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Intercontinental biogeography of subfamily Orontioideae (*Symplocarpus*, *Lysichiton*, and *Orontium*) of Araceae in eastern Asia and North America

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Abstract

Symplocarpus, *Lysichiton*, and *Orontium* (Orontioideae) are three of the few north temperate genera of the primarily tropical Araceae. *Symplocarpus* is disjunctly distributed in eastern Asia (3 spp.) and eastern North America (1 sp.); *Lysichiton* has an intercontinental discontinuous distribution in eastern Asia (1 sp.) and northwestern North America (1 sp.); and the monotypic *Orontium* is restricted to eastern North America. Phylogenetic analysis of the *trn*L–F and *ndhF* sequences supports (1) the monophyly of both *Symplocarpus* and *Lysichiton*, (2) the sister-group relationship of *Symplocarpus* and *Lysichiton*, and (3) the clade of *Orontium*, *Symplocarpus*, and *Lysichiton*. Although *Symplocarpus* shows a much wider disjunction than *Lysichiton*, the estimated divergence time of the former [4.49 ± 1.69 or 6.88 ± 4.18 million years ago (mya)] was similar to that of the latter (4.02 ± 1.60 or 7.18 ± 4.33 mya) based on the penalized likelihood and the Bayesian dating methods, respectively. Eastern Asia was suggested to be the ancestral area of the *Symplocarpus–Lysichiton* clade based on the dispersal–vicariance analysis. Our biogeographic results support independent migrations of *Symplocarpus* and *Lysichiton* across the Bering land bridge in the late Tertiary (Pliocene/late Miocene). Fossil evidence suggests Orontioideae dated back to the late Cretaceous in the temperate Northern Hemisphere (72 mya). The relative rate test shows similar substitution rates of the *trn*L–F sequences between the proto and the true aroids, although the latter has substantially higher species diversity. The proto Araceae perhaps suffered from a higher rate of extinction in the temperate zone associated with periods of climatic cooling in the Tertiary.

Keywords: Araceae; Disjunction; Eastern Asia; Eastern North America; *Orontium*; *Lysichiton*; *Symplocarpus*; Western North America

1. Introduction

The biogeographic disjunction between eastern Asia (EAS) and eastern North America (ENA) is a well-known intercontinental disjunct pattern in the Northern Hemisphere (Li, 1952; Manos and Donoghue, 2001; Tiffney, 1985a,b; Wen, 1999). The disjunction between EAS and western North America (WNA) is, however, less common in plants despite their closer physical connection via the Bering land bridge (Donoghue and Smith, 2004; Hong, 1993; Wu, 1983), which is at odds with the findings in Sanmartín et al. (2001) in the broad comparison of Northern Hemisphere animal groups. Divergence patterns between EAS and WNA are poorly understood in plants. Only a few genera have been examined for the evolution of the disjunction between EAS and WNA, including *Kelloggia* Torrey of Rubiaceae (Nie et al., 2005), *Oplopanax* (Miq.) Miq. (Lee and Wen, 2002) of Araliaceae, and *Glehnia* Schmidt ex Miq. of Apiaceae (Sun et al., 2004). *Kelloggia* and *Oplopanax* were each supported to be monophyletic and estimated to have diverged between the two continents ca. 5.42 and

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5.6 mya, respectively, while the two species of *Glehnia* were found to be biphyletic. A wide range of divergence times among the disjunct groups between EAS and North America (Donoghue et al., 2001; Wen, 1999) supports multiple origins of the patterns and the complexity of the disjunctions in the Northern Hemisphere.

Orontioideae is a subfamily of Araceae described by Mayo et al. (1997), which includes only three genera (*Symplocarpus* R.A. Salisbury ex Nuttall, *Lysichiton* Schott, and *Orontium* L.) and seven species. *Symplocarpus* and *Lysichiton* are excellent examples of species disjunctions between the Old and the New World showing both the EAS—ENA and the EAS–WNA disjunctions (Fig. 1). How the ENA endemic *Orontium* is related to the *Symplocarpus* and *Lysichiton* may represent one additional intercontinential disjunction. These genera are considered to be primitive groups in Araceae and were previously placed in Calloideae, which also includes *Calla* (Barabé and Forget, 1987; Grayum, 1990) or in Lasioideae (Bogner and Nicolson, 1991; Hay and Mabberley, 1991). Mayo et al. (1997) recognized four synapomorphies for Orontioideae: expanded nonlinear leaf blade, anatropous or hemianatropous ovules, endosperm sparse to absent, and base chromosome number $x = 13$. Orontioideae was sister to the monotypic subfamily Gymnostachydoideae (*Gymnostachys* R. Brown) from Australia based on cpDNA restriction site analysis, and *trn*L–F sequence data (French et al., 1995; Tam et al., 2004). Orontioideae and Gymnostachydoideae are referred to as the proto Araceae or proto aroids (Mayo et al., 1997). The remaining species of Araceae (ca. 3300 species) are known as the true Araceae or true aroids.

Species of *Symplocarpus* are rhizomatous perennials with a short and subglobose spadix hidden within a well-differentiated spathe. The three species: *S. foetidus* (L.) Salisb. ex W. Barton from ENA, *S. nipponicus* Makino, and *S. renifolius* Schott ex Tzvelev from EAS usually occur in swampy habitats (Mayo et al., 1997; Wen et al., 1996) (Fig. 1). Recently, a new species, *S. nabekuraensis* Otsuka & K. Inoue, was reported from northern Nagano of central Japan (Otsuka et al., 2002). *S. nipponicus* and *S. renifolius* from EAS were sometimes regarded as conspecific with *S. foetidus* from ENA (e.g., Fernald, 1950; Li, 1979; Wilson, 1960). However, Wen et al. (1996) reported that they can be easily distinguished from each other based on cpDNA restriction site data and morphology.

