

Molecular evidence for the sister relationship of the eastern Asia-North American intercontinental species pair in the *Podophyllum* group (Berberidaceae)

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Abstract. The presumed pair relationships of intercontinental vicariad species in the *Podophyllum* group (*Sinopodophyllum hexandrum* vs. *Podophyllum pelatum* and *Diphylleia grayi* vs. *D. cymosa*) were recently considered to be paraphyletic. In the present paper, the *trnL*-F and ITS gene sequences of the representatives were used to examine the sister relationships of these two vicariad species. A heuristic parsimony analysis based on the *trnL*-F data identified *Diphylleia* as the basal clade of the other three genera, but provided poor resolution of their interrelationships. High sequence divergence was found in the ITS data. ITS1 region, more variable but parsimony-uninformative, has no phylogenetic value. Sequence divergence of the ITS2 region provided abundant, phylogenetically informative variable characters. Analysis of ITS2 sequences confirmed a sister relationship between the presumable vicariad species, in spite of a low bootstrap support for *Sinopodophyllum hexandrum* vs. *Podophyllum pelatum*. The combined ITS2 and *trnL*-F data enforced a sister relationship between *Sinopodophyllum hexandrum* and *Podophyllum pelatum* with an elevated bootstrap support of 100%. Based on molecular phylogeny, the morphological evolution of this group was discussed. The self-pollination might have evolved from cross-fertilization two times in this group. The different pollination and seed dispersal systems of *Sinopodophyllum hexandrum* and *Podophyllum pelatum* resulted from their adaptations to different ecological habitats. The divergence time of *Sinopodophyllum hexandrum* - *Podophyllum pelatum* is estimated to be 6.52 ± 1.89 myr based on the ITS divergence. The divergence of this species pair predated or co-occurred with the recent uplift of the Himalayas 4-3 myr during the late Miocene and the formation of the alpine habitats. *Sinopodophyllum hexandrum* developed a host of specialized characters in its subsequent adaptation to the arid alpine surroundings. The present study confirmed the different patterns of species relationship between Asian-North American disjuncts. The isolation of plant elements between North America and eastern Asia must have been a gradual process, resulting in the different phylogenetic patterns and divergence times of the disjuncts.

Keywords: Berberidaceae; Disjunction; Eastern Asia; North American; Sister group *Podophyllum* group; Species pair.

Abbreviations: myr, million years ago; ITS, internal transcribed spacer of 18S-26S nuclear ribosomal DNA; *trnL*-F, *trnL* (UAA) 5' exon-*trnF* (GAA) exon region of chloroplast DNA.

Introduction

The disjunct distribution of morphologically similar plants between eastern Asia and North America has been a classical topic since the Linnean era. Many previous studies have contributed to a general documentation of this pattern in various plant groups (Li, 1952, 1972; Wu, 1983; Tiffney, 1985; Hong, 1993; Wen, 1998). This biogeographic pattern is currently recognized for approximately 91 genera (Hong, 1993), and numerous presumable spe-

cies pairs have been proposed between the two geographical areas (Li, 1952, 1972). These vicariad species pairs have recently received great attention because phylogenetic analysis, based mainly on molecular data, has shown most of them to be paraphyletic (Wen et al., 1996, 1998; Wen, 1998, 1999; Prince and Parks, 1998) or polyphyletic (Qiu et al., 1995; Soltis and Kuzoff, 1995; Soltis et al., 1995). Furthermore, the disjuncts in the phylogeny also showed various patterns. In *Nyssa* (Cornaceae), Wen and Stussy (1993) detected two phylogenetic connections between Asia and the Americas, but in *Hamamelis* (Hamamelidaceae), two Asian species comprised the basal clade, and four North American species formed the other monophyletic clade (Wen and Shi, 1999). Xiang et al. (1998) revealed that four studied genera (*Cornus*, *Boykinia*, *Tiarella* and *Trautvetteria*) showed this pattern of biogeo-

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graphic relationships among species. Species from eastern Asia are sister to species from North America.

