate	NA	NA	1	1
I. echinospora	Alaska, USA. *H-K Choi 2008-1* (AJOU)	IEJ	Densely echinate	Laevigate	22	15	15	15
I. maritima	Alaska, USA. *H-K Choi 2008-16* (AJOU)	IMC	Echinate–cristate	Aculeate	44	17	6 (53)[b]	17
I. coreana	Jeonranam-do, Korea. *C Kim 2006-73* (AJOU)	ICK	Cristate	Echinate	66	NA	1	1
I. taiwanensis	Taipei, Taiwan. *H-K Choi 2007-17* (AJOU)	ITT	Tuberculate	Echinate	22	NA	1	1
I. hallasanensis	Jeju-do, Korea. *H-K Choi 2006-114* (AJOU)	IHK	Echinate	Echinate	44	NA	1	1
I. jejuensis	Jeju-do, Korea. *H-K Choi 2006-104* (AJOU)	IJK	Rugulate	Echinate	44	NA	1	1
I. japonica	Honshu, Japan. *H-K Choi 2007-116* (AJOU)	IJJ	Reticulate	Laevigate	66	NA	1	1
I. sinensis	Wuhan, China. *H-K Choi* and *HR Na 2005-94* (AJOU)	ISC	Cristate	Echinate	44	NA	1	1
Total						42	38 (85)	49

AJOU Ajou University Herbarium, *K* KEW Herbarium, *NA* not applicable for the determination of chromosome number and AFLP marker

[a] Classification of mega- and microspore ornamentation followed by Hickey (1986) and Musselman (2003), respectively

[b] The number of cloned sequences of *I. maritima* used in nr ITS analysis is given in parentheses

30 cycles of 1 min at 94°C (denaturation), 1 min at 55°C (annealing), and 1 min at 72°C (extension), and a final extension step of 10 min at 72°C. Sequencing of the nrITS PCR products from the two diploid species *I. asiatica* and *I. echinospora* were performed on both 5′ and 3′ DNA strands with the use of the amplification primers ITS1 and ITS4 (White et al. 1990). Owing to ambiguity of the nrITS sequences for the tetraploid species *I. maritima*, we cleaned up the PCR products from six individuals using the Qiaquick Gel Extraction kit (Qiagen, Inc.) and then cloned the products into a TOPcloner TA V2 vector (Enzynomics Co.). Ligation of the purified nrITS PCR products and subsequent transformation were done according to manufacturer's protocols (Enzynomics Co.). We sequenced six to 12 clones for each individual to recover as many heterogeneous sequences as possible. Plasmid DNAs were purified with the Qiaprep Spin Miniprep kit (Qiagen, Inc.) and sequenced on an ABI3730 automated sequencer (Applied Biosystems).

Three chloroplast regions were amplified and sequenced in 49 individuals from nine species as follows (see Table 1 for information about the species): (1) the *rbc*L region, (2) the internal transcribed spacer of cp DNA (cpITS; Goremykin et al. 1996), and (3) the *trn*S-*psb*C spacer region (Demesure et al. 1995).

For PCR amplification of the *rbc*L gene, two synthetic primers, *Isorbc*LF and *Isorbc*LR, were designed according to the reported sequences of *Isoëtes australis* (GenBank accession no. DQ294240) and *Isoëtes coromandelina* (GenBank accession no. DQ294242). The cpITS between the 23S, 4.5S, and 5S rRNA genes was amplified and sequenced with the use of primers described previously (Goremykin et al. 1996). The *trn*S-*psb*C spacer region was amplified with the use of a pair of universal primers (Demesure et al. 1995). In addition, *trn*S-*psb*CF22 and *trn*S-*psb*CR1106 were used as internal sequencing primers (see "Appendix 2" for primer sequences).

PCR amplification was performed with a PTC 200 thermocycler (MJ research, Watertown, MA, USA) with the following parameters: initial denaturation for 4 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C (*rbc*L and *trn*S-*psb*C spacer region) or 58°C (cpITS), and 1 min 30 s at 72°C, ending with a 10 min extension step at 72°C.

Amplified DNA samples were analyzed by electrophoresis on 1.4% agarose gel run in a 0.5× TBE buffer and detected by ethidium bromide staining. The PCR products were then purified with the use of the Qiaquick Gel Extraction kit (Qiagen, Inc., Valencia, CA, USA) and directly sequenced with the ABI PrismTM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Norwalk, CT, USA) and the ABI3730 automated sequencer (Applied Biosystems).