Lysichiton (the western skunk cabbage) contains only two species in swampy areas and wet forests of the northern Pacific coasts of EAS and WNA (Fig. 1). Species of *Lysichiton* are rhizomatous perennials with a sub-cylindric spadix, a long stipe and a white or yellow spathe. The genus was previously considered to be monotypic containing only *L. camtschatcensis* (L.) Schott, which is now the valid name for the EAS species (Mayo et al., 1997). *L. camtschatcensis* occurs only in Japan and the Russian Far East. *L. americanus* Hultén & H. St. John is widespread and common in wet forests and muskegs from Alaska to northern California and ranges eastward in Canada as far as the Selkirk Mountains (Hultén, 1968). The two species are morphologically similar, and differ primarily in the spathe color (white in *L. camtschatcensis*, and yellow in *L. americanus*, Mayo et al., 1997). *Orontium* (the golden club) is monotypic consisting of *O. aquaticum* L. from ENA. It is a perennial aquatic herb with deeply sunken rhizomes, oblong-elliptic simple leaves, a golden yellow spadix held above the water level, and an inconspicuous simple spathe (Klotz, 1992; Mayo et al., 1997; Wilson, 1960). It has been regarded as a morphologically isolated taxon (Grear, 1966) and its close relationship to *Symplocarpus* and *Lysichiton* has been suggested by the morphological cladistic analysis (Mayo et al., 1997), the cpDNA restriction site analysis

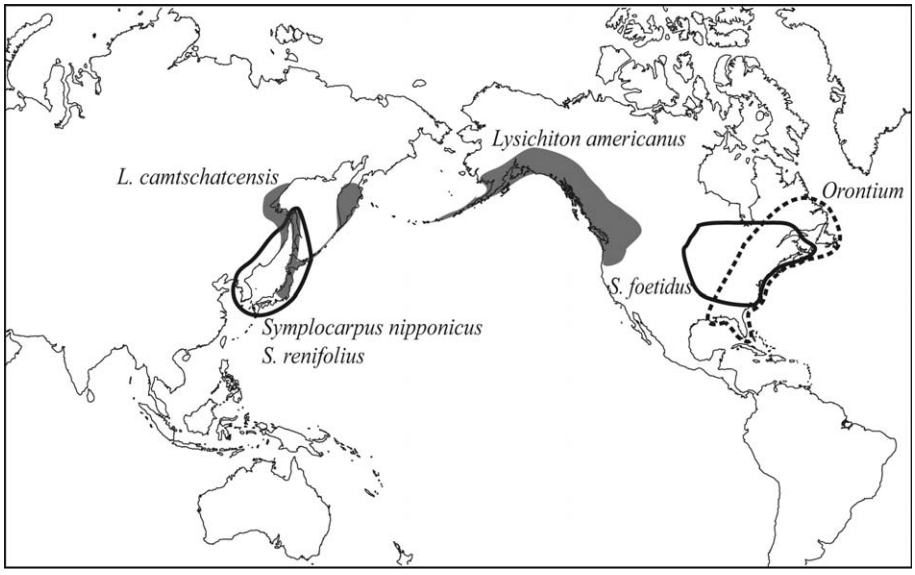

Fig. 1. Distribution of Orontioideae (*Symplocarpus*, *Lysichiton*, and *Orontium*) of Araceae showing disjunctions between eastern Asia and North America. The shaded area represents distribution of *Lysichiton*, area within the solid lines is for *Symplocarpus*, and the dashed lines are for *Orontium*.

(French et al., 1995), and the *trn*L–F sequence data (Tam et al., 2004).

Symplocarpus, *Lysichiton*, and *Orontium* (Orontioideae) are three of the eight genera which occur in the north temperate zone of Araceae (not including Lemnaceae), a predominantly tropical family comprised of more than 105 genera and 3300 species of herbs and vines, with *Arisaema* Mart. distributed in both temperate and tropical regions (Grayum, 1990; Mayo et al., 1997). Other genera include *Calla* L., *Pinellia* Ten., *Arum* L., and *Peltandra* Raf. Raven and Axelrod (1974) hypothesized Araceae to have a "West Gondwanalandic–Laurasian" distribution. Grayum (1990) suggested that Araceae may have "originated in temperate or subtropical Laurasia and then reached West Gondwanaland via Eurasia" perhaps in the late Cretaceous. Li (1986) also argued for a southern Laurasian origin for the family. Biogeographic analysis of Orontioideae from the proto aroids may provide important insights into the early evolution of Araceae and its diversification in the north temperate zone.

Objectives of the present study are to (1) construct the phylogenetic relationships of the subfamily Orontioideae and (2) estimate the divergence times of the biogeographically disjunct lineages and the ancestral areas of the clades to construct the biogeographic diversification history of this temperate subfamily in the Northern Hemisphere.

2. Materials and methods

2.1. Plant material, DNA extraction and amplification, and sequence alignment

We sampled all species from the Orontioideae except the newly described *Symplocarpus nabekuraensis* (Table 1). *Calla palustris* L., *Arisaema triphyllum* (L.) Torr., and *Arisaema* sp. of Araceae were also sequenced for the phylogenetic and biogeographic analyses in the broader context (Table 1), except that the *trn*L–F sequence of *Arisaema triphyllum* was obtained from GenBank (AY248958). Sequences of *Orontium aquaticum* L. (AY398577, AY224991), *Gymnostachys anceps* R. Brown (AY398579, AY191196), *Spathiphyllum wallisii* Regel (AY054738, AY007658), *Philodendron callosum* K. Krause (AY555163), *Dracontium polyphyllum* L. (AY054727), *Monstera obliqua* Miq. (AY398548), and *Pothos ovatifolius* Engl. (AY398573) from Araceae were obtained from GenBank in our analysis (Fig. 2). *Tofieldia glutinosa* (Michx.) Pers. (AY145337, AF547023) of Tolfieldiaceae from Alismatales was selected as the outgroup, because its close relationship with Araceae as shown in Chase et al. (2000).

