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PHYLOGENETIC ANALYSES AND BIOGEOGRAPHY OF TRILLIACEAE

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ABSTRACT

Trilliaceae are plants of North Temperate forests with a holarctic distribution and a high degree of endemism. Molecular phylogenetic analyses are presented in order to examine the tribal, generic, and species-level classification of the family. These molecular studies, and earlier morphological studies, support the placement of the genus *Pseudotrillium* as basal in the family and sister to the tribes Trillieae and Parideae, which are monophyletic sister groups. *Trillidium* (*Trillium*) *govanianum* provides an unresolved problem: morphologically it is included within Parideae, but molecular data place it within *Trillium* as sister to *T. undulatum*. Within tribe Trillieae, clades are noted that correspond to previous taxonomic groups. Within tribe Parideae the separation of *Paris* s.l. into *Daiswa*, *Kinugasa*, and *Paris* s.s. is strongly supported. In addition, some biogeographic correlations are noted, and phylogenetic distribution patterns are discussed.

Key words: biogeography, *Daiswa*, *Kinugasa*, molecular analyses, morphology, *Paris*, phylogeny, *Pseudotrillium*, Trilliaceae, *Trillidium*, *Trillium*.

INTRODUCTION

Because of their simple and distinctive morphology, Trilliaceae (Table 1) have been easy to circumscribe but difficult to place. Since initial recognition of the family as a unit by Chevallier (1827), its members have been placed in seven orders and as parts of five families (Table 2; Zomlefer 1996; Farmer 2000). Recent studies using a variety of molecular and morphological techniques (see Farmer & Schilling 2002 for summary) agree that Trilliaceae are monophyletic (Chase et al. 1993, 1995a, b; Davis 1995; Kato et al. 1995a, b; Stevenson and Loconte 1995).

The traditional view of generic limits in Trilliaceae has been the classification into the two Linnaean genera based on floral merosity: *Trillium* is trimerous, whereas *Paris* is 4- to 11-merous. Within *Trillium*, a major issue is whether the two subgenera (subgen. *Trillium* having a flower pedicel, subgen. *Phyllantherum* lacking a pedicel [Freeman 1969, 1975]) are monophyletic (summary in Zomlefer 1996). Two species within *Trillium* have more recently been segregated as monotypic genera: *Pseudotrillium* and *Trillidium*. *Pseudotrillium* was erected based its broad spotted petals, continuously elongating pedicel, and molecular evidence; *Trillidium* was described based on its tepaloid inflorescence, extrorse anther dehiscence, and trimerous phyllotaxy (see Table 1). Within *Paris*, the debate is whether to recognize a single broad genus (Hara 1969; Li 1984, 1998) or split it into three genera (Takhtajan 1983; Dahlgren et al. 1985; Tamura 1998). The most recent treatment of the family indicates that the two traditional subgenera of *Trillium* are not monophyletic, but there is morphological and molecular support for splitting *Paris* s.l. into three genera so that a total of six genera are recognized in the family: *Trillium*, *Trillidium*, *Pseudotrillium*, *Paris*, *Daiswa*, and *Kinugasa* (Farmer and Schilling 2002; see Tamura 1998; Fig. 1–6).

Trilliaceae are plants of North Temperate forests with a holarctic distribution (Takhtajan 1986; Fig. 7) and are thus

hypothesized to be Arcto-Tertiary in origin (Tamura 1998), referring to high latitude, Northern Hemisphere areas having an abundance of Tertiary fossils (Bufford and Spongberg 1983; see Engler 1879). Most contemporary botanists agree that this distribution consists of four disjunct areas: eastern North America, western North America, eastern Asia and southeastern Europe-Asia Minor (Wood 1972; Tiffney 1985b). Members of Trilliaceae occur in each of these regions north of Mexico as well as most of Europe.

Within the Tertiary relict disjunctions, the most widely studied of these patterns is that of eastern Asia and North America, particularly eastern North America (Gray 1846; White 1983; Tiffney 1985a; Hong 1993; Wen 1999; see Bufford and Spongberg 1983 for summary). Other patterns of distribution such as that between the eastern North America and the Old World (Fernald 1931) and the southern Appalachians and western North America (Wood 1971) have also been noted. Thorne (1972) and Raven (1972) discuss more general disjunct patterns in plants.

