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Research Article

New insights into the phylogeny and biogeography of subfamily Orontioideae (Araceae)

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Abstract Proto-Araceae, the earliest diverged lineage within the family Araceae, includes two subfamilies, Gymnostachydoideae (one species) and Orontioideae (eight species). Based on an extensive sampling (a total of 198 accessions) of six chloroplast non-coding regions (5799 aligned sites), we assessed phylogenetic relationships among the genera and species within subfamily Orontioideae and estimated the timing of intercontinental disjunct events in the Northern Hemisphere. Overall phylogenetic relationships among the genera were consistent with results from previous studies, but several new important findings were discovered, primarily within *Symplocarpus* Salisb. ex W. P. C. Barton. First, two major lineages within *Symplocarpus* were identified: one lineage included *S. foetidus* (L.) Salisb. ex W. Barton, *S. nabekuraensis* Otsuka & K. Inoue, and *S. renifolius* Schott ex Tzvelev (Japan), whereas the other included *S. nipponicus* Makino, *S. egorovii* N. S. Pavlova & V. A. Nechaev, and *S. renifolius* (Korea). *Symplocarpus renifolius* in Japan was tetraploid and closely related to the tetraploid *S. foetidus* in eastern North America. Populations of *S. renifolius* in Korea were confirmed to be diploid ($2n = 30$) and shared the most recent common ancestor with the other diploid species, *S. nipponicus*. Second, two recently described species, *S. nabekuraensis* and *S. egorovii*, were deeply embedded within *S. renifolius* in Japan and Korea, respectively, and their distinct taxonomic status requires further assessment. Finally, two intercontinental disjunction events in the subfamily, one in *Lysichiton* Schott between eastern Asia and western North America and the other in *Symplocarpus* between eastern Asia and eastern North America, were estimated to be between 4.5 and 1.4 million years ago (Pliocene and Pleistocene) and between 1.9 and 0.5 million years ago (Pleistocene), respectively.

Key words: chromosome number, intercontinental disjunction, *Lysichiton*, Orontioideae, proto-Araceae, *Symplocarpus*.

1 Introduction

In the Northern Hemisphere, four major areas are known to be involved with the intercontinental disjunct distributions of plant species: eastern Asia (EA), western North America (WNA), eastern North America (ENA), and Europe (EU) (Milne & Abbott, 2002; Donoghue & Smith, 2004). Of these four areas of disjunct distribution, the one between EA and ENA has attracted the most interest of phytogeographers and evolutionary biologists, and consequently numerous studies have been conducted since the mid-19th century (e.g., Raven, 1972; Raven & Axelrod, 1974; Boufford & Spongberg, 1983; Tiffney, 1985; Hong, 1993; Xiang et al., 1998; Manchester, 1999; Wen, 1999, 2001; Manos & Donoghue, 2001; Huang et al., 2013; Zhang et al., 2013). The striking similarity of the EA and the ENA flora was noted by Asa Gray, who provided a series of detailed comparisons of the floras in the two continents (Gray, 1840; Boufford & Spongberg, 1983; Wen, 1999). Surprisingly, not only taxa within the same genus or closely related genera are shared between these areas, but also climatic and ecological similarities (i.e., between the forests of Japan, central China, and the Appalachians in eastern North America), which support the reality of such a biological/biogeographic

connection (Boufford & Spongberg, 1983; Davidse, 1983). However, the disjunct distribution pattern between EA and WNA did not receive as much attention as that between EA and ENA (Donoghue & Smith, 2004), even though these areas are geographically much closer than that of EA and ENA (see review by Wen et al., 2016).

Remarkable progress in dating and biogeographic analyses has been made due to the discovery of relevant fossils, and the elucidation of the Earth-history events has made it possible for us to further explore the intercontinental disjunct distribution relationships (reviewed by Wen et al., 2010, 2016). Phytogeographic studies with ample fossil evidence suggest that floras of eastern Asia and North America were once connected continuously by land bridges (Graham, 1972; Hsü, 1983; Tiffney, 1985; Manchester, 1999; Wang & Manchester, 2000; Tiffney & Manchester, 2001). For example, although the spruce genus *Picea* A. Dietr. (Pinaceae) has been reported to have a complex biogeographic history as interspecific hybridization and mitochondrial DNA (mtDNA) introgression occurred commonly in *Picea*, the phylogenetic and fossil evidence were able to support that the genus originated in North America and dispersed from North America to Asia by way of the Bering land bridge in the Miocene and the Pliocene (Ran et al., 2015). On many occasions, the estimation of different migration timings of the eastern Asia and North America biogeographic distribution have given rise to multiple origins of intercontinental disjunctions (Wen, 1999; Donoghue et al., 2001; Wen et al., 2010). Also, as many plant distributions are greatly influenced by climatic factors, high latitude temperate floras declined in the Northern Hemisphere due to climate changes during the Cenozoic (Woodward, 1987; Criddle et al., 1994; Liu et al., 2007; Yang et al., 2007). For instance, Srivastava et al. (2018) have reported a great vegetation shift in the Miocene due to climate change using two paleofloras recovered from the Siwalik area of Nepal. Without knowing the current intercontinental distributions with sufficient support from morphological and phylogenetic evidence, it is difficult to answer questions concerning disjunct patterns and migration routes, such as: what are the genetic diversity and population structures of groups affected by intercontinental disjunct distribution? How do they contribute to the evolution of the overall genus?

For the Northern Hemisphere intercontinental disjunctions between EA–ENA and EA–WNA, two conceivable migration routes during the Tertiary and the Quaternary have been suggested: (i) Bering land bridges (Hopkins, 1967); and (ii) North Atlantic land bridges (McKenna, 1983). Wen (1999, 2001) and Wen et al. (2010) reviewed these biogeographic patterns based on the phylogenetic evidence that was available, estimating that the divergence times of the intercontinental disjunct distribution mostly fall in the time frame of approximately 5–30 Ma. Donoghue & Smith (2004) also analyzed the biogeographic relationships in the Northern Hemisphere by examining a number of genera, and their analyses suggested that EA to ENA movement occurred during the last 30 million years. However, most of these previous studies are based on a few molecular markers and/or morphology and often with insufficient sampling (Wen, 1999). A more detailed and accurate taxonomy of the disjunct groups is needed in order to gain insights into the origin and evolution of the EA–ENA and EA–WNA disjunct relationship (Wen, 1999; Wen et al., 2010).

An expanded sampling of lineages confirmed that most eastern Asian–North American disjunct lineages have the divergence time of disjunction in the Miocene (Wang et al., 2007; Nie et al., 2008; Jiao & Li, 2009; Triplett & Clark, 2010; Xie et al., 2010) with some exceptions (e.g., Harris et al., 2009). This supports the hypothesis that the Bering land bridge acted as a major connection for the intercontinental disjunction in the Northern Hemisphere. Recent studies have revealed a general pattern of relatively young crown group ages of each disjunct counterpart, but much older stem ages (e.g., Nie et al., 2006a; Xu et al., 2010). Wen et al. (2010) revealed that the directionality of migration/dispersal for eastern Asian–North American disjunction is commonly from the Old World to the New World (62%), much higher than New World to Old World (30%; mainly of conifers). However, the migration routes for approximately 23% of plant lineages studied (i.e., 23 lineages) could not be determined. Several factors, such as complex patterns of migration/dispersal, extinction, speciation, vicariance, and evolutionary convergence, can contribute to the difficulties in determining migration routes (Wen et al., 2010).

Subfamily Orontioideae Mayo, Bogner & P. C. Boyce is an early divergent lineage in Araceae Juss. (also known as the proto-Araceae, together with Gymnostachydoideae Bogner & Nicolson), and it comprises three genera: *Orontium* L., *Symplocarpus* Salisb. ex W. P. C. Barton, and *Lysichiton* Schott (Mayo et al., 1997). The genera *Symplocarpus* and *Lysichiton* are the best-known examples showing EA–ENA and EA–WNA disjunct distributions in the Northern Hemisphere, respectively. The golden-club genus *Orontium* includes only one species, *O. aquaticum* L., which occurs in eastern USA, primarily on the Atlantic and Gulf coastal plains and less frequently in the Appalachian region. It is a perennial aquatic herb with stout, deeply sunken rhizomes, distinctive blue-green velvety oblong-elliptic shaped leaves, and a golden yellow spadix with an inconspicuous simple spathe (Wilson, 1960; Klotz, 1992; Mayo et al., 1997). Although the monotypic genus *Orontium* appears to be a quite isolated taxon based on its morphological characters, several studies suggested its close relationship to *Symplocarpus* and *Lysichiton* (Grear, 1966; French et al., 1995; Mayo et al., 1997; Tam et al., 2004; Nie et al., 2006b; Cabrera et al., 2008; Cusimano et al., 2011; Cusimano et al., 2012; Nauheimer et al., 2012).