Genomic DNA was extracted from 15 mg silica-gel dried leaf material using the modified CTAB method of Doyle and Doyle (1987) or DNeasy plant mini kits (QIAGEN, Mississauga, Ont., Canada) following the manufacturer's instruction. Standard polymerase chain reaction (PCR) procedures were used to amplify the target gene region. The *trn*L–F region was amplified using primers "c" and "f" or in two shorter fragments using primers of "c" + "d" and "e" + "f" as described in Taberlet et al. (1991). The amplification of the *ndhF* gene followed the protocol of Bremer et al. (2002). The amplified products were then purified using the GELase Agarose (Epicentre Technologies, Madison, WI, USA) or polyethylene glycol (PEG) precipitation following the manufacturer's protocols. Cycle sequencing was conducted using BigDye3.1 reagents with an ABI 3100 or 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were then aligned with ClustalX version 1.83 (PC version,

Table 1
Voucher information of *Symplocarpus*, *Lysichiton*, *Orontium*, and other taxa used in the molecular analysis with GenBank accession numbers

Taxon	Voucher	Locality	*trn*L–F	*ndhF*
Symplocarpus foetidus (L.) Salisb. ex W. Barton	*Wen 6278* (US)	USA: Maryland	DQ400865	DQ400880
	Wen s.n. (A)	USA: Massachusetts	DQ400866	DQ400881
	Wen 6238 (US)	USA: Virginia	DQ400872	DQ400887
S. renifolius Schott ex Tzvelev	*Wen 2468* (US)	Japan: Nikko	DQ400868	DQ400883
	Yonekura 11570 (US)	Japan: Honshu	DQ400867	DQ400882
S. nipponicus Makino	*H. Ohashi 6702* (A)	Japan: Honshu	DQ400871	DQ400886
	Yoo s.n. (US)	Korea	DQ400869	DQ400884
	C.-W. Park s.n.	Korea	DQ400870	DQ400885
Lysichiton americanus Hultén & H. St. John	*Wen 7223* (US)	USA: Alaska	DQ400863	DQ400878
	L.P. Janeway 7927 (US)	USA: California	DQ400864	DQ400879
L. camtschatcensis (L.) Schott	*Soejima 1020* (US)	Japan: Hokkaido	DQ400861	DQ400876
	H. Ohashi 6701 (A)	Japan: Honshu	DQ400862	DQ400877
Orontium aquaticum L.	*Li 20554* (US)	USA: North Carolina	DQ400873	DQ400888
Calla palustris L.	*Wen* 7299 (F)	USA: Wisconsin	DQ400874	DQ400889
Arisaema sp.	*Nie & Meng 435* (KUN)	China: Yunnan	DQ400875	DQ400891
Arisaema triphyllum (L.) Torr.	*Wen 7294* (F)	USA: Illinois	—	DQ400890

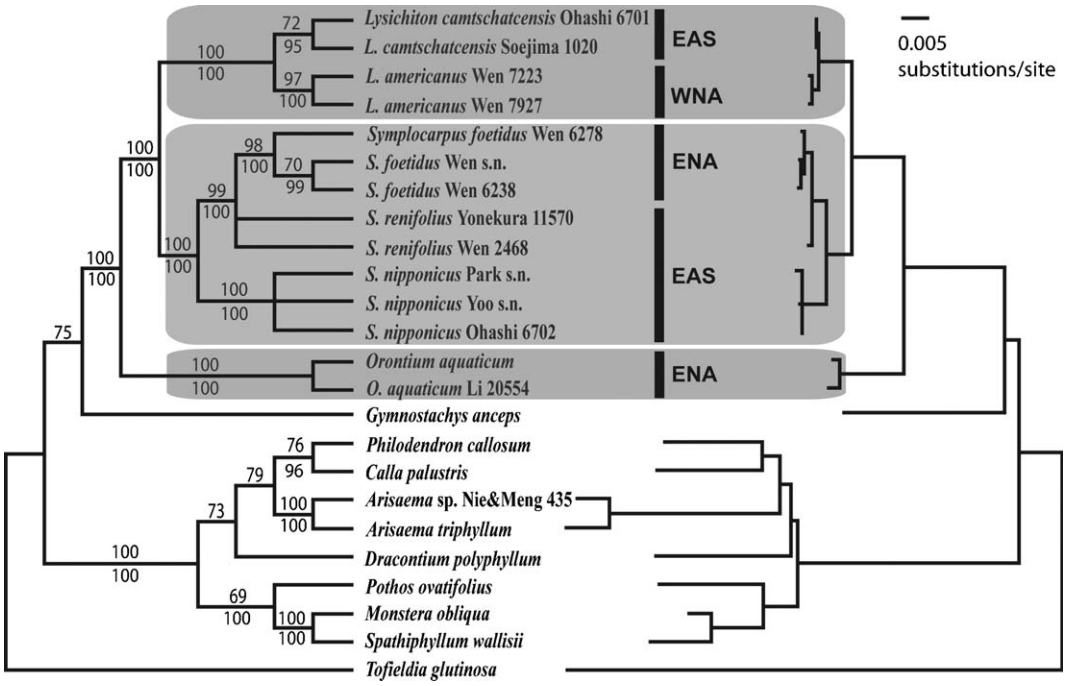

Fig. 2. Strict consensus (left) and maximum likelihood (right) trees of Orontioideae (Araceae) based on combined *trn*L–F and *ndhF* data (tree length = 1029 steps, CI = 0.85, RI = 0.89, and RC = 0.75). The bootstrap values in 1000 replicates are shown above the lines and the Bayesian Markov chain Monte Carlo (MCMC) posterior probabilities higher than 95% are indicated under the lines (EAS: eastern Asia, ENA: eastern North America, and WNA: western North America).