The *Podophyllum* group circumscribed here, comprises three or four genera in Berberidaceae: *Diphylleia*, *Dysosma*, *Podophyllum* and *Sinopodophyllum* (sometimes included within *Podophyllum*) according to different authors (Meacham, 1980; Loconte and Estes, 1989). This group was treated as a tribe or subfamily of the Berberidaceae and sometimes as a separate family, Podophyllaceae (Cronquist, 1988; Dahlgren, 1989; Takhtajan, 1997). In all phylogenetic analyses based on morphological and molecular data, the *Podophyllum* group formed a monophyletic group and was closely related to the other x=6 groups in the Berberidaceae (Loconte and Estes, 1989; Nickol, 1995; Kim and Jansen, 1995, 1996, 1998). Among the four genera, *Dysosma*, consisting of eight species, is restricted to China (Ying et al., 1993). In his monograph on *Diphylleia*, Ying et al. (1984) recognized three continuous vacariad species: *D. cymosa* from eastern North America, *D. grayi* from Japan, and *D. sinensis* from China. He asserted that the latter two species from eastern Asia were more closely related. In fact, the three species show very slight differences, especially between the Japanese and Chinese entities. Hong (1993) pointed out that "it is still a question whether *Diphylleia* should be treated as a single species or divided into two species," and he tentatively divided the eastern North American and eastern Asian entities into two separate varieties. Most workers treated them as a vacariad species pair (Li, 1952, 1972). Traditional *Podophyllum* is composed of two vacariad species: *P. peltatum* from eastern North America and *P. hexandrum* from the eastern Himalayas (Li, 1952, 1972; Takhtajan, 1997). Ying (1979) established a monotypic genus *Sinopodophyllum*, as a generic pair of *Podophyllum* based on *Podophyllum hexandrum*. The alpine shrub habitats of *Sinopodophyllum* provide further support for this treatment (Ying et al., 1993). Both *Dysosma* and *Sinopodophyllum* are self-pollinating while *Diphylleia* and *Podophyllum* are outcrossing (Ma and Hu, 1997). Consequently, Ma and Hu (1997) pointed out that the new genus *Sinopodophyllum* was more closely related to *Dysosma* than to *Podophyllum*, and *Podophyllum peltatum* might have originated from *Diphylleia cymosa* after a continental separation, which suggests only a single Asian-North American disjunction and no intercontinental

sister species in the *Podophyllum* group. This assumption has been partly confirmed by recent molecular research. The chloroplast DNA restriction sites phylogeny analysis revealed that *Diphylleia cymosa* and *D. grayi* are paraphyletic on the phylogeny tree, with *Diphylleia cymosa* forming a separate clade and *D. grayi* positioned at the base of the other clade including *Dysosma* and *Podophyllum* (Kim and Jansen, 1998).

In spite of the increasing data to show the presumed species pairs are not sister in various eastern Asia-eastern North American disjunct genera (see reviews by Wen, 1999), we feel that most phylogenetic analyses have been done based on a single molecular marker or morphology. The robustness of phylogenies needs to be tested. In the present paper, we explore the molecular sequence data to test the pair relationship of two pairs of presumed vicariad species between eastern Asia and eastern North America in the *Podophyllum* group of the Berberidaceae.

Materials and Methods

Plant species, their collection information and GenBank accession numbers are listed in Table 1. Total DNAs were isolated from silica-dried leaves following the hexadecyltrimethylammonium (CTAB) method of Doyle and Doyle (1987). The *trnL*-F (*trnL* (UAA) 5' exon-*trnF* (GAA) exon chloroplast DNA region) and ITS regions were amplified from purified genomic DNA using polymerase chain reactions. Primers used were as follows: *trnL*-F "c" and "f" (Taberlet et al., 1991), which amplified the intron, 3' exon and intergenic spacer, and primers "ITS4" and "ITS5" (White et al., 1990), which amplified ITS1, the 5.8S gene, and ITS2. The double-stranded PCR products were purified using 1% agarose gel following the protocol of the Wizard Kit, and 10 µl sequencing reactions were carried out in a Perkin-Elmer GenAmp model 9600 thermocycler using Applied Biosystems Inc. (ABI) Tag DyeDeoxy Terminator Cycle Sequencing Kit. Sequencing was done on an ABI 377 automated sequencer, and each region was sequenced for both strands. Sequences were assembled using CLUSTAL V (Higgins, 1994). The completed sequences were manually aligned prior to analysis. The boundaries of the ITS1 and ITS2 regions were determined based on the posted sequences of nrDNA in the Berberidaceae obtained from GenBank. The published sequences of the

Table 1. List of species and the accession numbers in GenBank for ITS and *trnL*-F sequences.