The western skunk cabbage genus, *Lysichiton*, includes two species, *L. americanus* Hultén & H. St. John in western North America and *L. camtschatcensis* (L.) Schott in East Asia. The genus was considered to constitute only a single species, *L. camtschatcensis*, prior to the recognition of the North American *L. americanus*. The two species of *Lysichiton* are morphologically similar, but they can be distinguished primarily based on the spathe color: bright yellow in *L. americanus* versus white in *L. camtschatcensis*. They are rhizomatous perennials and commonly found in low wet areas including swamps, wet woods, and along streams. The eastern Asian species, *L. camtschatcensis*, occurs only in Japan (from central and northern Honshu to Hokkaido) and the Russian Far East (including Sakhalin, Ussuri, Kamchatka, and the Kuril Islands). The western North American species,

L. americanus, is common in wet forests and muskegs from Alaska to northern California, and ranges eastward in northeastern British Columbia, Canada and northwestern Montana and northern Idaho, USA. Phylogenetic analyses (Nie et al., 2006b) showed reciprocal monophyly between the two intercontinental species of *Lysichiton* and a sister relationship between *Lysichiton* and *Symplocarpus*.

The genus *Symplocarpus* includes approximately five species and usually occurs in wet places and forest swamps: *S. renifolius* Schott ex Tzvelev, *S. foetidus* (L.) Salisb. ex W. Barton, *S. nipponicus* Makino, *S. nabekuraensis* Otsuka & K. Inoue, and *S. egorovii* N. S. Pavlova & V. A. Nechaev (Otsuka et al., 2002; Pavlova & Nechaev, 2005; Nie et al., 2006b). *Symplocarpus foetidus* is the only species widely distributed in ENA from Nova Scotia, southern Quebec and Minnesota to North Carolina and Tennessee. The remaining four species are found in EA. Two species, *S. renifolius* and *S. nipponicus*, have broad geographic distributions in EA (i.e., Japan, Korea, northeast China, and the Russian Far East), whereas the remaining two recently described species have narrower distribution (i.e., *S. nabekuraensis* in the northern Nagano Prefecture of central Japan and *S. egorovii* in the Primorye region, Russia) (Otsuka et al., 2002; Pavlova & Nechaev, 2005). The intercontinental disjunct species pair *S. foetidus* (ENA) and *S. renifolius* (EA) share flowering time in the early spring before the emergence of leaves and fruit ripening in the fall of the same year. Both *S. foetidus* and *S. renifolius* have been the subjects of study in exothermic spadices (Knutson, 1974; Wada & Uemura, 1994, 2000; Ito et al., 2004). *Symplocarpus egorovii* is similar to *S. foetidus* and *S. renifolius*, including exothermic spadices, but it can be distinguished from them by its distinct coniferous habitat and by its yellowish-white colored spathe (Pavlova & Nechaev, 2005). *Symplocarpus nipponicus* has smaller and more spherical inflorescences, and lacks precocious flowering. Its fruit ripens in the following spring (Koyama, 1983). The newly described species, *S. nabekeraensis*, has intermediate characteristics between *S. renifolius* and *S. nipponicus* in leaf emergence and flowering time, but it can be distinguished from the congeneric species by a rounder leaf shape and longer peduncles (Otsuka et al., 2002).

The chromosome numbers of Orontioideae species are known: *O. aquaticum* ($2n = 26$), *L. americanus* ($2n = 28$), *L. camtschatcensis* ($2n = 28$), *S. foetidus* ($2n = 60$), *S. renifolius* ($2n = 60$), *S. nabekuraensis* ($2n = 60$), *S. egorovii* ($2n = 30$), and *S. nipponicus* ($2n = 30$) (basic number $x = 15$) (Mulligan, 1965; Grear, 1966; Sokolovskaya & Probatova, 1985; Petersen, 1989; Flora of North America Editorial Committee, 1993; Iwatsubo & Otsuka, 2005; Probatova et al., 2008, 2012).

Several molecular phylogenetic studies have been carried out to resolve species relationships within the subfamily Orontioideae. Based on the seven populations of the three species, Wen et al. (1996) used 20 restriction endonucleases to investigate chloroplast DNA (cpDNA) variation of the genus *Symplocarpus*, and they found the earliest divergence of *S. nipponicus* within the genus and a sister relationship between *S. foetidus* from ENA and *S. renifolius* from EA. Moreover, they inferred a single origin for exothermic spadices in *Symplocarpus* (Wen et al., 1996). The phylogenetic study of Nie et al. (2006b) focused on the subfamily Orontioideae, and sampled all three genera (*Symplocarpus*, *Lysichiton*, and *Orontium*). Using the *trnL–F* intergenic spacer region and the *ndhF* gene, they inferred the monophyly of subfamily Orontioideae and a sister relationship between *Symplocarpus* and *Lysichiton* (Nie et al., 2006b). In addition, they supported the monophyly of both *Symplocarpus* and *Lysichiton* and found a close relationship between the disjunct species pair of *S. foetidus* from ENA and *S. renifolius* from EA, and *L. americanus* from WNA and *L. camtschatcensis* from EA. The eastern Asian species *S. nipponicus* was sister to the clade of *S. foetidus–S. renifolius*. The sister relationship of intercontinental species pair in *Symplocarpus* has been supported by a palynological study, with *S. renifolius* closer to *S. foetidus* than to *S. nipponicus* (Lee et al., 2010). *Symplocarpus nabekuraensis*, which appears to have intermediate characteristics between *S. renifolius* and *S. nipponicus*, is closer to *S. renifolius* than to *S. nipponicus* based on two cpDNA regions (*trnG* intron and 3′ to *rps2*) (Kitano et al., 2005). These findings are consistent with the observation of general similarities in leaf morphology, leaf emergence, and fruiting season (Otsuka et al., 2002).

The timing of the intercontinental disjunct event in Orontioideae has been proposed. Wen et al. (1996) estimated the intercontinental disjunct event of *Symplocarpus* (between *S. renifolius* and *S. foetidus*) to be 6.1 Ma during the late Miocene, using cpDNA restriction site data. Later, Nie et al. (2006b) estimated divergence times of major clades in subfamily Orontioideae based on the penalized likelihood and Bayesian dating methods. The Bayesian dating methods inferred the crown age of *Symplocarpus* as 20.65 Ma, and the divergence time of *S. renifolius* and *S. foetidus* was estimated to be 6.88 Ma. The crown age and the divergence time of disjunction event in *Lysichiton* was 7.18 Ma according to the Bayesian dating methods of Nie et al. (2006b). The Bering land bridge was inferred to be the most plausible dispersal route for the intercontinental disjunct species within *Symplocarpus* as well as *Lysichiton* because their divergence times are inferred to be in the Miocene (Wen, 1999; Nie et al., 2006b; Wen et al., 2010).

Although the previous phylogenetic and biogeographic studies established the basic framework to understand the intercontinental disjunct events in Orontioideae, the limited sampling both at the species and the population levels have hindered the progress to fully elucidate the phylogenetic and biogeographic patterns in the subfamily. For example, with the uncertain phylogenetic relationships of *S. renifolius* from Japan and Korea, the divergence time was estimated solely based on the cpDNA sequence divergence between *S. renifolius* from Japan and *S. foetidus* from ENA (Wen et al., 1996). The most recent phylogenetic results of Orontioideae by Nie et al. (2006b) were based on two to three accessions for each species. Finally, the previous studies did not allow us to evaluate the taxonomic significance or distinctiveness of two recently described species (*S. egorovii* and *S. nabekuraensis*), which were recognized primarily based on subtle morphological and ecological differences. Therefore, in this study, we thoroughly sampled all eight species in multiple populations of Orontioideae (a total of 198 accessions) and undertook molecular phylogenetic analyses and molecular dating. We

hope to: (i) determine phylogenetic relationships among the eight species of Orontioideae based on the extensive sampling; (ii) assess the taxonomic status of the two recently described species in *Symplocarpus*; and (iii) estimate divergence times of the intercontinental disjunct event(s) within Orontioideae.