Thompson et al., 1997) and followed by manual adjustments. The *trn*L–F regions with ambiguous alignment were excluded from the analysis. Gaps were either coded as presence (1) and absence (0) in our study or treated as missing data. When it was difficult to decide upon homology, the gap or indel was coded with a question-mark according to Eriksson et al. (2003).

2.2. Phylogenetic analysis

Phylogenetic analyses were performed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (Rannala and Yang, 1996). Parsimony analyses used heuristic searches with 10 random taxon addition replicates in PAUP* 4.0b10 (Swofford, 2003). The bootstrap support (BS) for the clades (Felsenstein, 1985) revealed in the maximally parsimonious tree(s) (MPTs) was examined with 1000 bootstrap replicates with the heuristic search options using parsimony. The TVM + G model of DNA substitutions for the maximum likelihood analysis was determined using Modeltest version 3.6 (Posada and Buckley, 2004; Posada and Crandall, 1998). Bayesian inference was conducted using MrBayes version 3.0 (Huelsenbeck and Ronquist, 2001) with the model as estimated above. The MCMC algorithm was run for 2,000,000 generations with four incrementally heated chains, starting from random trees and sampling one out of every 100 generations. Internodes with posterior probabilities $\geqslant$95% were considered statistically significant.

2.3. Biogeographic analysis

We initially estimated the divergence times using a large data set of *trn*L–F sequences sampled across Araceae because many *trn*L–F sequences are available from GenBank. Due to problems associated with the zero-length branches of clades in *Lysichiton* and *Symplocarpus*, we used the combined *trn*L–F and *ndhF* data in the biogeographic analysis of the Orontioideae. This clade also has reliable fossil data to serve as calibration points (see fossil introduction below). The maximum likelihood tree (Fig. 3) was used in the age estimates. The likelihood ratio test (Felsenstein, 1988) suggested that rate constancy in this data set was not supported as clocklike. We therefore employed both the penalized likelihood (PL, Sanderson, 2002) and the Bayesian dating methods (Thorne and Kishino, 2002; Thorne et al., 1998) to estimate divergence times.

Penalized likelihood is a semiparametric smoothing method and permits deviations from a molecular clock. It uses cross-validations to determine the best level of clock enforcement (''smoothing'') with the given data (Sanderson, 2002, 2003). The cross-validation analysis and time estimates of nodes were performed under a truncated newton algorithm with the program r8s version 1.60 (Sanderson, 2003). Confidence intervals around the age estimates were calculated using nonparametric bootstrapping (Baldwin and Sanderson, 1998).

Bayesian approach uses a probabilistic model to describe the change in evolutionary rates over time and uses the MCMC procedure to derive the posterior distribu-

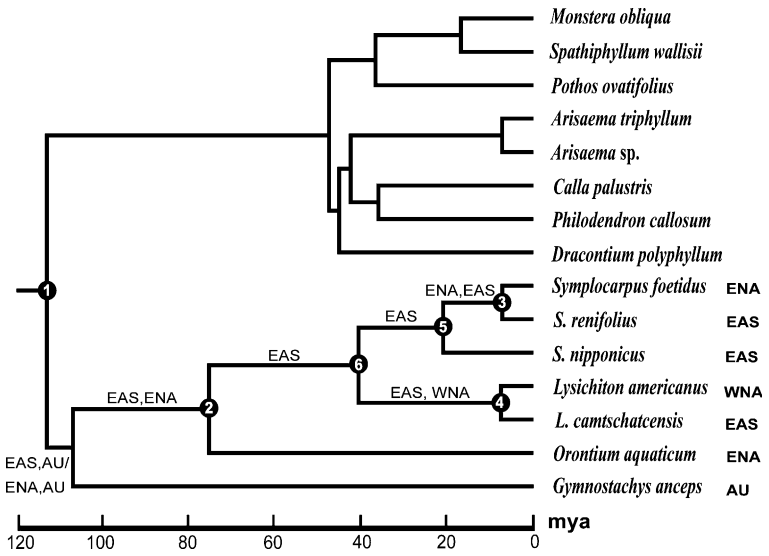

Fig. 3. Chronogram of Orontioideae and other representative taxa from Araceae based on the maximum likelihood tree of *trn*L–F and *ndhF* data, with results of the dispersal–vicariance (DIVA) analysis of the proto aroids. Divergence times were estimated using the Bayesian method with internal age constraints enforced (node 1 was constrained to 120 mya, and node 2 to 72 mya). The optimal distributions are shown above the line; equally optimal distributions are separated with a slash (EAS: eastern Asia, WNA: western North America, ENA: eastern North America, and AU: Australia).

tion of rates and time (Thorne et al., 1998; Thorne and Kishino, 2002; Yang and Yoder, 2003). This parametric approach relaxes the assumption of a strict molecular clock with a continuous autocorrelation of substitution rates across the phylogeny and allows the use of several calibrations/time constraints. The MCMC parameters were: 10,000 Markov chain samples, 100 cycles, and 100,000 burn-in cycles. The mean of the prior distribution for the time separating the ingroup root from the present was set to 100 with a standard deviation of 50. The prior distribution for the rate at the ingroup root node (rtrate) was set to 0.008, based on Thorne's recommendation that it be calculated by dividing the median distance between the ingroup root and the ingroup tips obtained from Estbranches by the time unit. The standard deviation of rtrate was set to 0.004. The largest value of the time unit between the root and the tips was set to 120 mya. Brownmean was set to 0.01, following the manual's recommendation. The standard deviation on brownmean (brownsd) was also set to 0.01. We repeated each analysis twice to ensure that the Markov chains were long enough to converge.