Species	DNA source/voucher	GenBank Accession No. <i>trnL</i> -F/ITS
<i>Podophyllum peltatum</i> L.	USA, Cult.; Mo. Bot. Gard., no voucher	AF325904/AF328964
<i>Sinopodophyllum hexandrum</i> (Royle) Ying	China, Liu Jianquan 9917	AF325905/AF328965
<i>Diphylleia grayi</i> F. Schmidt	China, Liu Jianquan 9918	AF325906/AF328966
<i>Dysosma majorensis</i> (Gagnep.) Hsiao et Y.H. Chen	China, Cult.; no voucher	AF325907/AF328967
<i>Dysosma versipellis</i> (Hance) M. Cheng	China, Chen et al. 96072	AF325908/AF328968
<i>Berberis silvataroucana</i> Schneid.	China, Chen et al. 960586	AF325909/AF328969
<i>Epimedium pubescens</i> Maxim	China, Feng Min & Yang Qiner 95026	AF325910/AF328970
<i>Caulophyllum robustum</i> Maxim	China, Chen et al. 960601	AF325911
<i>Nandia domestica</i> Thunb.	China, Chen et al. 960325	AF325912

trnL-F region in the GenBank were used to identify the boundaries of the *trnL* intron and *trnL*-F intergenic spacer. The materials of *D. cymosa* were unavailable to us. We were unable to amplify the target DNA using specimens kept in the herbaria, and the ITS2 sequences of *D. cymosa* for analysis in the present paper were downloaded from GenBank.

Phylogenetic relationship analyses were conducted with PAUP, Version 4.0 (Swofford, 1999). Parsimony analyses were performed by heuristic searches with TBR branch swapping, MULPARS option, ACCTRAN optimization, and random sequence addition replicates. Gaps were treated as missing data or “fifth state.” Relative support for the clades identified by the parsimony analysis was assessed by bootstrapping (Felsenstein, 1985), with 1,000 replicates for each matrix. Sequence divergence and standard errors between a species pair were estimated for total substitution using the Jukes-Cantor model, available on MEGA 1.0 (Kumar et al., 1993). Time of divergence was calculated as the value of DNA sequence divergence divided by twice the sequence rate (Li, 1997).

Results

The aligned *trnL*-F region of nine sampled species consists of 954 bp, including the intron, *trnL*3' exon, and *trnL*-F intergenic spacer. There are 16 indels, ranging in size from 1 bp to 38 bp. Most of them are informative. The major identified clades are the same when the gaps were treated as either missing data or the fifth state. There were 196 informative sites when gaps were treated as missing. For the outgroup selection, in the first *trnL*-F data analysis, we chose *Nandiana* as the outgroup because all morphological and molecular data suggested its basal position in the Berberidaceae (Nickol, 1995; Kim and Jansen, 1996, 1998). One of six most-parsimonious trees with 291 steps (C.I.=0.969; R.I.=0.971) is shown in Figure 1. The monophyly of the *Podophyllum* group was also identified in the *trnL*-F tree as has been evidenced by many molecular and morphological analyses (Loconte and Estes, 1989; Kim and Jansen, 1995, 1996, 1998). *Diphylleia* comprised a clade, as the basal to the other three genera. Interrelationships within the three genera could not be resolved.

The length of ITS 1 in the taxa surveyed varied from 228 to 234 bp with a higher base variation between 12% and 47%. An examination of the aligned matrix and the Paup analysis indicated that most variable bases in the ITS1 region are parsimony-uninformative with no phylogenetic value. We concur with Kim and Jansen (1996) that ITS1 is not suitable for phylogenetic analysis in the Berberidaceae. However, most variations in the ITS2 sequences are phylogenetically informative. Therefore, we mainly used ITS2 data to assess the sister relationships of the presumable species pairs. The aligned ITS2 region consists of 248 bp. A total of 144 sites were variable, 100 of which were phylogenetically informative when gaps were treated as missing. Because *Berberis* has the chromosome of $x=7$, we chose it as the outgroup to analyze.