2 Material and Methods

2.1 Taxon sampling

Our collection of plant material in the field included a total of 198 accessions representing all members of the subfamily Orontioideae (195 accessions) and one outgroup genus *Gymnostachys* R. Br. (three accessions) of the subfamily Gymnostachydoideae, also known as proto-Araceae. Gymnostachydoideae is endemic to eastern Australia (New South Wales) and is sister to the Orontioideae (Nie et al., 2006b; Cusimano et al., 2011) (Table S1). For subfamily Orontioideae, we sampled extensively, covering all geographic ranges for all members from both eastern Asia and North America. We included two populations of the monotypic eastern North American, *Orontium*, from Florida and Alabama (two accessions from each population). For the disjunct genus *Lysichiton*, we included a total of 15 and 18 accessions of *L. camtschatcensis* and *L. americanus*, respectively. The representative populations of the two species covered broad geographic regions: Alaska, Oregon, Washington, and California for *L. americanus*, and Miyagi, Aomori, Nagano, and Niigata in Japan for *L. camtschatcensis.*

We made exceptional effort to include as many accessions as we could for all members of *Symplocarpus*. We included 15 accessions of *S. foetidus* in eastern North America, and sampled from northeastern (Maine) to the Midwest (Wisconsin and Ohio). For two narrowly occurring and recently described species of *Symplocarpus*, we sampled nine accessions for *S. nabekuraensis* from Japan and three from *S. egorovii* from the Russian Far East. *Symplocarpus nipponicus* occurs widely in regions of eastern Asia, and we sampled a total of 29 populations with 53 accessions: Korea (eight populations and 24 accessions) and Japan (21 populations with 29 accessions). Finally, we sampled a total of 78 accessions for *S. renifolius*, which occurs in eastern Asia: Korea (42 accessions), Japan (30 accessions), and the Russian Far East (six accessions). All voucher specimens were deposited at the Herbarium of Sungkyunkwan University (SKK), Korea and Tohoku University (TUS), Japan (Table S1).

2.2 DNA extraction, polymerase chain reaction amplification, and DNA sequencing

Total genomic DNA was extracted from 20 mg silica-gel-dried leaf material using the DNeasy plant mini kits (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. For each accession, six chloroplast intergenic spacers (i.e., primer sequences from Shaw et al., 2007: *atpI–atpH*, *psbJ–petA*, *rpl32–trnL*, *trnL–F*, *trnQ–rps16*, and *trnS–ycf9*) were amplified using polymerase chain reaction (PCR). These regions were chosen based on their relatively high level of variation and successful amplification after a broad survey of the regions. Total DNA was diluted (1:10) with TE buffer and the DNA regions were amplified in a total volume of 20 μL: 6 μL Master-Mix (10× Tricine Taq buffer, 10 mmol/L dNTPs and ddH_2O), 0.5 μL dimethyl sulfoxide, 1 μL each primer at 0.02 μmol, 0.2 μL of 1 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 2 μL diluted template DNA. The PCR amplification was carried out using the following program: initial denaturation at 95°C for 5 min followed by 37 cycles of denaturation at 95°C for 1 min, primer annealing at 54°C to 56°C for 1 min, and primer extension at 72°C for 2 min, followed by a final extension step of 10 min at 72°C. After the amplification process, PCR products were checked by 1% agarose gel and purified using the purification kit ExoSAP-IT (USB, Cleveland, OH, USA). The purified PCR products were sequenced at Geno Tech (Daejeon, Korea) using the same PCR primers. For each region, contigs were assembled and edited with Sequencher 4.7 (Gene Codes, Ann Arbor, MI, USA) and Geneious 6.1.6 (Biomatters, Auckland, New Zealand). The sequences were aligned with CLUSTALX (Thompson et al., 1994; Larkin et al., 2007), and manually adjusted using Mesquite version 2.75 (Maddison & Maddison, 2011). The sequences obtained in this study are deposited in GenBank (Table S2).

2.3 Phylogenetic analysis

We tested the congruence of the DNA regions by applying the incongruence length difference test (Farris et al., 1994) in PAUP* 4.0b10 (Swofford, 2003), and there was no significantly different signal between the data partitions ($P = 1.0$). Therefore, we combined all six cpDNA regions and undertook maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses.

The MP analysis was carried out using heuristic search options in PAUP* 4.0b10 (Swofford, 2003): equal weighed characters, one tree held per replicate, Multrees on, and tree bisection–reconnection in branch swapping. The bootstrap analysis was undertaken with 1000 replicates using the same heuristic search options (Felsenstein, 1985). The ML analysis was undertaken with RAxML version 7.0.4 (Stamatakis, 2006) and raxmlGUI (Silvestro & Michalak, 2011) using the generalized time reversible model (Tavaré, 1986) with a gamma-distributed rate variation (Yang, 1993) (GTR + G substitution model) found by 1000 bootstrap replicates under the same model (Felsenstein, 1985). The model was selected by the Akaike information criterion (Akaike, 1974) based on the model test using Modeltest version 3.7 (Posada & Crandall, 1998). Bayesian inference was carried out using the Markov chain Monte Carlo (MCMC) method (Yang & Rannala, 1997) in the program MrBayes version 3.2 (Ronquist et al., 2012). The BI phylogeny was reconstructed from two independent sets of Markov chains using a GTR + G substitution model. The MCMC algorithm had a length of 40 000 000 generations that was sampled every 1000 generations; the average standard deviation of the split frequencies reached below 0.01, which indicates that two runs reached stationary distribution (Ronquist et al., 2012). The first 25% of samples were discarded as burn-in, and the post burn-in majority rule consensus tree was created with posterior probability.

2.4 Molecular dating

Divergence times were estimated by the Bayesian method (Drummond et al., 2006) using the program BEAST version

1.8.2 (Drummond et al., 2012). The XML file for the analysis was prepared in the Bayesian Evolutionary Analysis Utility (BEAUTi). Bogner et al. (2005) found the fossil record of *Albertarum pueri* from Alberta, which has morphological similarity to Orontioideae. However, this fossil has a few features that are inconsistent with that subfamily according to Kvacek & Smith (2015). They found the earliest records of orontioid leaves were from the Campanian (72.1–83.6 Ma) of Austria. Therefore, we used 72 Ma as the minimum constraint (using lognormal distribution, mean = 0, standard deviation = 1) to the stem age of the subfamily, following the guidelines for the fossil constraint (Donoghue & Benton, 2007; Ho & Phillips, 2009; Sauquet, 2013). It is worth noting that several fossil taxa have been reported within Orontioideae and were suggested to be the possible calibration candidates (Bogner et al., 2007), and they are slightly younger than our calibration (between 65.5 and 70 Ma) (Nauheimer et al., 2012). However, we excluded them because they lack diagnostic features to assign them to specific lineages within the subfamily (Smith, 2013; Iles et al., 2015). We generated marginal likelihoods using path sampling and stepping stone sampling to compare between Yule process speciation prior and birth–death prior. As the birth–death prior setting provided a higher log marginal likelihood (–15774.76 compared to –15825.90 with the Yule process speciation prior), we used the GTR + G model, the birth–death model (an appropriate model for groups that have undergone significant extinction; Condamine et al., 2015), and lognormal relaxed clock (Drummond et al., 2006).

The analyses were run for 50 000 000 generations with a sample frequency of 5000 generations. Tracer version 1.5 (Rambaut & Drummond, 2007) was used to check over the posterior distribution of all statistics. All effective sample size values were well above 200, which is considered to be a recommended threshold to indicate a stationary probability (Drummond et al., 2012). To obtain an estimate of the phylogenetic tree with mean divergence time and 95% highest posterior density (HPD) intervals, the program TreeAnnotator version 1.8.2 (http://beast.bio.ed.ac.uk/TreeAnnotator) was used for the reconstruction of the maximum credibility tree with posterior probability limit of 0.5 setting, after removing the first 25% of trees in each run as burn-in (Drummond et al., 2012).

2.5 Ancestral state reconstruction

The ancestral area reconstruction (AAR) and estimation of patterns of geographic distribution of Orontioideae were undertaken using statistical dispersal vicariance analysis (Ronquist, 1997; Yu et al., 2010), Bayesian binary MCMC (Ronquist & Huelsenbeck, 2003), and dispersal–extinction–cladogenesis (Ree & Smith, 2008) implemented in the program Reconstruct Ancestral State in Phylogenies version 3.1 (RASP) (Yu et al., 2015). The distributional range of taxa was categorized into four areas: A, Australia; B, western North America; C, eastern North America; and D, East Asia. The selected areas were coded as unordered character states. *Gymnostachys anceps* R. Br. was used as the outgroup. We have limited the maximum numbers of combined areas to two in all three analyses.