Phylogenetic Analysis

Consensus sequences were assembled for each individual by the Codoncode Aligner software (http://www.codoncode.com). Multiple-sequence alignment was done by the Clustal X program (Thompson et al. 1997). Sequence alignment was checked and edited by hand to minimize software artifacts. The boundaries of the nrITS were determined by comparison to already known sequences (Hoot et al. 2006; Schuettpelz and Hoot 2006). Gaps were excluded from the alignment but each indel was recorded as a binary character according to the "simple gap coding" method (Simmons and Ochoterena 2000). Sequence divergences were estimated on the basis of pairwise genetic distance (*p*-distance). The phylogenetic reconstruction of the sequences was performed by maximum parsimony (MP) methods with the PAUP* program 4.0 (Swofford 2002), and all characters were coded as having equal weight. Most parsimonious trees were searched with a heuristic algorithm comprising tree bisection-reconnection, branch swapping, MULPARS, and the alternative character state. Strict consensus trees were constructed from most of the parsimonious trees. Bootstrap analyses (1,000 pseudoreplicates) were conducted to examine the relative level of support for individual clades on the cladograms of each

Table 2 Polymorphic bands generated by each of three pairs of primers in three *Isoëtes* species from Hokkaido and Alaska regions

No.	Primer pairs	Primer sequence (5′–3′)	Total bands	Polymorphic bands	Polymorphism (%)
1	E-CAAC/M-ACTG	GACTGCGTACCATTCAAC/ GATGAGTCCTGAGTAACTG	192	183	95.3
2	E-CACT/M-ACTC	GACTGCGTACCATTCACT/ GATGAGTCCTGAGTAACTC	201	194	96.5
3	E-CATG/M-ACGT	GACTGCGTACCATTCATG/ GATGAGTCCTGAGTAACGT	192	189	98.4
Mean			195.0	188.7	96.8
Total			585	566	–

Table 3 Genetic distance matrix based on AFLP markers using pairwise estimated values of *p*-distance within and between three species in Hokkaido and Alaska regions

Species	*I. asiatica*	*I. echinospora*	*I. maritima*
I. asiatica	0.292 (0.033)		
I. echinospora	0.296 (0.030)	0.102 (0.039)	
I. maritima	0.373 (0.018)	0.271 (0.021)	0.061 (0.018)

Standard deviations are indicated in parentheses

search (Felsenstein 1985). Five parsimony analyses were performed: an analysis of the nrITS, the *rbc*L region, cpITS, *trn*S-*psb*C spacer region, and a combined analysis of the three chloroplast DNA regions. Before combining the three chloroplast data sets, congruence was assessed by comparing the MP bootstrap trees for each data set and by conducting the partition homogeneity test (Farris et al. 1995) with the PAUP* program and 1,000 heuristic search replications. All of the sequences used in this study are available on the GenBank ("Appendix 1").

Results

AFLP Analysis

We scored 585 fragments, of which 566 (96.8%) were polymorphic across 42 individuals of three *Isoëtes* species

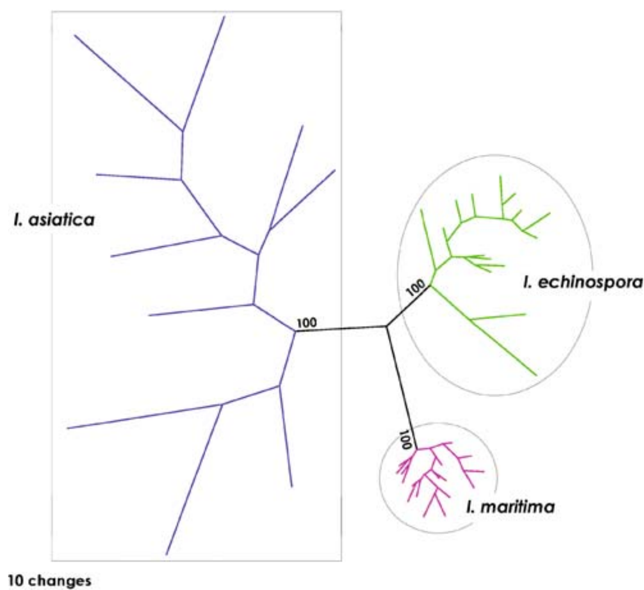

Fig. 1 Neighbor-joining dendrogram of 42 individuals from three *Isoëtes* species in Hokkaido and Alaska based on 566 polymorphic AFLP markers generated using PAUP* 4.0 (Swofford 2002). Individuals of *Isoëtes* species form unique clusters according to their group based on taxonomy. Numbers associated with nodes indicate significant support from bootstrap value (≥80%)