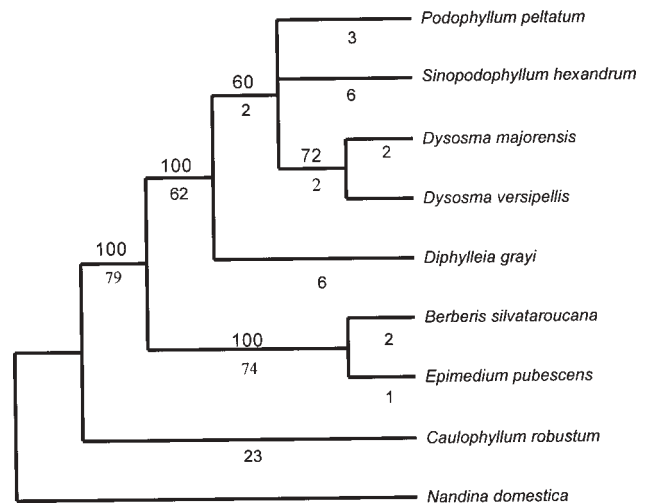


Figure 1. One of six equally parsimonious trees showing the relationships among samples of Berberidaceae based on *trnL*-F DNA sequence data, gaps were treated as missing. Bootstrap support and number of base changes are shown above and below the branches, respectively.

Berberis, however, always nested within the *Epimedium* clade. The *trnL*-F data analysis had indicated the close relationship of *Berberis* and *Epimedium*. So we designated *Jeffersonia* as the outgroup according to the unrooted analysis. The parsimony analysis of ITS2 data generated three shortest trees of 253 steps, a CI of 0.81, and an RI of 0.76 when treating gaps as missing. The topology of the tree did not change data when gaps were coded as the fifth state. One randomly chosen tree and the bootstrap supports for each clade can be found in Figure 2. The monophyly of the *Podophyllum* group is well identified. The interrelationships within the group are also discerned. *Diphylleia* formed a distinct basal clade. The sister relationship of *Diphylleia grayi* and *D. cymosa* was strongly supported with an 89% bootstrap value. In the other clade, three species of *Dysosma* clustered together. The pair relationship of *Sinopodophyllum* and *Podophyllum* was supported by a relatively low bootstrap value of 68%. The parsimony analysis based on the combined ITS2 and *trnL*-F data generated two most-parsimonious trees of 490 steps, with a CI of 0.951 and a RI of 0.924 (Figure 3). The bootstrap support for the sister relationship of *Sinopodophyllum* and *Podophyllum* was elevated to 100%.

Despite the controversy regarding the molecular evolution rate (Wilson et al., 1990; Gaut et al., 1992, 1993, 1996), a molecular clock may still be useful for estimating divergence time if it can be calibrated with fossil records and correlated with other molecular evidence (Xiang et al., 1998). Another species pair of Berberidaceae, *Caulophyllum robustum*-*C. thalictroides*, split between eastern Asia and North America has been calibrated by a combination of ITS divergence, allozyme divergence, and fossil record (Lee et al., 1996). The three estimated divergence times are almost concordant and are, respectively,