2.6 Chromosome counting

Newly formed roots were collected from *S. renifolius* from Mt. Geumbyeong, Mt. Samak, and Mt. Ungil in Korea, and were potted in the experimental garden of Sungkyunkwan University (Suwon, Korea). Root tip samples were collected and pretreated with 0.002 mmol/L 8-hydroxyquinoline aqueous solution for 1 h at 25°C, and fixed in a mixture of acetic acid and absolute ethyl alcohol (1:3) for 1 h at 4°C. They were macerated in 1 mol/L HCl at 60°C for 15 min and stained by a drop of 1.0% orcein (Chroma CO.) for 24 h. The slides were prepared by the squash technique. Chromosome images were captured using a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany).

3 Results

3.1 Phylogenetic analysis

The six concatenated cpDNA regions generated a data matrix of 5799 bp in length with 765 parsimony-informative sites (PS): *atpI–atpH* (971 bp, 104 PS), *psbJ–petA* (1099 bp, 103 PS), *rpl32–trnL* (1145 bp, 212 PS), *trnL–F* (1137 bp, 117 PS), *trnQ–rps16* (995 bp, 197 PS), and *trnS–ycf9* (452 bp, 32 PS). The insertion and deletion (indel) length varied from a single base pair in *trnQ–rps16* up to 70 bp in *trnL–F*. Of a total of 18 indels, 10 were non-homoplasious supporting two major lineages (Clades A and B) in *Symplocarpus* (Fig. 1).

All three analyses (MP, ML, and BI) generated an identical tree topology concerning the nodes of interest (threshold for that support >50%); the ML tree is presented in Fig. 1. The MP analysis found 24 equally most parsimonious trees with a tree length of 886 steps, a consistency index of 0.9436 (0.9419 excluding uninformative sites), a retention index of 0.9946, and a rescaled consistency index of 0.9385. With *Gymnostachys* from eastern Australia as the outgroup, the eastern North American monotypic genus *Orontium* diverged first within subfamily Orontioideae. The next diverged clade (100% parsimony bootstrap support (PBS), 100% likelihood bootstrap support (LBS), 1.00 posterior probability (PP) contained two reciprocally monophyletic genera, *Lysichiton* (100% PBS, 100% LBS, 1.00 PP) and *Symplocarpus* (100% PBS, 100% LBS, 1.00 PP). The two species of *Lysichiton*, *L. americanus* from western North America and *L. camtschatcensis* from eastern Asia, were reciprocally monophyletic. There were few resolutions within *L. camtschatcensis* and the polytomy prevented assessment of phylogenetic relationships among populations in Japan. However, within *L. americanus*, the clade containing populations from California (with one exceptional accession from Oregon, OR4) was sister to the clade containing the remaining accessions sampled from Washington, Alaska, and Oregon (Fig. 1).

We discovered several novel and important findings within genus *Symplocarpus*. First, our results showed a deep split of the genus into two major lineages of Clade A and B (100% PBS, 100% LBS, 1.00 PP for each lineage) (Fig. 1). Clade A included *S. foetidus* in ENA (Clade I; 100% PBS, 100% LBS, 1.00 PP), *S. renifolius* in Russia and Japan, and *S. nabekuraensis*. Clade B consisted of *S. nipponicus* in Korea and Japan, *S. renifolius* in Korea, and *S. egorovii* in the Russian Far East. *Symplocarpus renifolius* and *S. nipponicus* were not

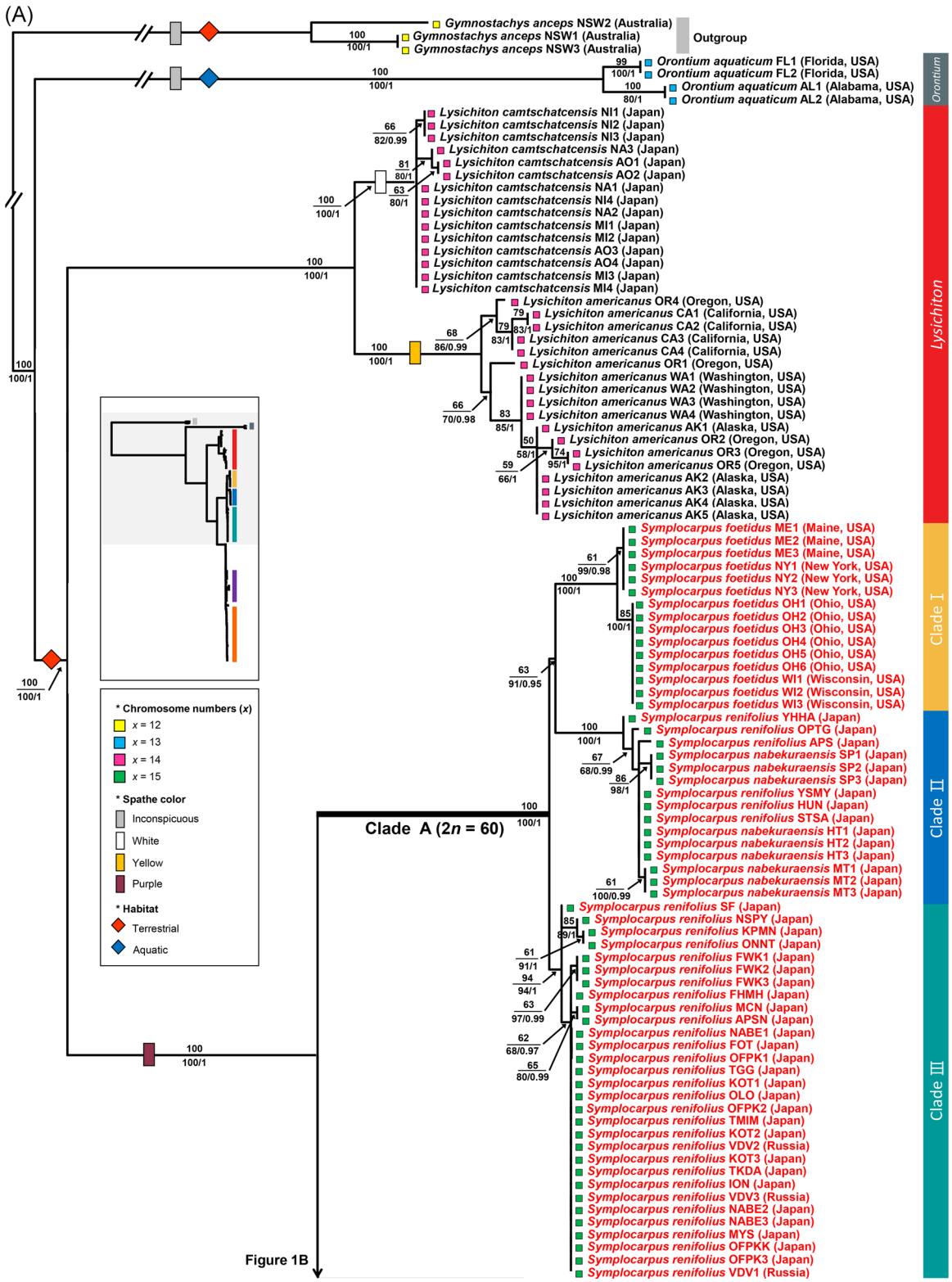

Fig. 1. Maximum likelihood (ML) tree of proto-Araceae based on six concatenated chloroplast non-coding regions. The number above the branch is parsimony bootstrap support; the numbers below represent maximum likelihood bootstrap support/Bayesian inference posterior probability. Chromosome numbers, spathe color, and habitat are indicated in the figure. Red colored accessions are tetraploids. **A**, Upper half of the phylogenetic tree. **B**, Lower half of the phylogenetic tree.