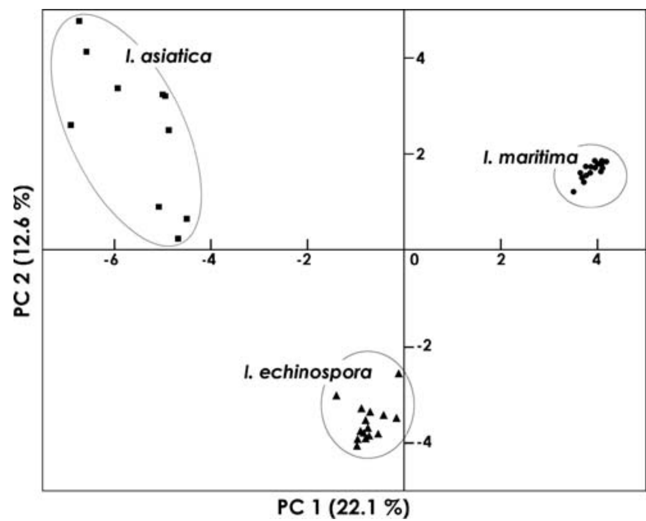

Fig. 2 Relationship of the 42 individuals from three *Isoëtes* species visualized by PCA of AFLP markers. The two first axes represent 34.9% of the total variation

in Hokkaido and Alaska (Table 2). All the sampled individuals could be distinguished by distinct AFLP phenotypes. The number of polymorphic fragments generated had a similar range in the different primer pairs, from 183 by E-CAAC/M-ACTG to 194 by E-CACT/M-ACTC. Furthermore, the percentage of polymorphic bands from the three different primer pairs was similar (Table 2). Of the 566 polymorphic bands, 230 were specific for *I. asiatica*, 43 for *I. echinospora*, and 39 for *I. maritima*.

The mean genetic distance (*p*-distance) and standard deviation between species were calculated (Table 3). Genetic distances among the three species ranged from 0.271 between *I. echinospora* and *I. maritima* to 0.373 between *I. asiatica* and *I. maritima*.

In the NJ analysis, three major clusters were separated with the highest bootstrap support (100%). Overall, three distinct clusters were revealed by group-based taxonomy. However, individuals within each species were shown to have no subgroup (Fig. 1). In our PCA analysis, the first two principal components accounted for 22.1% and 12.8% of the total variance, respectively. In the PCA performed for each of the individual of species, the same groups as in results from the NJ method are revealed (Fig. 2).

nrITS Analysis

The nrITS data set (ITS1+5.8S+ITS2) comprising 42 accessions from 29 species contains 696 characters, including 47 simple gap coding characters; 279 characters were variable and 217 were phylogenetically informative. The nrITS sequence data of all the individual plants of *I. echinospora* were identical. However, relatively higher

levels of nucleotide diversity were observed in *I. asiatica* (*p*-distance, 0.000–0.005).

Individuals of *I. asiatica* from Hokkaido and Kamchatka were separated into three distinct sequence types: (1) one type (IAH1, 2, 4, 8, and 9) was identical to *I. echinospora*, (2) another type comprised the individuals (IAH 3, 5, 7, 10, and IAK), and (3) the third type included one individual (IAH6). The 11 sequence types were detected in 53 clones from six individuals of *I. maritima*. These 11 types showed two distinct sequence groups, i.e., one group (types 1–5; *maritima[A]*) forming a clade with *I. echinospora* and *I. asiatica* and the other group (types 6–11; *maritima[B]*; Fig. 3 and "Appendix 1" for types information of *I. maritima*).

The 16 equal MP cladograms were generated with a total length of 405 steps, a consistency index (CI) of 0.817, a retention index (RI) of 0.917, and a rescaled consistency index (RC) of 0.750. The strict consensus tree is shown in Fig. 3 with bootstrap percentages. Three species, *I. asiatica*, *I. echinospora*, and some accessions of *I. maritima*, formed a clade (but with moderately supported [bootstrap value=73%]). This monophyletic group was a strong affinity to *Isoëtes hawaiiensis* from North America (bootstrap value=100%) instead of East Asian *Isoëtes* species (Fig. 3).