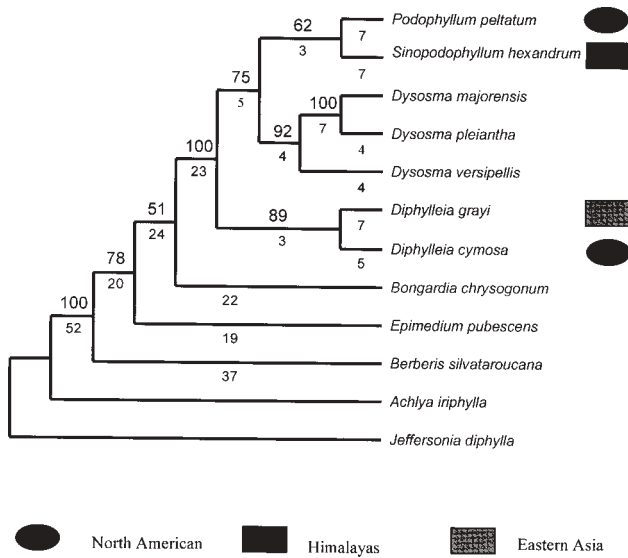


Figure 2. One of three equally parsimonious trees showing the relationships of samples in the *Podophyllum* group based on ITS2 DNA sequence data, gaps were treated as missing. Bootstrap support and number of base changes are shown above and below the branches, respectively.

3.3 myr (million years before present), 6.0-6.5 myr, and 7-8 myr, all falling in the Late-Miocene. We used the ITS divergence rate of this species pair, 3.9×10^{-9} per site per year to estimate the divergence of two species pairs in the present study. Based on the 0.0465 ± 0.0141 value of the ITS2 sequence divergence of *Diphylleia cymosa* - *D. grayi*, the divergence time is estimated to be 5.95 ± 1.80 myr. The ITS2 sequence divergence of *Sinopodophyllum hexandrum* - *Podophyllum peltatum* is 0.0509 ± 0.0148 . Based on this divergence, the divergence time of *Sinopodophyllum hexandrum* - *Podophyllum peltatum* is estimated to be 6.52 ± 1.89 myr. This estimate, however, is subject to several sources of error. First, ITS may not evolve at a clocklike rate because it is not a coding area. Secondly, the estimated substitution rates of the ITS region vary greatly, from 3.5×10^{-10} in the Winteraceae (Suh et al., 1993) to 7.8×10^{-9} per site per year in *Dendroseris* of the Asteraceae (Sang et al., 1994, 1995). Nevertheless, this approximate estimate continues to be useful in understanding tempos of plant historical biogeography. We are convinced that the divergence time of *Sinopodophyllum hexandrum* - *Podophyllum peltatum* calculated here is accurate because it is inconsistent with the divergence time, 6.94 ± 3.19 myr, an estimate based on the *rbcL* sequence divergence by Xiang et al. (2000).

Discussion

The presumable species pairs between eastern Asia and eastern North America were found not to have sister relationships in various groups, e.g. *Hammelis* (Hamamelidaceae, Wen and Shi, 1999), *Hydrangea* sect.

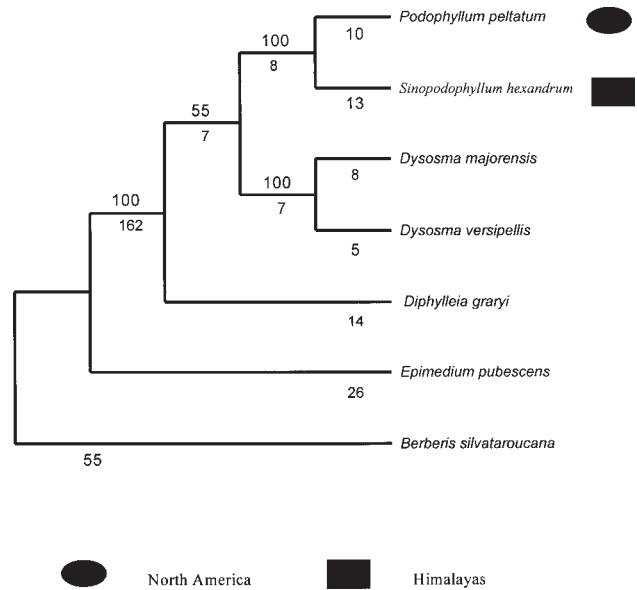


Figure 3. One of two equally parsimonious trees showing the pair relationships of *Sinopodophyllum* and *Podophyllum* based on the combined ITS2 and *trnL-F* DNA sequence data, gaps were treated as missing. Bootstrap support and number of base changes are shown above and below the branches, respectively.