Fossils of Araceae are known from seeds, fruits, leaves, pollen, and infructescences (Gregor and Bogner, 1984, 1989; Mayo et al., 1997; Bogner et al., 2005; Wilde et al., 2005). Our study focused on the proto Araceae, especially on Orontioideae, which has a reliable fossil infructescence showing similarities to, but distinct from *Symplocarpus* from the late Cretaceous (the Campanian Stage) of Canada. Each fruit of the fossil has three seeds with a ribbed testa (Bogner et al., 2005). But in extant *Symplocarpus* the berries are one-seeded and the testa is smooth (Wilde et al., 2005). It was described as *Albertarum pueri* and assigned to Orontioideae with an absolute age of approximately 72 mya. In both penalized likelihood and Bayesian methods, the Orontioideae clade (clade 2 in Fig. 3) was thus constrained to a minimal age of 72 mya; and the root of Araceae (clade 1 in Fig. 3) was constrained to 120 mya based on the fossil species *Mayoa portugallica*, a highly characteristic in aperturate, striate fossil pollen described from the Early Cretaceous (110–120 my) of Torres Vedras in the Western Portuguese Basin (Friis et al., 2004). It was assigned to the tribe Spathiphylleae (subfamily Monsteroideae) of Araceae.

We used the dispersal–vicariance (DIVA) analysis (Ronquist, 1997) to infer the biogeographic diversification of *Symplocarpus* and *Lysichiton* together with other proto Araceae (including *Orontium* and *Gymnostachys*), based on the maximum likelihood topology (Fig. 3). DIVA reconstructs ancestral areas by minimizing the number of vicariance, dispersal, and extinction events needed to explain the distribution patterns. We were especially interested in inferring the ancestral areas of the intercontinentally disjunct *Symplocarpus* (EAS or ENA) and *Lysichiton* (EAS or WNA). Four areas of endemism were circumscribed according to the geographic distribution of the proto Araceae (Fig. 3): (A) EAS, (B) ENA, (C) WNA, and (D) Australia. We used DIVA 's default cost matrix, which assigns a cost of 1 to dispersal and extinction events, and 0 to vicariance. Because there are no modern proto aroid species distributed in multiple areas of endemism, we constrained our search to a maximum of two inferred ancestral areas by following the suggestion of Ronquist (1996, 1997) and Donoghue et al. (2001). The DIVA analysis was implemented with the computer program DIVA version 1.1 (Ronquist, 1996).

2.4. Relative rate test between proto and true aroids

The proto and the true aroids demonstrate drastically different species diversity, with the primarily tropical true aroids comprising about 3300 species, and the temperate proto aroids with seven species. We are particularly interested in whether extinction is responsible for the low species diversity in the proto aroids. We thus performed the relative rate tests between the two lineages (Li and Bousquet, 1992; Takezaki et al., 1995) using the program RRTree (Robinson et al., 1998; Robinson-Rechavi and Huchon, 2000) based on both the Jukes–Cantor one-parameter (Jukes and Cantor, 1969) and Kimura two-parameter distances (Kimura, 1980). Results from these two distances are almost similar to each other. A data set of 117 noncoding *trn*L–F sequences broadly sampled from Araceae was used for the relative rate test (see Appendix A). Our sampling included six of the seven species of the proto aroids, and 107 samples of the true aroids representing all major groups of Araceae (Mayo et al., 1997; Keating, 2003; Tam et al., 2004). Because of the very long branch length and the possibly higher rate of DNA substitutions in *Lemna* (now in Araceae, previously in Lemnaceae; Rothwell et al., 2004), we also performed an alternate test excluding *Lemna* from the data set.

In general, it is preferred that the closest outgroup should be used in the relative rate test (Robinson et al., 1998). Araceae are closely related to Tofieldiaceae (Chase et al., 2000; Tam et al., 2004) and we thus used *T. glutinosa* as the outgroup for the relative rate test. We also explored the use of alternative outgroups. In a second analysis, *Potamogeton pusillus* L. from Alismatales was used as an alternative outgroup. Furthermore, we did the relative rate test with both *T. glutinosa* and *P. pusillus* as the outgroups.

3. Results

The alignment of *trn*L–F sequences of *Symplocarpus* and *Lysichiton* with the outgroups generated a data matrix of 1097 positions excluding 207 bp (from position 355 to 561) because of difficulties in aligning that part of the sequences. Nineteen indels were coded as separate binary characters. The length of the indels varied from a single base up to 117 base pairs (bp). Sequences of the proto and the true aroids also differed in two large indels in the *trn*L–F region. A large deletion of 117 bp was detected in the proto aroids (excluding *Gymnostachys*) at positions 932–1048. Another deletion was found in the true aroids (55 bp, at positions 96–150). The *ndhF* matrix comprised 2074 aligned positions. Because there is no recombination within the chloroplast genome, we combined the two cpDNA data sets (*trn*L–F and *ndhF*). A partition homogeneity test between the two data sets also supports combining the two data sets ($P = 0.96$). The combined data set had 3190 positions, 403 of which were parsimony informative. When gaps were treated as missing data, three MPTs were generated with a length of 1029 steps, a consistency index (CI) of 0.85 (CI excluding uninformative characters = 0.76), a retention index (RI) of 0.89, and a rescaled consistency index (RC) of 0.75. The strict consensus tree with BS and posterior probability for each clade was shown in Fig. 2. The phylogenetic analysis supported the monophyly of *Symplocarpus* (BS = 100%), *Lysichiton* (BS = 100%), and the *Symplocarpus–Lysichiton* clade (BS = 100%). The close relationship of the two disjunct species of *Symplocarpus* (*S. renifolius* from EAS and *S. foetidus* from ENA) was also well supported (BS = 99%), and this *S. foetidus–S. renifolius* clade was sister to the eastern Asian *S. nipponicus* (Fig. 2).