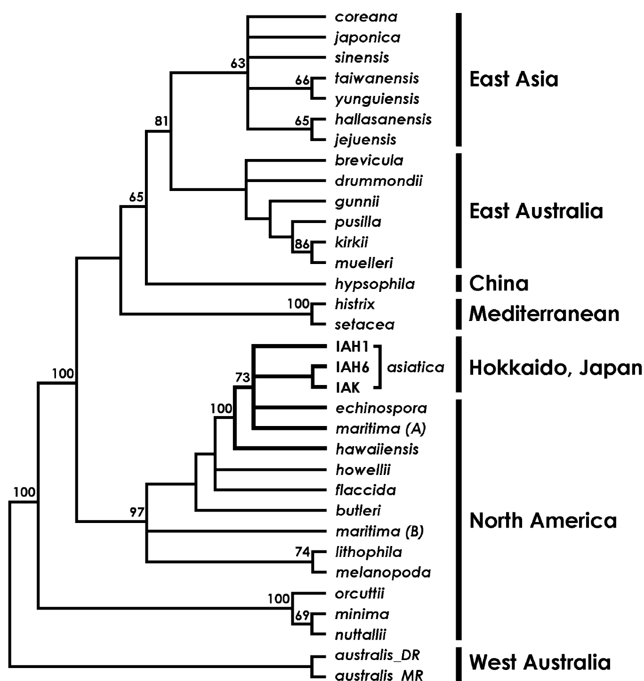

Fig. 3 Strict consensus tree of 16 most parsimonious trees inferred from nrITS sequence data. Tree length is 405 steps of equally weighted nucleotide substitutions and gaps, CI=0.817, RI=0.917. NrITS phylogeny recognized *I. asiatica* as having a strong affinity with *I. echinospora*, *I. maritima*, and *I. hawaiiensis* in North America (*bold lines*). *Numbers above branches* are bootstrap values (≥60%). Population abbreviations of *I. asiatica* are given Table 1

Chloroplast DNA Analysis

On the basis of the three regions of chloroplast DNA encompassing 2,571 bp in length, 22 substitutions were detected among 49 individuals from nine *Isoëtes* species (Table 4). However, all the individuals within each of the three species (*I. asiatica*, *I. echinospora*, and *I. maritima*) had identical sequences in three cp DNA regions. Of the three cp DNA regions surveyed, two regions showed no polymorphism among *I. asiatica*, *I. echinospora*, and *I. maritima*. However, the *trn*S-*psb*C spacer region contained two substitutions: one was T/A at site 869 and the other was C/A at site 959 between *I. asiatica* and two species of Alaska (Table 4).

The four data sets subjected to phylogenetic analysis were (1) the *rbc*L gene data set, 998 bp in length; (2) cpITS data set, 588 bp in length; (3) *trn*S-*psb*C spacer data set, 985 bp in length; and (4) the combined data set, 2,571 bp in length containing 49 individuals from nine species. The parameters of the most parsimonious trees obtained from the four data sets are presented in Table 5. All four MP analyses resulted in a single most parsimonious tree with CI=RI=1.00 (Table 5). Four MP trees support the monophyly of the three species, *I. asiatica*, *I. echinospora*, and *I. maritima* from Hokkaido, Kamchatka, and Alaska regions. Furthermore, the phylogenetic tree based on the combined data set, as determined by the MP method, reveals that *I. asiatica* is divergent from two Alaskan species, *I. echinospora* and *I. maritima* (Fig. 4).

Discussion

Because of the conserved morphology among *Isoëtes* species, species delimitation and inferring phylogenetic relationship have been problematic (Hickey 1986; Taylor and Hickey 1992). Morphology provides few characteristics (e.g., spore surface ornamentation) that can be used to determine the species boundaries and to reconstruct phylogenetic relationships (Kott and Britton 1983; Hickey 1986; Britton et al. 1999). Therefore, a number of systematists have used various forms of DNA fragment analysis (e.g., RAPD and AFLP) or DNA sequence data to solve the problems of species delimitation, phylogenetic relationships, and the determination of hybrid origins in the genus *Isoëtes* (Hoot and Taylor 2001; Taylor et al. 2004; Hoot et al. 2006; Kim et al. 2009). It is known that the topologies derived from different genes sometimes differ from each other (Cao et al. 1994). Because analyses of single genes can lead to an erroneous tree, it is important to carry out analyses that are based on as many different genes as possible and to synthesize the results. Our phylogenetic analysis included nrITS and three cp gene sequences and is therefore considered to provide a more reliable phyloge-