Hydrangea (Hydrangeaceae, Soltis et al., 1995), *Gleditsia* (Fabaceae, Schnabel and Wendel, 1998), *Aralia* (Araliaceae, Wen et al., 1998), *Panax* (Araliaceae, Wen and Zimmer, 1996) and *Triosteum* (Caprifoliaceae, Gould and Donoghue, 2000). In most cases, however, the Asian species are sister to their North American species counterparts. This was also found to be the case for the species pairs in the *Podophyllum* group according to recent research by Ma and Hu (1997).

Contrary to the previous paraphyletic hypotheses of the species pair in the *Podophyllum* group (Ma and Hu, 1997; Kim and Jansen, 1998), the present study supports the sister relationship of species pairs, although the conclusion needs to be further tested. We are more confident of their pair relationship after examining specimens and comparing morphological characters. *Diphylleia grayi* and *D. cymosa* share the following common characters: fibrous roots, many-flowered cyme or umbel, small and white flowers, valve-like dehiscent basifixed anthers, conspicuously spiny pollens, and small, dark blueberry-like fruits. Petiole lengths in the upper leaves vary greatly, as do the number of flowers and ovules, the hairs on the leaf, and inflorescence within and among populations. Separating two species is still difficult without the collection site information. They have sometimes been treated as one single species (Hong, 1993). Placing *D. cymosa* with members of the *Podophyllum*-*Sinopodophyllum*-*Dysosma* clade is unimaginable. We have not found a morphological synapomorphy to unite it with them. *Sinopodophyllum* and *Podophyllum* show more differentiation in morphology (Ying, 1979), as evidenced by sequence data, but they still share the following characters:

large and single pink flower, lobed leaf, 2B karyotype and no wide stamen connectives. *Sinopodophyllum* is different from *Podophyllum* in having crass thick rhizomes, precocious flowers, 6 stamens, tetrad and spinulate pollens, and selfing mating systems (Ma and Hu, 1997). *Podophyllum* has slender rhizomes, peltate leaves, 12-18 stamens, single and reticulate pollens, and outcrossing mating systems. We found that most of these characters are autoapomorphies of *Sinopodophyllum* or *Podophyllum*, which might contribute to the taxonomic treatment (Ying, 1979), but not to phylogenetic relationships. They resulted from the different ecological adaptation, especially the breeding system, because this character is more sensitive to habitat pressure (Takhtajian, 1997). The self-pollination shared by both *Sinopodophyllum* and *Dysosma* may have undergone convergent evolution under similar habitat selection pressure.

The species differentiation of the *Podophyllum* group and the acquisition of specialized characters of *Sinopodophyllum hexandrum* are reinforced by the biogeographical evidence of the Himalayas. The last rapid uprising of the Himalayas began about 4-3 myr in the late Miocene (Shi et al., 1998). The divergence time estimated for two species pairs (6.52 ± 1.89 myr for *Sinopodophyllum hexandrum* - *Podophyllum pelatum* and 5.95 ± 1.80 myr for *Diphylleia cymosa* - *D. grayi*) predated or almost co-occurred with the uplift of the Qinghai-Tibet plateau. From the Mid-Miocene to the Late-Miocene, the Himalayas were still covered by subtropical forests, and the average altitude of the Qinghai-Tibet Plateau had not reached 1,000 meters. The alpine shrub vegetation appeared 3-2 myr with the recent uplift of the plateau (Shi et al., 1998). The extant close relatives of *Sinopodophyllum*, *Podophyllum*, *Dyphilleia*, and *Dysosma* still occur now under the subtropical forests (Ying, 1979). Therefore, the ancestor of *Sinopodophyllum* might have grown in such surroundings and then diverged with *Podophyllum pelatum*. With the recent uprising of the Himalayas and the appearance of the alpine shrub habitats, it is not unimaginable that *Sinopodophyllum hexandrum* developed a series of specialized characters in order to adapt to the new alpine surrounding, which made it look different from *Podophyllum pelatum*.