Results of both the PL and Bayesian divergence time estimates are presented in Table 2 and the chronogram based on the Bayesian method is shown in Fig. 3. Using the smoothing value of 100 as obtained from the cross-validation procedure in the r8s program, the PL analysis estimated the divergence between *S. renifolius* and *S. foetidus* (node 3) to be 4.49 ± 1.69 mya, and 4.02 ± 1.60 mya for the disjunction of *L. americanus* and *L. camtschatcensis* (node 4 in Fig. 3). Their divergence times were estimated to be 6.88 ± 4.18 mya for node 3 and 7.18 ± 4.33 mya for node 4 with the Bayesian method. The crown group of *Symplocarpus* was 15.54 ± 2.52 or 20.65 ± 6.44 mya, which is older than that of *Lysichiton* (4.02 ± 1.60 or 7.18 ± 4.33 mya). The divergence between *Symplocarpus* and *Lysichiton* was 30.68 ± 3.66 (PL) or 40.41 ± 7.63 (Bayesian) mya.

The DIVA analysis suggested an initial diversification of *Symplocarpus* and the *Symplocarpus–Lysichiton* clade in EAS, with subsequent and independent movement into ENA and WNA (Fig. 3). The North American species from the two genera were thus suggested to have migrated from EAS, independently via the Bering land bridge, which was a viable migration route at that time in the late Tertiary, whereas the North Atlantic land bridge (Tiffney, 1985b) was no longer available for plants by then.

The program RRtree was used to test the differences in substitution rates of the 117 *trn*L–F sequences between the proto and the true aroid clades. Using *T. glutinosa* as the outgroup (Appendix A), the two groups showed a significant difference in the nucleotide substitution rates, with the weighted mean distance (K) of 0.0757286 for the proto aroids and 0.104403 for the true aroids (difference in K values (dK) = 0.0286747, $dK/SD = 2.51884$, $P = 0.01$). When *Lemna* was excluded from the true aroid clade, no significant difference was detected between the two clades, (K value as 0.110616 for the proto aroids, and 0.127343 for the true aroids; $dK = 0.0109493$, $dK/SD = 1.52764$, $P = 0.13$). With *P. pusillus* as the outgroup, the relative rates between the two groups were more similar (with *Lemna* included, K value as 0.175168 for the proto aroids, and 0.186862 for the true aroids; $dK = 0.0116949$, $dK/SD = 0.838657$, $P = 0.40$; with *Lemna* excluded, K value as 0.174868 for the proto aroids, and 0.175552 for the true aroids; $dK = 0.000683447$, $dK/SD = 0.053877$, $P = 0.96$). Results of the analysis with both *T. glutinosa* and *P. pusil-*

Table 2
Penalized likelihood (PL) and Bayesian estimates of divergence times with standard deviations

Node number in Fig. 3	Node	PL	Bayesian
1	The root of Araceae	120	113.20 ± 5.16
2	Orontioiderae	72	75.31 ± 2.95
3	*Symplocarpus foetidus–S. renifolius*	4.49 ± 1.69	6.88 ± 4.18
4	*Lysichiton*	4.02 ± 1.60	7.18 ± 4.33
5	*Symplocarpus*	15.54 ± 2.52	20.65 ± 6.44
6	*Symplocarpus–Lysichiton*	30.68 ± 3.66	40.41 ± 7.63

Constraints were placed on the root of Araceae (120 mya) and the Orontioideae node (72 mya) based on fossil evidence.

lus as the outgroup also showed that the proto and the true aroids had similar relative rates of evolution (with *Lemna* included, K value as 0.130717 for the proto aroids, and 0.149461 for the true aroids; $dK = 0.018744$, $dK/SD = 1.47803$, $P = 0.14$; with *Lemna* excluded, K value as 0.145792 for the proto aroids and 0.153573 for the true aroids; $dK = 0.00778089$, $dK/SD = 0.663067$, $P = 0.51$).

4. Discussion

4.1. Phylogenetic relationships in Orontioideae

Despite the great geographical distance, the EAS *S. renifolius* formed a clade with the ENA *S. foetidus* rather than with the EAS *S. nipponicus* (BS = 99%, Fig. 2). Based on cpDNA restriction site data, Wen et al. (1996) also found that *S. nipponicus* was highly divergent from its EAS relative, *S. renifolius* (1.82%), and the North American *S. foetidus* (2.58%). Morphologically *S. nipponicus* is distinguished from the other two species by having much smaller and obtuse (vs. acute) leaves with relatively long petioles (Wen et al., 1996). Furthermore, *S. foetidus* and *S. renifolius* flower in the early spring before the appearance of leaves and fruit in the summer of the same year, whereas *S. nipponicus* flowers after the appearance of leaves and the fruits ripe in the following spring (Mayo et al., 1997; Wen et al., 1996; Wilson, 1960).

Lysichiton camtschatcensis and *L. americanus* are well supported to form a clade in our analysis (BS = 100%). The two species are disjunctly distributed between northeastern Asia and northwestern North America, regions physically near the Bering land bridge (Fig. 1). They are morphologically similar and were sometimes considered to be a single species (Hultén and St. John, 1931; Mayo et al., 1997; Nicolson, 1981). *Lysichiton* populations from EAS and WNA differ primarily in their spathe color, with the EAS *L. camtschatcensis* having a white spathe and the North American *L. americanus* with a yellow spathe.

The sister-group relationship of *Symplocarpus* and *Lysichiton* is well supported in our analysis (Fig. 2). The two genera have similar floral characters and mephitic odor, lack laticifers, and occupy similar swampy habitats (Barabé and Forget, 1987; Barabé and Labrecque, 1984; Grayum, 1990; Mayo et al., 1997; Rosendahl, 1911; Wilson, 1960). Such a close relationship was also suggested by the *trn*L–F (Tam et al., 2004) and the cpDNA restriction site data (French et al., 1995). *Symplocarpus* is distinguished from *Lysichiton* by the former's subglobose spadix with a short stipe embedded in the spathe (vs. a subcylindric spadix with a highly elongated stipe not embedded in the spathe), a 1-locular (vs. 2-locular) ovary, and its absence of a seed coat (Barabé and Forget, 1987; Mayo et al., 1997; Wilson, 1960).