Table 4 Distribution of polymorphic sites in the *rbc*L, ITS, and *trnS*-*psbC* spacer region of chloroplast DNA

Species	*rbc*L								cpITS	*trnS*-*psbC*											
	65	386	504	560	592	616	813	908	79	9–10	109	141	305	473	629	642	693	706	869	871	959
IAH	*T*	*C*	*C*	*C*	*C*	*C*	T	*T*	*T*	GA	*C*	*A*	*T*	G	A	A	A	*C*	*T*	C	*C*
IAK	*T*	*C*	*C*	*C*	*C*	*C*	T	*T*	*T*	GA	*C*	*A*	*T*	G	A	A	A	*C*	*T*	C	*C*
IEJ	*T*	*C*	*C*	*C*	*C*	*C*	T	*T*	*T*	GA	*C*	*A*	*T*	G	A	A	A	*C*	A	C	A
IMC	*T*	*C*	*C*	*C*	*C*	*C*	T	*T*	*T*	GA	*C*	*A*	*T*	G	A	A	A	*C*	A	C	A
ICK	C	T	A	T	T	T	T	C	C	GA	G	G	C	G	A	A	A	A	A	C	A
ITT	C	T	A	T	T	T	T	C	C	GA	G	G	C	G	A	A	A	A	A	C	A
IHK	C	T	A	T	T	T	T	C	C	GA	G	G	C	G	A	A	A	A	A	C	A
IJK	C	T	A	T	T	T	T	C	C	GA	G	G	C	G	A	A	A	A	A	T	A
IJJ	C	T	A	T	T	T	G	C	C	GA	G	G	C	A	A	A	A	A	A	C	A
ISC	C	T	A	T	T	T	T	C	C	TC	G	G	C	G	T	T	T	A	A	C	A

Numbers refer to the positions in the aligned matrix of *rbc*L, cpITS, and *trnS*-*psbC* regions. Data in italics represented the phylogenetically informative substitution for *I. asiatica* (IAH and IAK), *I. echinospora* (IEJ), and *I. maritima* (IMC)

netic relationship of *Isoëtes* species from Hokkaido, Kamchatka, and Alaska regions.

I. asiatica is easily distinguished from other East Asian species by several morphological characters (Table 1), including two-lobed corm, the presence of velum, and echinate megaspore (Watanabe et al. 1996; Takamiya et al. 1997). The distinct species status of *I. asiatica* is also supported by AFLP analysis of six *Isoëtes* species from East Asia, including *Isoëtes taiwanensis*, *Isoëtes coreana*, *Isoëtes jejuensis*, *Isoëtes hallasanensis*, *I. japonica*, and *I. asiatica* species (Kim et al. 2009). Although *I. asiatica* is similar to the *I. echinospora* complex, which is a group of circumpolar taxa with echinate megaspores and uniformly $2n=22$ (Love 1962), *I. asiatica* can be distinguished from *I. echinospora* in having a broader velum that covers two thirds to three fourths of the sporangium, coarser spinules on the megaspores, and smoother microspores (Makino 1904). Because the taxon appears morphologically distinct, it is often treated as a subspecies or species (Makino 1914; Love 1962). In this study, we found that *I. asiatica* has a higher number of specific AFLP markers than *I. echinospora* and *I. maritima*, suggesting that the gene flow between the Japanese population of *I. asiatica* and the Alaskan populations of *I. echinospora* and *I. maritima* is very limited. In NJ dendrogram and PCA analysis based on AFLP markers among *I. asiatica*, *I. echinospora*, and *I. maritima* from Hokkaido and Alaska regions, the three taxa showed distinct clustering according to group-based taxonomy (Figs. 1 and 2). Therefore, *I. asiatica* is morphologically distinct and AFLP analysis indicates that this species is also genotypically distinct.