Within Trilliaceae there are some interesting distributional patterns: there are no *Trillium* in Europe; no members of Parideae are found in the New World; polyploids are restricted to the Old World; the only documented hybrids are in Asia; and the only sessile-flowered species are in the New World. In addition, there is only one globally widespread species, *Paris quadrifolia* (which is found from Ireland to Mongolia and Siberia); there are two widespread genera, *Paris* and *Trillium*; but there are three narrowly endemic genera, *Pseudotrillium*, *Kinugasa*, and *Trillidium*. The traditional sister species groups that are often found in Arcto-Tertiary distributions also exist: the Erectum Group (discussed later) exhibits the traditional eastern North America–Asia pattern (Fernald 1931; Raven 1972; Thorne 1972; Wood 1972; Xiang et al. 1998), while both the Grandiflorum Group (discussed later) and the sessile flowered species of *Trillium* (subgen. *Phyllantherum* Raf.) exhibit the western North America–eastern North America pattern (Wood 1971).

Table 1. Generic types of Trilliaceae. Numbers after the genus name refer to numbers of species: National Flora (e.g., Flora of China [Liang and Soukup 2000]; Flora of North America [Case 2002]; Tamura 1998; Farmer and Schilling 2002). All species have a single flower subtended by a whorl of leaves on an otherwise naked stem.

Genus, number of species, and type	Description
<i>Trillium</i> L. (43/43/41)	Trimerous phyllotaxy; flowers sessile (subgen. <i>Phyllantherum</i>) or pedicellate (subgen. <i>Trillium</i>).
<i>T. cernuum</i> L.	
<i>Paris</i> L. (25/14/14)	4- to 12-merous phyllotaxy; flowers pedicellate.
<i>P. quadrifolia</i> L.	
<i>Daiswa</i> Raf. (NA/10/10)	4- to 12-merous phyllotaxy; flowers pedicellate.
<i>D. polyphylla</i> (Smith) Raf.	
<i>P. polyphylla</i> Smith	
<i>Trillidium</i> Kunth (NA/NA/1)	Trimerous phyllotaxy; tepaloid inflorescence; petiolate leaves; pedicellate flowers.
<i>Td. govanianum</i> (Wall. in Royle) Kunth	
<i>T. govanianum</i> Wall. in Royle	
<i>Kinugasa</i> Tatew. & Sutô (NA/1/1)	7- to 10-merous phyllotaxy; showy white sepals; pedicellate flowers.
<i>K. japonica</i> (Franch. & Sav.) Tatew. & Sutô	
<i>Td. japonicum</i> Franch. & Sav.	
<i>Pseudotrillium</i> S. Farmer (NA/NA/1)	Trimerous phyllotaxy; broad spotted petals; petiolate leaves; pedicellate flowers.
<i>Pst. rivale</i> (S. Watson) S. Farmer	
<i>T. rivale</i> S. Watson	

Table 2. Historical placement and composition of genera associated with Trilliaceae. Liliaceae is assumed to be in Liliales. 1 = *Medeola* as *Gyroomia*, 2 = *Medeola*, 3 = *Scolioopus*, 4 = *Demidovia*, 5 = Listed in synonymy for *Trillium*, but mentioned in text, 6 = *Clintonia*, 7 = *Daiswa*, *Kinugasa*, and *Paris* s.s., 8 = *Trillium* and *Trillidium*, 9 = *Pseudotrillium*. Note that Watson and Dallwitz (1991b, 1996) refer to DELTA data sets (Watson and Dallwitz 1991a).