The monophyly of the subfamily Orontioideae (including *Orontium*, *Symplocarpus*, and *Lysichiton*) is well supported (BS = 100%, Fig. 2). The subfamily also has two large indels in the *trn*L–F sequences compared with the true aroids. A large 117 bp deletion (at positions 932–1048) is found in the *trn*L–F spacer of the proto aroids, but not in the true aroids. Another 55 bp deletion (at positions 96–150) in the *trnL* intron occurs in all true aroids, but absent in the proto aroids. The monophyly of Orontioideae is also supported by the following synapomorphies: expanded nonlinear leaf blade, anatropous or hemianatropous ovules, endosperm sparse to absent, and base chromosome number $x = 13$ (Mayo et al., 1997).

Although *Gymnostachys* forms a clade with other proto aroids, it shares two large indels (as described above) with the true aroids. Its sister relationship with the Orontioideae is supported with BS of 75% in our analysis of the combined *trn*L–F and *ndhF* sequences when gaps were coded as binary characters (Fig. 2), and only 55% when gaps were treated as missing data. Tam et al. (2004) reported a BS of 97% for the *Gymnostachys*–Orontioideae clade with the *trn*L–F sequences, but they coded indels as missing data. *Gymnostachys* may be an evolutionary link of the proto and true aroids. It is morphologically highly specialized in the proto aroids with linear leaves, parallel venation, and a unique flowering shoot of a complex synflorescence. This synflorescence consists of 3–6 (7), short, perennating floral sympodia seperated from each other by a distinct peduncular axis with each sympodium subtended by a leaf-like bract. Mayo et al. (1997) pointed out that the monophyly of the proto Araceae including *Gymnostachys* needs to be tested. Our results also support the inclusion of *Gymnostachys* in the proto aroids, but with a relatively low bootstrap support.

4.2. Divergence times, migration route, and direction

The divergence between the two intercontinental sister species of *Symplocarpus* (*S. foetidus* and *S. renifolius*) is

estimated to be 4.49 ± 1.69 and 6.88 ± 4.18 mya based on PL and Bayesian dating with the combined *trn*L–F and *ndhF* sequences, respectively. A similar estimate was obtained by Wen et al. (1996) as 6.1 mya based on cpDNA restriction site data with the molecular clock approach. The Bering land bridge seems to be the most likely migration route, which was available for floristic exchanges until about 3.5 mya (Hopkins, 1967). The disjunction of *Lysichiton* between EAS (*L. camtschatcensis*) and WNA (*L. americanus*) was estimated to be 4.02 ± 1.60 and 7.18 ± 4.33 mya. The disjunction in *Lysichiton* is apparently related to the Bering land bridge with a close disjunct distribution around the northern coastal regions of the North Pacific, including Japan, Russian Far East, and northern part of WNA (Fig. 1). The divergence times of *Symplocarpus* are similar to those of *Lysichiton* estimated by both PL (4.49 ± 1.69 vs. 4.02 ± 1.60 mya) and Bayesian dating (6.88 ± 4.18 vs. 7.18 ± 4.33 mya).

Similar divergence times for *Symplocarpus* and *Lysichiton* in the late Tertiary suggest that both lineages migrated intercontinentally via the Bering land bridge. The two sister genera overlap in distribution in EAS such as in northern Japan and the Russian Far East (Fig. 1), whereas they show a discontinuous distribution in eastern (*Symplocarpus*) and western North America (*Lysichiton*). The DIVA analyses supported EAS as the ancestral area for *Symplocarpus* as well as for the *Symplocarpus–Lysichiton* clade (Fig. 3). They subsequently migrated into North America through the Bering land bridge, consistent with the previously proposed hypothesis that the two genera have an Old World origin (Grear, 1966; Rosendahl, 1911). *Symplocarpus* was suggested to be a more ancient immigrant into North America than that of *Lysichiton* based on their slow rate of seed dispersal by small rodents (Rosendahl, 1911; Wada and Uemura, 1994) and a wider disjunct distribution of *Symplocarpus* than that of *Lysichiton* (Fig. 1). Both genera, however, have similar estimated divergence times.

In general, EAS harbors higher species diversity and possesses many more ancient taxa than ENA (Qian and Ricklefs, 2000, 2004). The Old World is usually considered as the place of origin for many of the disjunct taxa in these two regions (Wen, 1999; Xiang et al., 2004). Our study of *Symplocarpus* and *Lysichiton* also supports an Old World origin of the two lineages with their migrations via the Bering land bridge. The New World, however, was suggested to be the ancestral area for several groups such as the EAS–ENA *Phryma* (Nie, Sun, Olmstead, Beardsley, and Wen, in review). The DIVA analysis of *Ribes* (Grossulariaceae) also supports a WNA origin for its subgenus *Grossularia*, with subsequent dispersal to EAS giving rise to a well-supported Asian clade in section *Grossularia* (Schultheis and Donoghue, 2004). Biogeographic analysis of *Fraxinus* suggests a New World origin (Jeandroz et al., 1997). Both Asia and the New World have been documented as ancestral areas for some disjunct plant groups in these two regions (see Wen, 1999, 2001).