Although sampling within Kamchatka population of *I. asiatica* was not enough to draw any phylogenetic relationship within this species, one individual of *I. asiatica* from Kamchatka has a virtually identical nrITS and three chloroplast DNA sequences to those found in Hokkaido population (Figs. 3 and 4). These results suggest that recent long dispersal between populations of *I. asiatica* in different areas within suitable habitats has occurred. The most likely explanation for the long distance dispersal of *I. asiatica* is transport by means of waterfowl, either by spores carried in the gut or by spore containing mud adhering to the feathers or legs of birds. Indeed the dispersal of *Isoëtes* species has occurred not only by the movement of water but also by means of animal (Taylor and Hickey 1992; Liu et al. 2004; Hoot et al. 2006; Chen et al. 2008).

On the basis of spore morphology, habit, and habitat, Huang et al. (1992) proposed that the diploid species *I. taiwanensis* of Taiwan is probably close to *I. asiatica*. Furthermore, Liu et al. (2004) suggested that *I. asiatica* is the parental species for hexaploid *I. japonica* of Japan based on their distribution patterns and chromosome

Table 5 Sequence characteristics and information about the most parsimonious tree (MPT) obtained from parsimony analyses

Data set	*rbc*L	cpITS	*trn*S-*psb*C	Combined
Number of taxa (individuals)	9 (49)	9 (49)	9 (49)	9 (49)
Total characters	998	588	985	2571
Number of informative characters	7	1	6	14
Number of MPT	1	1	1	1
Number of steps	8	1	13	22
Consistency index (CI)[a]	1.00	1.00	1.00	1.00
Retention index (RI)	1.00	1.00	1.00	1.00

[a] The consistency index is calculated excluding uninformative characters

numbers. However, our nrITS and cp phylogeny showed that *I. asiatica* formed a monophyletic group with *I. echinospora* and *I. maritima* from Alaska instead of *Isoëtes* species from East Asia (Figs. 3 and 4). That is, our phylogenetic analyses recognized at least two lineages in East Asia (i.e., *I. asiatica* and the remaining East Asian species). Therefore, we do not concur with Liu et al. (2004) in proposing that *I. asiatica* is probably the ancestral diploid of the polyploids occurring in East Asia.

The well-supported sister relationship of *I. asiatica* and North American *Isoëtes* species raises interesting biogeographical questions. Based on fossil records, Liu et al. (2004) suggested that *I. asiatica* might be an intermediate species between East South Siberia and North American species and more ancient that *I. echinospora*. However, it is a more parsimonious assumption that *I. asiatica* should have been derived from North America (Fig. 3) rather than *I. echinospora* that originated in East Asia. This phylogeographical scenario is offered as a hypothesis that needs further testing. Future work will include additional sampling from East South Siberia and western USA, as well as the use of analytical methods to provide a temporal framework for the diversification of *Isoëtes* species.