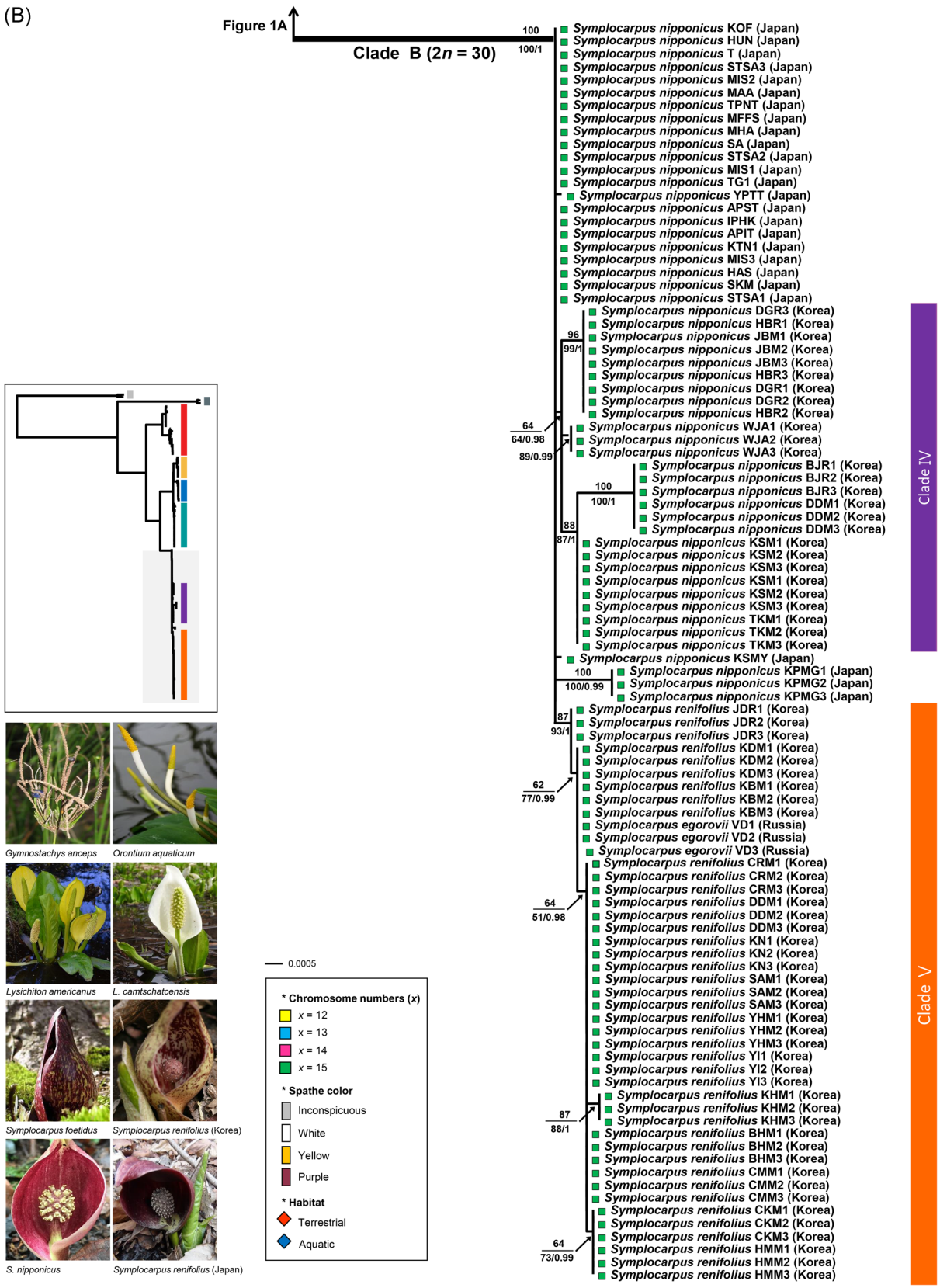

Fig. 1. Continued

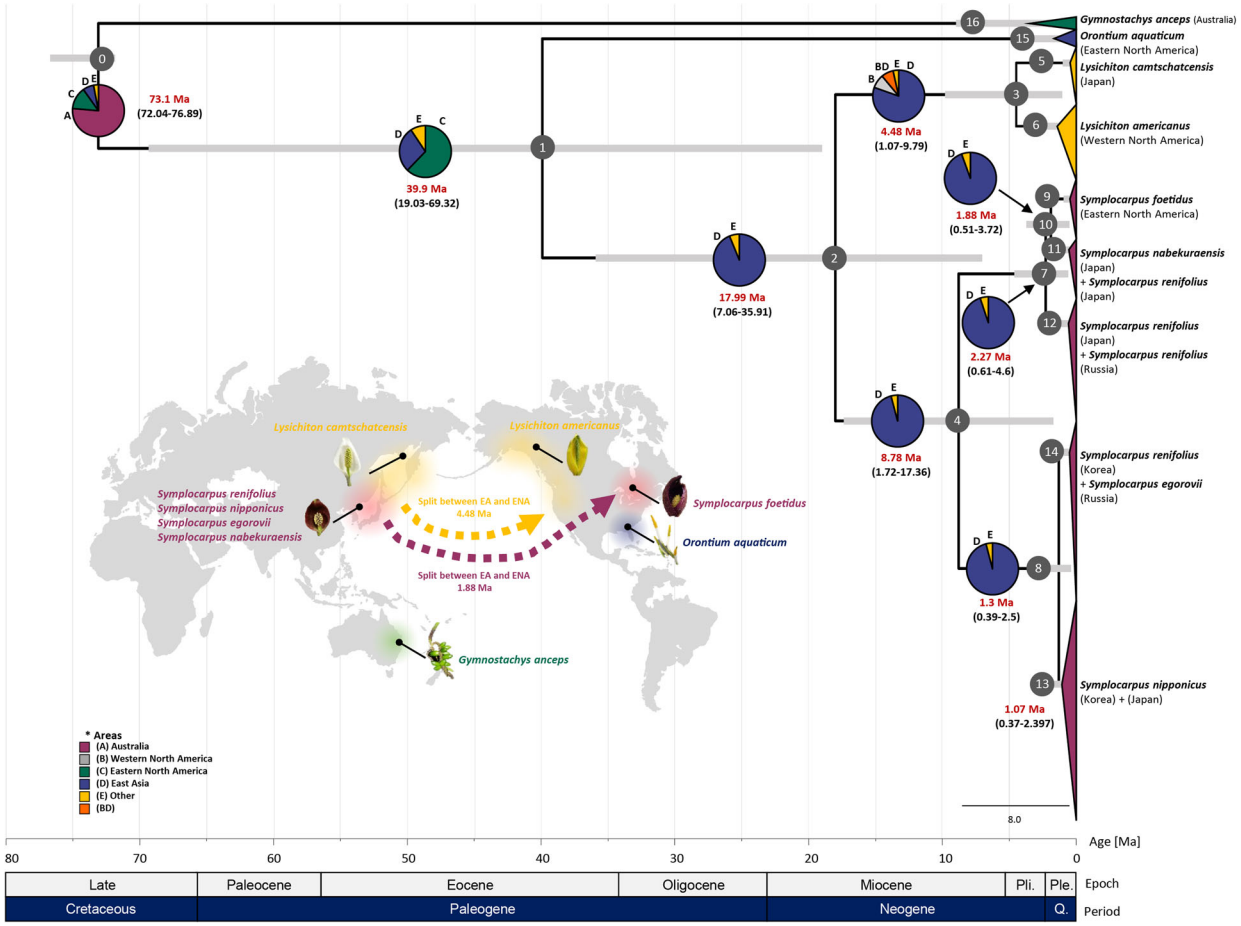

Fig. 2. Chronogram and ancestral area reconstruction of Orontioideae based on six concatenated chloroplast non-coding regions. Blue bars at major nodes indicate 95% highest posterior density intervals. The areas of ancestral area reconstruction analysis are indicated (Bayesian binary Markov chain Monte Carlo): **A**, Australia; **B**, western North America; **C**, eastern North America; and **D**, east Asia.

monophyletic. Clade II (100% PBS, 100% LBS, 1.00 PP), which included *S. renifolius* from regions of the East Sea/Sea of Japan and *S. nabekuraensis* from Japan, was sister to the ENA *S. foetidus* (100% PBS, 100% LBS, 1.0 PP). Clade III (94% PBS, 94% LBS, 1.00 PP) included two populations of *S. renifolius* from the Russian Far East (Nadezhdinsky and Vladivostok) and the remaining *S. renifolius* populations in Japan (Pacific Ocean side). Within Clade B, the recently described *S. egorovii* from the Russian Far East was deeply embedded within the clade containing *S. renifolius* (Clade V; 87% PBS, 93% LBS, 1.00 PP). *Symplocarpus nipponicus* from Korea formed a monophyletic group (Clade IV; 64% PBS, 64% LBS, 0.98 PP), whereas those from Japan did not (Fig. 1).