In Orontioideae, the *Symplocarpus–Lysichiton* clade is sister to *Orontium* (Fig. 2), which is monotypic and restricted to ENA. An intercontinental disjunction is thus supported between the ENA *Orontium* and the ancestral lineage of the *Symplocarpus–Lysichiton* clade, with the latter's ancestral area in EAS (see our DIVA results). Fossil evidence suggests the subfamily Orontioideae dated back at least to the late Cretaceous in the temperate Northern Hemisphere (72 mya, Bogner et al., 2005). Within the subfamily, our estimates also suggested a very ancient divergence (30.68 ± 3.66 and 40.41 ± 7.63 mya) between the ENA *Orontium* and the *Symplocarpus–Lysichiton* clade based on the PL and Bayesian methods, respectively (Table 2). *Arisaema* is one of the few examples of the true aroids distributed in both the temperate and tropical regions (Li, 1980). Renner et al. (2004) suggested that *Arisaema* had entered the New World twice during the Miocene–Oligocene from the Old World based on multiple chloroplast data sets (Renner et al., 2004). Our study and other recent work in Araceae (e.g., Renner et al., 2004) have shown that the family had a highly complicated intercontinental biogeographic history in the Northern Hemisphere.

4.3. Extinction of proto Araceae in the north temperate region

The Araceae are primarily tropical with only eight genera occurring in the north temperate zone (Grayum, 1990), three of which belong to the proto Araceae group: *Symplocarpus*, *Lysichiton*, and *Orontium*. The other five belong to the true Araceae: the ENA *Peltandra*, the EAS *Arisaema* and *Pinellia*, the Eurasian *Arum*, and the circumboreal *Calla*.

Our relative rate test suggests similar rates ($P = 0.13$) of nucleotide substitutions in the *trn*L–F region between the true and the proto aroids with the outgroup of *T. glutinosa*. However, when *Lemna* was included in the data set, the relative rates between the proto aroid clade and the true aroids—*Lemna* clade were significantly different ($P = 0.01$). This significant difference may be due to the much higher sequence substitution rates in *Lemna* as shown in the molecular phylogenetic study by Rothwell et al. (2004). With the anomalous *Lemna* excluded, the evolutionary rates of *trn*L–F sequences in the proto and the true aroids showed no significant difference ($P = 0.13$), in spite of the fact that the true aroids have drastically higher species richness than the proto aroids (ca. 3300 vs. 6).

Although the relationship between rates of speciation and molecular evolution is still unclear (Barraclough et al., 1996), several recent studies showed a positive correlation between species numbers and the rate of neutral nucleotide substitutions in flowering plants (Savolainen and Chase, 2003). The more species-rich plant families usually have an increased rate of neutral substitutions in both plastid and nuclear genes in comparison with their sister families (Barraclough and Savolainen, 2001). Another example is in Lentibulariaceae, in which the *Utricularia–*

Genlisea clade is substantially more species-rich and morphologically more divergent than its sister lineage, *Pinguicula*. Jobson and Albert (2002) found that the former has much higher nucleotide substitution rates across seven loci (the *trn*L intron, the second *trn*L exon, the *trn*L–F intergenic spacer, the *rps*16 intron, *rbc*L, *cox*I, and 5.8S rDNA). The positive correlation between rates of ITS evolution and species diversity was also found in the 10 EAS–ENA disjunct genera compared (Xiang et al., 2004).

Results of the relative rate tests may be influenced by taxonomic sampling biases in both ingroups and outgroups (Bromham et al., 2000). Robinson et al. (1998) suggested the inclusion of as many distantly related ingroup sequences as possible with topological weighting. They also favored including sequence of only the nearest outgroup. In our analysis, we included six of the seven species of the proto aroids and selected as many sequences as possible for the true aroids. Our sampling represented 45 of the ca. 100 genera and all seven subfamilies (Mayo et al., 1997; Tam et al., 2004). We also used topology weighting and the nearest outgroup (see Section 2).

Evolutionary rates may also be influenced by biological attributes of the lineages under comparison, such as generation time, population size, and speciation rate (Bousquet et al., 1992; Gaut et al., 1992; Laroche and Bousquet, 1999; Laroche et al., 1997; Whitcher and Wen, 2001; Wilson et al., 1990). All Araceae members are perennial herbs and the effects of generation time, population size, and speciation rate should be slight on the relative rates of nucleotide substitution among the aroid lineages. Although we cannot rule out the impact of incomplete sampling and other biological factors, our results support the hypothesis of the similar evolutionary rates between the proto and the true aroids.

Under the scenario of a similar evolutionary rate between the proto and the true aroids, species diversity of the proto aroids is expected to be similar to that of the true aroids and much higher than what they have today. We thus hypothesize that the contrasting difference in the species richness of the proto and the true aroids may be due to a greater rate of extinction in the proto aroids during the Tertiary. Paleoclimatic evidence suggests that the tropical conditions were farther north with relatively warm temperature in the Northern Hemisphere during several periods of climatic optima from the late Cretaceous to the middle Tertiary (e.g., early Eocene, late Oligocene, and middle Miocene; Graham, 1999; Zachos et al., 2001). The temperate or subtropical Laurasia was suggested to be an important region for the early development of the Araceae (Grayum, 1990). Fossil evidence also suggests the early occurrence of Araceae in the north temperate region, such as the infructescence fossil of the Orontioideae from Canada (72 mya, Bogner et al., 2005). Taxa of Araceae as well as a wide range of other evergreen plants were recorded even in the high northern latitudes in the Tertiary (Grear, 1966; Wolfe, 1975, 1977). During the several periods of climatic cooling and the southward retreat of tropical conditions in the Tertiary, many of the proto aroids most likely became extinct in the temperate regions whereas the sister lineage (the true aroids) became well developed in the tropics.

The disjunctions of temperate taxa in the Northern Hemisphere are usually considered to have resulted from extinctions of a more widely distributed flora in the Tertiary, the "boreotropical flora," or the mixed mesophytic forest (Tiffney, 1985a,b; Wolfe, 1975; Wen, 1999). Our relative rate test also supports more extensive extinctions of the proto aroids in the temperate zone during the Tertiary. Our result is consistent with the hypothesis of the relict disjunction of these Asian and North American disjunct plants (Wen, 2001).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.03.012.

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