Reference	Date	Included genera			Family	Order
		<i>Trillium</i>	<i>Paris</i>	Other		
de Jussieu	1789				Liliaceae	
Dumortier	1829	X	X	1	Paridaceae	Paridales
Endlicher	1836–1840	X	X	2, 4	Smilacaceae (Parideae)	
Lindley	1846	X	X	4, 5	Trilliaceae	Dioscoreales
Kunth	1850	8	X		Smilacineae	
Watson	1879	8	X	2, 3	Liliaceae (Trillieae)	
Bentham & Hooker	1883	X	X	2, 3, 6	Liliaceae (Medeoleae)	
Engler	1888	X	X	2	Liliaceae (Parideae)	
Dalla Torre & Harms	1908	X	X	2, 3	Liliaceae (Parideae)	
Hutchinson	1926	X	X	2, 3	Trilliaceae	Liliales
Rendle	1930	X	X		Liliaceae	
Cronquist	1968				Liliaceae	
Takhtajan	1959				Trilliaceae	Dioscoreales
Melchior	1964	X	X	2	Liliaceae (Parideae)	
Huber	1969				Trilliaceae	Stemonales
Takhtajan	1969				Liliaceae	
Willis	1973	X	X	2, 3	Trilliaceae	
Dahlgren	1975				Trilliaceae	Stemonales
Huber	1977				Trilliaceae	Roxburghiales
Takhtajan	1980	X	X		Trilliaceae	Smilacales
Dahlgren et al.	1985	X	X	3	Trilliaceae	Dioscoreales
Takhtajan	1987	X	7		Trilliaceae	Dioscoreales
Watson & Dallwitz	1991b	X	X	2, 3	Trilliaceae	Dioscoreales
Brummitt & Powell	1992	X	7	3	Trilliaceae	
Thorne	1992	X	X		Trilliaceae	Liliales
Noltie	1994	8	X		Trilliaceae	
Stevenson & Loconte	1995				Trilliaceae	Stemonales
Watson & Dallwitz	1996	X	7		Trilliaceae	Dioscoreales
Takhtajan	1997				Trilliaceae	Trilliales
APG	1998				Melanthiaceae	Liliales
Farmer & Schilling	2002	8	7	9	Trilliaceae	Liliales
APG II	2003				Melanthiaceae	Liliales

Fig. 1–6.—Type species for each genus within Trilliaceae.—1. *Trillium cernuum*.—2. *Paris quadrifolia*.—3. *Daiswa polyphylla*.—4. *Trillidium govanianum*.—5. *Kinugasa japonica*.—6. *Pseudotrillium rivale*. Photography by G. C. Denton (1, 4), J. M. McClements (2), B. E. Wofford (3), V. G. Soukup (5), and S. B. Farmer (6); all images used by permission.

Both subgenera of *Trillium* have a long history of study (Small 1897; Gleason 1906; Barksdale 1938; Freeman 1969, 1975; Ihara and Ihara 1978, 1982), but the genus has only recently begun to be studied in a phylogenetic context (Kato et al. 1995a, b; Kazempour Osaloo et al. 1999; Kazempour Osaloo and Kawano 1999; Farmer and Schilling 2002). Formal subgroups have not been proposed for the pedicellate *T.* subgen. *Trillium*, but two informal subgroups are used based on texture and aging characteristics of the petals: the Grandiflorum Group (characterized by delicate textured, undulate-margined petals that age to pink) and the Erectum Group

(characterized by more coarsely textured petals without undulate margins that age to brown) (Gleason 1906; Barksdale 1938; Ihara and Ihara 1978, 1982; Patrick 1984). However, studies that use informal groups include other characters such as pistil morphology (Gleason 1906; Barksdale 1938; Ihara and Ihara 1978). Often considered a part of the Grandiflorum Group, the Delostylis Group was recognized by Rafinesque (1819) to refer to species with an apparent style between the ovary and three slender stigmas. As defined by Rafinesque, this group comprises four species: *Trillium catesbaei*, *T. nivale*, *T. persistens*, and *T. pusillum*. All of these

Fig. 7.—Distribution map for members of Trilliaceae (after Samejima and Samejima [1987] and Li [1998]).

species are from the southern Appalachians and southeastern United States, with the exception of *T. nivale*, which ranges from Pennsylvania westward to Montana, including distributions in South Dakota, Nebraska, and Missouri.

MATERIALS AND METHODS

Sampling Strategy

Seventy species were considered in earlier morphological analyses of Trilliaceae (Farmer 2000; Farmer and Schilling 2002). Samejima and Samejima (1987), Li (1984, 1998), and Takhtajan (1983) served as primary taxonomic sources although species more recently recognized were also included. The *Flora of China* (Liang and Soukup 2000) was used as the source for currently recognized Parideae taxa.

The first molecular analysis, using ITS data alone, was based on 38 accessions from GenBank sequence data (Table 3). The second analysis, combining ITS and *matK* data, focused on the Delostylis Group of *Trillium*. All 54 members of this group were selected (including varieties, geographical variants, and closely related species) (Table 3).