3.2 Molecular dating

The estimated divergence times among major lineages of Orontioideae calibrated by fossil record of orontioid leaves (72 Ma; Kvacek & Smith, 2015) and results from the Bayesian estimations are shown in Fig. 2 and Table 1. The major divergence event of Orontioideae between *Lysichiton* and *Symplocarpus* was estimated to be 17.99 Ma (95% HPD, 7.06–35.91 Ma; node 2). The crown age of *Lysichiton* (node 3) was 4.48 Ma (95% HPD, 1.07–9.79 Ma), and this indicates the divergence time of intercontinental disjunct distribution between EA and WNA was in the Pliocene or Pleistocene. The crown age of *Symplocarpus* (node 4) was estimated to be 8.776 Ma, in the Miocene (95% HPD, 1.72–17.36 Ma). The crown age of Clades A and B were estimated to be 2.27 Ma, early Pleistocene (95% HPD, 0.61–4.6 Ma; node 7) and 1.3 Ma, early Pleistocene (95% HPD, 0.39–2.5 Ma; node 8), respectively. The estimated divergence time between *S. foetidus* in ENA and *S. renifolius* and *S. nabekuraensis* in Japan was inferred at 1.88 Ma, Pleistocene (95% HPD, 0.51–3.72 Ma; node 10). All other divergence time estimates are shown in Table 1. We also ran the analysis using Yule process speciation prior (Table S3), but the divergence time estimation results were significantly older than the birth–death model results.

3.3 Ancestral area reconstruction

According to the ancestral area reconstructions (Fig. 2; Table 1), Bayesian binary MCMC suggests the eastern Asian origin (marginal probability 82% and 99%) of both *Lysichiton* and *Symplocarpus* (nodes 3 and 4). The intercontinental

Table 1 Summary of estimated divergence times and ancestral area reconstruction of subfamily Orontioideae using the birth–death model

	Divergence time estimation		Ancestral area reconstruction		
Node	Median (Ma)	95% HPD (Ma)	BBM	S-DIVA	DEC
Node 0 (proto-Araceae)	73.1008	72.0397–76.8911	A 0.7635 C 0.1382 D 0.0684 Other 0.0299	AD 0.5 AC 0.5 	AC 0.3083 AD 0.2192 CD 0.1363 C 0.1022D 0.0717 AB 0.0506BC 0.0506
Node 1 (Orontioideae)	39.8961	19.0278–69.3186	C 0.6218 D 0.2846 Other 0.0936	CD 1.0	C 0.2190BC 0.1228 D 0.0853
Node 2 (*Symplocarpus–Lysichiton*)	17.9877	7.0591–35.9099	D 0.9380 Other 0.0620	D 1.0	D 0.3642 CD 0.3559 BD 0.2799
Node 3 (*Lysichiton*)	4.4818	1.0745–9.787	D 0.8202 B 0.0871 BD 0.0771 Other 0.0156	BD 1.0	BD 1.0
Node 4 (*Symplocarpus*)	8.776	1.7233, 17.3644	D 0.9879 Other 0.0121	D 1.0	D 0.5394 CD 0.4606
Node 5 (*L. camtschatcensis*, Japan)	0.4438	0.0456–0.9835	D 0.9993 Other 0.0007	D 1.0	D 1.0
Node 6 (*L. americanus*, western North America)	1.4133	0.3301–2.8852	B 0.9614 Other 0.0386	B 1.0	B 1.0
Node 7 (Clade A)	2.2716	0.6145–4.6008	D 0.9856 Other 0.0144	D 1.0	CD 0.8279 D 0.1721
Node 8 (Clade B)	1.2952	0.3916–2.502	D 0.9976 Other 0.0024	D 1.0	D 1.0
Node 9 (*S. foetidus*, eastern North America)	0.4698	0.0589–0.9418	C 0.9102 CD 0.0733 Other 0.0165	C 1.0	C 1.0
Node 10 (*S. foetidus*, eastern North America and *S. renifolius* and *S. nabekuraensis*, Japan)	1.8844	0.5109–3.7201	D 0.9450 Other 0.0550	CD 1.0	CD 1.0
Node 11 (*S. renifolius* and *S. nabekuraensis*, Japan)	0.5339	0.0951–1.0516	D 0.9978 Other 0.0022	D 1.0	D 1.0
Node 12 (*S. renifolius*, Japan)	0.5742	0.0928–1.2489	D 0.9990 Other 0.0010	D 1.0	D 1.0
Node 13 (*S. nipponicus*)	1.0747	0.3704–2.3946	D 0.9980 Other 0.0020	D 1.0	D 1.0
Node 14 (*S. renifolius*, Korea and *S. egorovii*, Russia)	0.5027	0.1068–0.9328	D 0.9986 Other 0.0014	D 1.0	D 1.0

Continued

Table 1 Continued

Node	Divergence time estimation		Ancestral area reconstruction		
	Median (Ma)	95% HPD (Ma)	BBM	S-DIVA	DEC
Node 15 (*Orontium aquaticum*, eastern North America)	1.6527	0.2006–4.087	D 0.9967 Other 0.0033	C 1.0	C 1.0
Node 16 (*Gymnostachys anceps*, Australia)	3.4567	0.5688–8.9557	D 0.9967 Other 0.0033	A 1.0	A 1.0

Distributional range of taxa: A, Australia; B, western North America; C, eastern North America; and D, East Asia. BBM, Bayesian binary Markov chain Monte Carlo; DEC, dispersal–extinction cladogenesis; HPD, highest posterior density; S-DIVA, statistical dispersal–vicariance analysis.

disjunction events in *Lysichiton* (nodes 3 and 6) and *Symplocarpus* (nodes 9 and 10) were estimated to be from EA to WNA and from EA to ENA, respectively. The statistical dispersal–vicariance analysis and dispersal–extinction cladogenesis also supported the eastern Asian origin of *Symplocarpus*, but the origin of *Lysichiton* was uncertain.

3.4 Chromosome number of *S. renifolius* in Korea

Root tip samples from populations of *S. renifolius* from Mt. Geumbyeong, Mt. Samak, and Mt. Ungil in Korea had the diploid chromosome number of $2n = 30$ during mitotic metaphase spread (Fig. 3).

4 Discussion

4.1 Phylogenetic relationships within the subfamily Orontioideae

The two genera of Orontioideae, *Lysichiton* and *Symplocarpus*, served as important examples of intercontinental disjunct distribution patterns in the Northern Hemisphere. However, limited sampling in previous molecular phylogenetic studies and poor resolution at the species level (Wen, 1999; Kitano et al., 2005; Nie et al., 2006b) led to weak biogeographic inferences (Wen et al., 1996; Xiang & Soltis, 2001; Nie et al., 2006b). The current study represents the most comprehensive phylogenetic study on the subfamily Orontioideae with an extensive species-level sampling. As expected, the sister relationship of *Orontium* to the rest of the Orontioideae and the monophyly of each genus (*Lysichiton* and *Symplocarpus*) were strongly supported (Fig. 1).

The phylogenetic analysis based on six concatenated cpDNA non-coding regions supported two major lineages within *Symplocarpus* (Clades A and B; Fig. 1). The deep divergence of two major lineages within the genus was never detected in the previous studies (Wen et al., 1996; Kitano et al., 2005; Nie et al., 2006b). Clade A consists of *S. foetidus* from ENA and *S. renifolius* and *S. nabekuraensis* from Japan. The three species in Clade A are tetraploids ($2n = 60$) (Sokolovskaya & Probatova, 1985; Iwatsubo & Otsuka, 2005; Bogner & Petersen, 2007; Cusimano et al., 2011). Surprisingly, the populations of *S. renifolius* from Korea belong to Clade B, containing *S. nipponicus* from both Korea and Japan and *S. egorovii*, a narrow endemic to the Russian Far East. In contrast, two of the three species in Clade B, *S. nipponicus* and *S. egorovii*, are diploids ($2n = 30$) (Probatova et al., 2008, 2012). The chromosome number of *S. renifolius* from Korea was unknown and its unusual phylogenetic position in the strongly supported clade with other diploid species raised a possibility of the diploid nature of *S. renifolius*. Therefore, we counted the chromosome number of *S. renifolius* from Korea and determined that it had a diploid chromosome number of 30 (Fig. 3). The results thus support two major clades of *Symplocarpus* corresponding to the tetraploid and the diploid lineages, Clades A and B, respectively (Fig. 1). This phylogenetic pattern suggests several scenarios with regard to the origin of *S. renifolius*. After the divergence from its sister genus *Lysichiton*, a common ancestor of *Symplocarpus* split into two lineages likely in the Miocene (Fig. 2). One lineage from the diploid common ancestor ($2n = 30$)