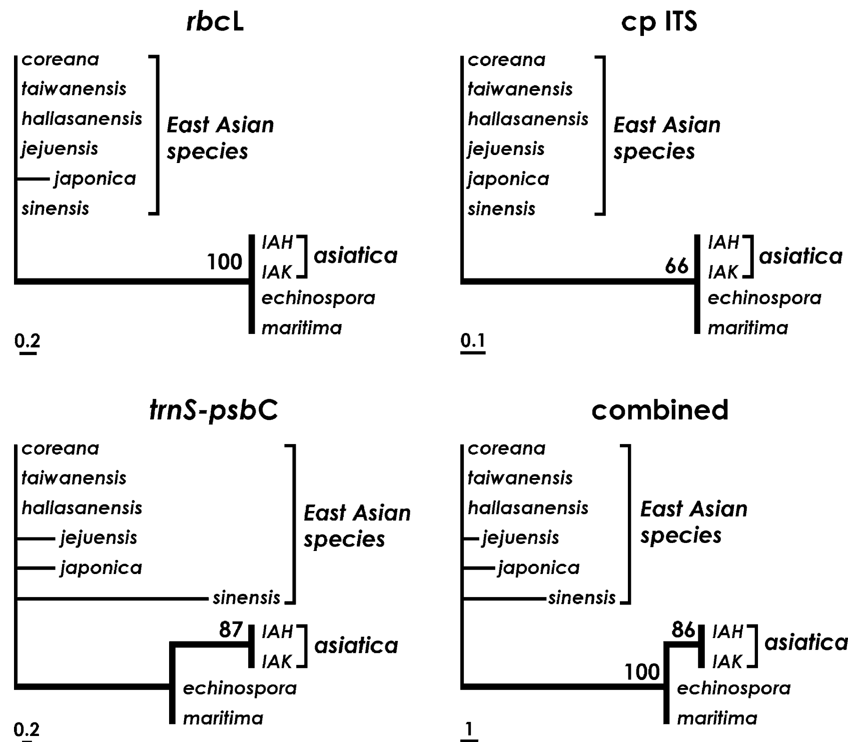

Fig. 4 The single most parsimonious tree derived from the *rbc*L, cpITS, and *trn*S-*psb*C spacer region and the combined three regions of data for chloroplast DNA (see Table 5 for tree information). On the basis of all four chloroplast DNA sequence analyses, *I. asiatica* of Hokkaido–Kamchatka formed a sister group with *I. echinospora* and *I. maritima* of Alaska (*bold lines*). *Numbers above the branches* are bootstrap values (≥60%). Branch lengths are proportional to number of substitutions. Population abbreviations of *I. asiatica* are given in Table 1

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Appendix 1

GenBank accession numbers for *Isoëtes* DNA sequences used in this manuscript

Species	Abbreviation	Accession number			
		nrITS	cp DNA		
			*rbc*L	cpITS	*trn*S-*psb*C
I. asiatica	IAH1	**FJ785161**	**FJ785182**	**FJ785192**	**FJ785196**
	IAH6	**EU348559**			
	IAK	**FJ785162**	**FJ785183**	**FJ785193**	**FJ785197**
I. echinospora	IEJ	**FJ785160**	**FJ785184**	**FJ785194**	**FJ785198**
I. maritima	IMC_Type 1	**FJ785166**	**FJ785185**	**FJ785195**	**FJ785199**
	IMC_Type 2	**FJ785167**			
	IMC_Type 3	**FJ785163**			
	IMC_Type 4	**FJ785164**			
	IMC_Type 5	**FJ785165**			
	IMC_Type 6	**FJ785172**			
	IMC_Type 7	**FJ785173**			
	IMC_Type 8	**FJ785170**			
	IMC_Type 9	**FJ785171**			
	IMC_Type 10	**FJ785168**			
	IMC_Type 11	**FJ785169**			
I. australis		DR, DQ284989			
		MR, DQ284990			
I. brevicula		AY641098			
I. butleri		DQ479974			
I. coreana		EU348548	**FJ785176**	**FJ785186**	EU348498
I. drummondii		DQ284993			
I. flaccida		DQ479979			
I. gunnii		DQ479980			
I. hallasanensis		EU348554	**FJ785178**	**FJ785188**	EU348504
I. hawaiiensis		DQ479982			
I. histrix		DQ284994			
I. howellii		DQ479983			
I. hypsophila		AY641099			
I. japonica		EU348558	**FJ785180**	**FJ785190**	EU348508
I. jejuensis		EU348551	**FJ785179**	**FJ785189**	EU348501
I. kirkii		AY641100			
I. lithophila		DQ479986			
I. melanopoda		DQ284996			
I. minima		DQ479989			
I. muelleri		DQ479990			
I. nuttallii		DQ284997			
I. orcuttii		DQ284998			
I. pusilla		DQ479993			
I. setacea		DQ285000			
I. sinensis		EU348563	**FJ785181**	**FJ785191**	EU348513
I. taiwanensis		EU348561	**FJ785177**	**FJ785187**	EU348511
I. yunguiensis		GQ175877			

New sequences determined in this study are in bold

Appendix 2

Primer sequences and their sources

Primers	Sequences (5′–3′)	Sources
ITS1	GGAAGTAAAAGTCGTAACAAGG	White et al. 1990
ITS4	TCCTCCGCTTATTGATATGC	White et al. 1990
CprbcLF	AGACACCGATATTCTGGCGGCGTT	This study
CprbcLR	GGTGTCCCAAAGTTCCACCACCGA	This study
cpITS2	CCGGATAACTGCTGAAAGCATC	Goremykin et al. 1996
cpITS3	TCCTGGCGTCGAGCTATTTTTCC	Goremykin et al. 1996
*trn*S	GGTTCGAATCCCTCTCTCTC	Demesure et al. 1995
*psb*C	GAGAGAGGATTCGAACC	Demesure et al. 1995
*trn*S-*psb*CF22	TTCCGTCGAAGGATCATCGCAT	This study
*trn*S-*psb*CR1106	TTGCTAGGTGTTGGTGCTCTC	This study