The commonest phylogenetic relationship pattern of eastern Asia and North America disjunct distribution genera is that eastern Asian species are sister to the North American species (Wen, 1999; Xiang et al., 1998 and refs therein). This pattern was easily explained by the long-standing hypothesis that the disjunction between eastern Asia and North America represents the fragmentation of a once-continuous plant community (Li, 1952; Wood, 1972). The isolated taxa respectively evolved into the present eastern Asian species and North American species. In fact, more phylogenetic patterns reflect different vicariance events (Xiang et al., 1998, 2000; Wen, 1999). For example, in the section *Rytidospermum* of *Magnolia*, Qiu et al. (1995) revealed the American species *M. tripetala* is sister to the Asian species. There are two phylogenetic connections between Asia and the Americas in *Nyssa* (Wen

and Stuessy, 1993). In the present study, more than one phylogenetic connection between eastern Asia and North America was also detected in the *Podophyllum* group.

Tiffney (1985) suggested five major periods for the disjunct distribution between eastern Asia and North America: the Pre-Tertiary, the early Eocene, the late Eocene-Oligocene, the Miocene and the later Tertiary-Quaternary. Because of the lack of fossil records, this assumption was rarely tested for some groups. The estimated divergence times for the two species pairs of the Berberidaceae in the present study, 6.52 ± 1.89 myr and 5.95 ± 1.80 myr, are between the late Miocene and the early Pliocene. Xiang et al. (2000) examined the divergence time of eleven species pairs of eastern Asian-North American disjuncts based on the *rbcL* sequence divergence, which suggested different divergence times of the disjuncts from the late Tertiary stage to the Quaternary stage. However, all the divergence times fall within the period assumption of Tiffney (1985) on the disjunct distribution. Furthermore, these molecular data are supported by the paleontological evidence. The mixed mesophytic forest was widely distributed the Miocene stage, and plant elements between North America and eastern Asia were frequently exchanged via the North Atlantic Bridge and continued via the Bering Strait at the Pliocene and early Quaternary stages (Wolfe, 1985; Cunningham and Collins, 1994). As pointed out by Graham (1993), the change from free exchange to complete isolation between North America and eastern Asia must have been a gradual process that stretched from the Miocene to Quaternary. This process resulted in the different phylogenetic patterns and divergence times for different disjuncts.

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分子證據支持足葉草群（小檗科）中東亞-北美間斷種對的姊妹對應關係

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足葉草群中的兩個東亞-北美間斷種對 (*Sinopodophyllum hexandrum* vs. *Podophyllum pelatum* and *Diphylleia grayi* vs. *D. cymosa*) 最近被分別認為是並系。本文利用 *trnL-F* 和 ITS 序列檢查了種對間的姊妹對應關係。*trnL-F* 序列分析表明 *Diphylleia* 位於其餘三個屬的基部，但這三個屬內部的分支關係未得到分辨。ITS1 在該群中雖然變異較大，但具有系統資訊的位元點較少，因而無系統學價值。ITS2 序列具有豐富的系統學意義的變異位元點。基於 ITS2 序列的分支分析支援以前東亞-北美間斷種對的姊妹對應關係，儘管 *Sinopodophyllum hexandrum* vs. *Podophyllum pelatum* 得到的鞋帶支持較低。綜合 ITS2 和 *trnL-F* 序列的分支分析將 *Sinopodophyllum hexandrum* 和 *Podophyllum pelatum* 的姊妹關係支援率提高到了 100%。根據得到的分子系統發育關係，討論了該群的形態性狀演化。從異花到自花授粉可能在該群中進化了兩次。*Sinopodophyllum hexandrum* 和 *Podophyllum pelatum* 傳粉和種子傳播的差異可能是來自它們對不同生態環境的適應。它們的分歧時間大約是 6.52 ± 1.89 myr，早於或與喜馬拉亞的隆升和高寒生境的形成同步。*Sinopodophyllum hexandrum* 在適應極端高寒生境中產生了系列特化性狀。本研究進一步證實了東亞-北美間斷類群中存在各式的系統發育式樣。東亞-北美植物類群之間斷是一個逐漸的過程，該過程的延續性造成了在間斷類群中存在的各種系統發育式樣和不同的分歧時間。

關鍵詞：間斷；東亞；北美；種對；姊妹關係；足葉草群；小檗科。