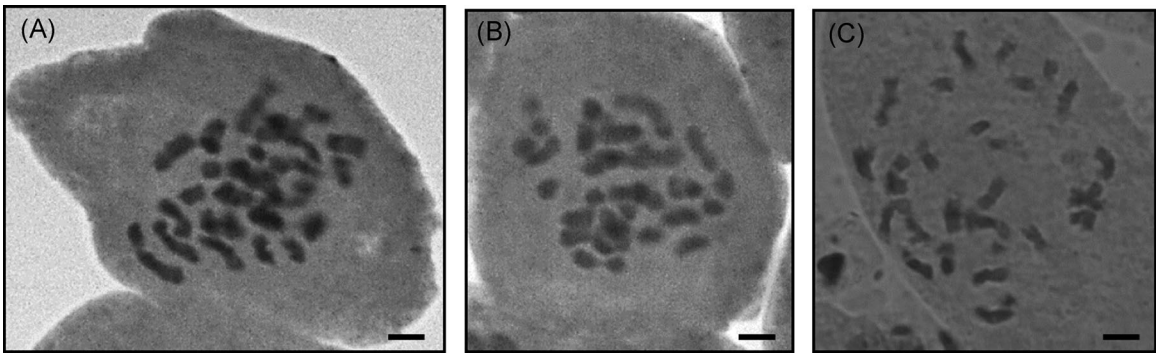

Fig. 3. Somatic chromosomes of *Symplocarpus renifolius.* **A**, *S. renifolius* (2*n* = 2*x* = 30) from Mt. Geumbyeong, Korea. **B,** *S. renifolius* (2*n* = 2*x* = 30) from Mt. Samak, Korea. **C**, *S. renifolius* (2*n* = 2*x* = 30) from Mt. Ungil, Korea. Scale bar = 0.1 μm.

(Cusimano et al., 2011), that is, Clade B, includes all diploids of *S. nipponicus*, *S. egorovii*, and *S. renifolius* in Korea. *Symplocarpus egorovii* was deeply embedded within *S. renifolius*, suggesting that the taxa should be merged. The other lineage, Clade A, underwent a polyploidization event in the early Pleistocene (node 7), and subsequently gave rise to the tetraploid *S. renifolius* (Fig. 2). After the polyploidy event, the *S. foetidus* in ENA and *S. nabekuraensis* in Japan were derived from the East Asian ancestor during the Pleistocene (node 10). Therefore, the species delimitations are incorrect and further research is needed.

Clade I consisted of all individuals of *S. foetidus* in eastern North America. Studies of Wen et al. (1996) and Nie et al. (2006b) showed that *S. foetidus* is monophyletic and is sister to *S. renifolius* from eastern Asia. However, the current study clearly indicated that the monophyletic *S. foetidus* (Clade I) is sister to Clade II containing the tetraploid populations of *S. renifolius* and *S. nabekuraensis* from Japan, rendering the tetraploid *S. renifolius* to be paraphyletic (Fig. 1). Although the cpDNA restriction site data (Wen et al., 1996) clearly distinguished *S. foetidus* in ENA from *S. renifolius* in EA (0.61% sequence divergence), species delimitation needs further investigation (Fernald, 1950; Li, 1979; Koyama, 1983). Nie et al. (2006b) also did not support the monophyly of *S. renifolius* as *S. foetidus* was embedded within the clade. We found divergent populations of *S. foetidus* from the east coast area (New York and Maine) and the Midwest area (Wisconsin and Ohio), separated by the northern Appalachian Mountains (i.e., the east coast subregion and the Midwest subregion of North America) to be differentiated. Many animal and plant species have shown a pattern of discontinuity across the Appalachian Mountains (Sewell et al., 1996; Church et al., 2003; Zamudio & Savage, 2003; Joly & Bruneau, 2004). This pattern of discontinuity in *S. foetidus* could be explained by the refugia theory (Soltis et al., 2006), but additional studies of *S. foetidus* based on a much broader sampling is required to test this hypothesis. Given the sister relationship between Clades I and II with moderate bootstrap/PP support, we cautiously hypothesize that the ENA *S. foetidus* might be derived from an ancestor closely related to the west coast populations of *S. renifolius* in Japan.

With our current sampling of molecular markers, the accessions of *S. nipponicus* did not form a clade but a grade (Fig. 1). Wen et al. (1996) showed the monophyly of *S. nipponicus* based on two samples collected from Korea and Japan. In addition, Nie et al. (2006b), based on the two same samples of Wen et al. (1996) and one additional sample from Korea, have shown the monophyly of *S. nipponicus*, but the relationship between populations from Japan and Korea was unresolved. Our study, based on a very thorough sampling scheme of Asian *Symplocarpus*, shows that the populations from Korea constitute a monophyletic group, whereas the ones from Japan do not form a clade (Fig. 1). The non-monophyly of the Japanese populations could be due in part to the slow substitution rate of the chloroplast markers from the recent divergence. However, the slow rate of cpDNA evolution might not be the sole explanation for the lack of support of the monophyly of *S. nipponicus* in Japan. Future studies based on different molecular markers (i.e., single- or low-copy nuclear genes) are needed. In addition to resolving relationships of *S. nipponicus* between Japan and Korea, it is also necessary to determine the phylogenetic relationships among lineages in Clade B to better understand the species delimitation and pollination syndromes (Fig. 1). We observed that most populations of *S. nipponicus* in Korea are found in high altitudes of an eastern province (i.e., Kangwon Province) along the main ridge of the Korean peninsula, which stretches across the Taebaek Mountains, with one exceptional population in the southern part (i.e., Jeollabuk Province). Aizawa et al., (2012) used the mtDNA diversity pattern of *Pinus koraiensis* Siebold & Zucc. in eastern Asia as the evidence for a glacial refugium to the pine populations of the species in Japan. The phylogeographic pattern of East Asian *Neolitsea sericea* (Blume) Koidz. (Lauraceae) seems to also support this refugia theory (Lee et al., 2013). Unlike the study of Aizawa et al. (2012), in which the Korean pine (*Pinus koraiensis*) are represented by a single mtDNA haplotype, compared to much greater level of mtDNA diversity in the Japanese populations, this study found similar levels of cpDNA diversity in the populations of *S. nipponicus* in Japan and Korea. Given the comparable number of chlorotypes found between populations in Japan and Korea, our preliminary data do not provide support for the refugial theory for *S. nipponicus*. Rather, our results support the idea that the Korean peninsula could have been one of the important refugia during the Quaternary (Kim et al., 2013). A phylogeographic study of *S. nipponicus* based

on extensive sampling is required to better understand the biogeographic history of this species in East Asia.

The taxonomic status of *S. egorovii* remains uncertain based on this study. The cpDNA phylogeny showed that *S. egorovii* is embedded within the strongly supported clade containing the diploid populations of *S. renifolius* in Korea (Clade V; Fig. 1). Pavlova & Nechaev (2005) described *S. egorovii* as a new species based on its distinct morphological characteristics and its habitat. However, the chromosome number of *S. egorovii* ($2n = 30$) is the same as that of *S. renifolius* in Korea, whereas *S. renifolius* in Japan is tetraploid. In addition, our preliminary morphological and ecological comparisons between the two species suggested that they could be considered as conspecific populations. Further morphological and molecular investigations on diploid populations of *S. renifolius* from Korea and the Russian Far East and *S. egorovii* from southern Russian Far East could shed light into species circumscription of the recently described species of *Symplocarpus*.

The genus *Lysichiton* was well supported to be monophyletic (Fig. 1). However, we did not detect any significant phylogenetic structure within either of the two species using our DNA markers. This result could be due to inclusion of fewer populations compared to the ones sampled for *Symplocarpus*. The accessions of *Lysichiton* sampled in California formed a clade with moderate support. We suggest that the Californian population might have been isolated from other western North American populations by the Klamath Mountains; some climate differences were noted between California (mostly hot-summer Mediterranean) and Oregon and Washington (warm-summer Mediterranean and humid subtropical) according to the Köppen–Geiger climate classification (Peel et al., 2007). A detailed phylogeographic study could enlighten the intracontinental diversification history of *Lysichiton*.

4.2 Estimated divergence times and historical biogeography of Orontioideae

For the first time, all species of Orontioideae with extensive sampling were included for molecular dating (Fig. 2). We found the stem node to be 73.1 Ma (72.04–76.89 Ma, 95% HPD) and the crown node at 39.9 Ma (19.03–69.32 Ma, 95% HPD). These are much younger ages than Nie et al. (2006b), but within the range of the results of Nauheimer et al. (2012) who used multiple fossils in the Araceae. This coincides with the Eocene–Oligocene cooling period (Liu et al., 2009). During the Eocene–Oligocene cooling, thermophilic evergreen taxa became restricted to favorable sites in Europe and North America and thus it is plausible that the range contraction due to global cooling was responsible for the splitting of the genera (Tiffney & Manchester, 2001). The split between *Lysichiton* and *Symplocarpus* was estimated to be 17.99 Ma (7.06–35.91 Ma, 95% HPD) (node 2; Fig. 2). The common ancestor of these two genera was inferred to have originated from eastern Asia. These results are congruent with the prediction of the origin of the two species (*S. foetidus* and *L. americanus*) in North America (Nie et al., 2006b). Wen et al. (2010) reviewed the biogeographic disjunctions of seed plants, and suggested that many temperate forest species originated and distributed in Asia, with subsequent migration to other continents. Moreover, it is suggested that the direction of migration is predominantly from EA to ENA in the last 30 million years (Nie et al., 2006a; Wang et al., 2007; Jiao & Li, 2009; Xie et al., 2010). Based on our molecular dating analysis, we hypothesized that the divergence of two main lineages within genus *Symplocarpus*, which correspond to different ploidy levels (diploid vs. tetraploid), began during the Miocene (node 4; Fig. 2). The crown age of the two *Lysichiton* species was also in the early Pliocene (node 3; Fig. 2), suggesting that this should be considered as a major split within the genus. It is congruent with the previously known age (7.18 Ma; Nie et al., 2006b; Wen et al., 2016), and it agrees with the hypothesis of migration from EA to WNA by way of the Bering land bridge, which worked as a migration path between the continents of Asia and North America.

During the Miocene, Orontioideae underwent major speciation events (Fig. 2). Wen et al. (2010) mentioned that the EA–ENA disjunct groups have a general pattern of relatively young crown ages, but much older stem ages, and our study also supports their findings. A number of previous geological studies have found that the climate during the Miocene was more humid and warmer than today (Zachos et al., 2001; Shevenell et al., 2004; Steppuhn et al., 2006; Knorr et al., 2011). The gradual warming into the early Miocene resulted in the expansion of several thermophilic and evergreen lineages in the Northern Hemisphere (Mai, 1995; Wing, 1998; Jiménez-Moreno et al., 2010). With an extensive sampling strategy, Zuo et al. (2017) also estimated the age of intercontinental disjunction of *Panax* L. in the mid-Miocene and inferred that the core clade of *Panax* originated from EA and migrated into ENA. Based on the phylogenetic structure, the possible polyploidization event of *Symplocarpus* in Clade A occurred in eastern Asia between 8.78 and 2.27 Ma (node 4 and node 7, respectively). After the polyploidization event, the intercontinental disjunct event (between node 9 and node 10) might have led to the origin of *S. foetidus* in the Pliocene. Within Clade B that comprises *S. renifolius*, *S. egorovii*, and *S. nipponicus*, a major split could have played a significant role in the speciation with physiological and morphological adaptation into early flowering (March to May) and larger spathes (node 14), and late flowering (July to August) and smaller spathes (node 13). However, although node 8 is well supported, node 13 lacks the support. We suggest that this relationship is not yet resolved. A single origin for the exothermic spadices in *Symplocarpus* was suggested based on previous cpDNA restriction site analysis (Wen et al., 1996); the direction of pollination mechanism shift is purportedly from ancestral non-exothermic spadices in *S. nipponicus* to exothermic spadices in *S. renifolius*. However, our study suggests that it is uncertain whether the common ancestor of *Symplocarpus* had exothermic spadices and the number of evolutionary origin of pollination shift from the non-exothermic to the exothermic spadices or vice versa. If we assume that the common ancestor had non-exothermic spadices, it requires two independent evolutionary gains of exothermic spadices (i.e., one in the diploid lineage of *S. renifolius* from Korea in Clade B and the other in the tetraploid lineage of *S. renifolius*, *S. nabekuraensis*, and *S. foetidus* in Clade B) (Fig. 1). However, it is also possible that the common ancestor of subfamily

Orontioideae (*Symplocarpus*, *Lysichiton*, and *Orontium*) had non-exothermic spadices and *S. nipponicus* either reverted back to the ancestral characteristics or retained the symplesiomorphic characteristics after the origin of exothermic spadices in *S. renifolius*. This scenario also requires two evolutionary steps. It is critical to investigate carefully the pollination mechanism of the sister genus *Lysichiton* as well as genus *Orontium*, which is sister to the clade of *Lysichiton* and *Symplocarpus*, to fully understand the evolution of exothermic spadices in the subfamily Orontioideae.

4.3 Different ploidy levels in Orontioideae

Within Orontioideae, polyploidy was previously reported in the genus *Symplocarpus* (Blair, 1975; Sokolovskaya & Probatova, 1985; Iwatsubo & Otsuka, 2005). Polyploidy in *Symplocarpus* could explain the emergence of more species in the genus as compared to other genera in Orontioideae (Wood et al., 2009). However, among these polyploids, *S. renifolius* seemed to represent two different lineages. *Symplocarpus renifolius* in Korea shared its most common ancestor with the diploid species *S. nipponicus*. This raised the question of whether *S. renifolius* in Korea has the same chromosome numbers as the ones in Japan. We hypothesized that *Symplocarpus* has two major evolutionary lineages according to the ploidy levels: tetraploid ($2n = 60$) and diploid ($2n = 30$). We suggest that *S. renifolius* in Korea ($2n = 30$) and *S. renifolius* in Japan ($2n = 60$) have distinct origins. Several reproductive traits also differ between the Japanese and the Korean populations of *S. renifolius*, for example, the spathe color patterns of *S. renifolius* in Korea include irregular spots, lines, and splotches in shades of green, maroon, and purple, whereas the spathe of *S. renifolius* in Japan is uniformly dark purple or red without the irregular spots or lines (Lee JS, 2014, unpublished data). In addition, *S. renifolius* in Japan has a somewhat elongated spadix, which is congruent with the species description found in *Flora of China* (http://efloras.org), whereas *S. renifolius* in Korea has a round or spherical spadix. The flower number and size on spadix also differ; populations of *S. renifolius* in Japan have smaller and more numerous flowers on spadix than the ones in Korea. The increased ploidy level has an immediate effect resulting in larger cell sizes than those of diploids (Stebbins, 1971). Morphologically, a larger cell and larger genome often correlate with larger flower size and seeds (Garbutt & Bazzaz, 1983). Further detailed morphological study is needed to support the differentiation of the two distinct lineages identified based on the cpDNA phylogeny. Also, the possibility of coexistence of diploid and tetraploid *S. renifolius* in Korea and Japan could not be ruled out and thus further cytological investigation is required.

5 Conclusions

Phylogenetic relationships among the eight species in subfamily Orontioideae were elucidated based on the extensive sampling of 198 accessions from six regions in this study. Phylogenetic analysis based on six concatenated cpDNA non-coding regions supports two major lineages within *Symplocarpus*, which corresponded to differences in the ploidy level (tetraploid vs. diploid). The hypothesis of intercontinental disjunct distribution of *Symplocarpus* and *Lysichiton* mediated by the Bering land bridge from EA to ENA/WNA was supported, and the timing of the disjunct event was estimated to be in the Pliocene and the Pleistocene. *Symplocarpus renifolius* in Japan is more closely related to *S. foetidus* in ENA than to its conspecific populations in EA. The two recently described species, *S. nabekuraensis* from Japan and *S. egorovii* from the Russian Far East, are deeply embedded within *S. renifolius* in Japan and *S. renifolius* in Korea, respectively. *Symplocarpus renifolius* in Korea shares its most recent common ancestor with the diploid *S. nipponicus* in Korea and Japan instead of the conspecific populations in Japan. Additional morphological and cytological studies are required to better understand the taxonomic distinction between populations of *S. renifolius* in Japan and Korea. Finally, the several novel findings in this study require further confirmation based on independent nuclear gene genealogies and/or population genetics approaches.

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12498/suppinfo:

Table S1. Specimens included in this study, with locality and voucher information. Vouchers were deposited at Sungkyunkwan University (SKK), Tohoku University (TUSG), and Australian National herbarium (CANB).

Table S2. GenBank accession numbers.

Table S3. Summary of estimated divergence times and ancestral area reconstruction using Yule process speciation model.