Molecular markers.—DNA sequence information for two gene regions was available from GenBank for a subset of taxa used in the morphological analysis (Table 3). The two molecular data sets were the Internal Transcribed Spacer (ITS) region (ITS1, 5.8S, ITS2 sequences) of nuclear ribosomal DNA, and the chloroplast DNA sequences for the maturase (*matK*) gene. Because ITS and *matK* sequence data were already available for species within the family, these two regions were chosen for initial sequencing of additional taxa. All ancillary data sets and sequence alignments are available from the author.

Outgroup selection.—Despite the fact that Xerophyllaceae are the nearest relatives of Trilliaceae, (Chase et al. 1995b, 2000; Davis 1995; Davis et al. 1998; Fuse and Tamura 2000; Rudall et al. 2000; Zomlefer et al. 2001), taxa within Melanthiaceae s.l. are not satisfactory outgroups for Trilliaceae (Farmer 2000) due to both morphological and molecular divergence making assessment of character homologies difficult. I therefore used *Pseudotrillium rivale* as a functional outgroup based on preliminary analyses (Farmer and Schilling 2002; Fig. 8, 9).

DNA Extraction, PCR, and Sequencing Protocols

For the analysis of the Delostylis Group, genomic DNA was extracted from plant material using the DNeasy Plant

Kit (QIAGEN Inc., Valencia, California, USA) from frozen, desiccated, or herbarium specimens (Table 3). PCR amplification and sequencing was carried out using the primers in Table 4. The PCR reactions were performed in 20 μ l reactions containing 13.5 μ l sterile water, 2.0 μ l 10 $\times$ PCR buffer, 1.8 μ l 2.5 mM MgCl₂, 0.4 μ l 0.2 mM dNTPs in equimolar ratio, 1 unit Taq polymerase (Eppendorf North America, Westburg, New York, USA), 0.4 μ l 0.5 mM each primer, 0.4 μ l BSA and 1 μ l genomic DNA. The ITS protocol was described by Sun et al. (1994), used primers ITS4 and ITS5, and proceeded as follows: initial denaturing at 94°C for 3 min, followed by 35 cycles of a 94°C denaturing step for 60 sec, a 58°C annealing step of 60 sec, and a 72°C extension step for 120 sec followed by an additional extension step for 5 min at 72°C to complete any unfinished DNA strands. The *matK* protocol (Fuse and Tamura 2000) used primers trnK-710F and matK-8R and proceeded as follows: initial denaturing at 94°C for 2 min, followed by 40 cycles of a 94°C denaturing step for 60 sec, a 50°C annealing step of 60 sec, and a 72°C extension step for 90 sec followed by an additional extension step for 3 min at 72°C to complete any unfinished DNA strands. After PCR, the samples were purified with ExoSAP-IT (USB Corp., Cleveland, Ohio, USA) before sequencing. In all cases, both strands of the PCR products were sequenced using the ABI PRISM Dye Terminator cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, California, USA) per manufacturer's instructions. To sequence the ITS region, primers ITS4 and 17SE were used; to sequence the *matK* region primers trnK-710F, matK-1470R, matK-1470F, and matK-8R were used. After the sequencing reaction, the samples were purified with Sephadex (Sigma-Aldrich, St. Louis, Missouri, USA) using Centri-Sep tubes (Princeton Separations, Adelphia, New Jersey, USA) and vacuum dried. The dried samples were delivered to the sequencing facility where they were sequenced on an ABI Prism 3100 automated sequencer (Perkin-Elmer Applied Biosystems) at the University of Tennessee Molecular Biology Resource Facility.

Sequence Manipulation and Alignment

The sequences were manually contiged (where necessary) and aligned using BioEdit (Hall 1997–2005, 1999). The ITS sequences obtained from GenBank (Kazempour Osaloo and Kawano 1999) consisted of 648 base pairs (of which 199 were variable for Trilliaceae); the *matK* sequences obtained from GenBank (Kazempour Osaloo et al. 1999) comprised

1578 base pairs, 84 of which were variable for Trilliaceae. The data matrices from the previous analyses were deposited at TreeBase; the final matrices from these analyses will also be deposited at TreeBase at the completion of the project. All aligned data matrices, tree files, and supporting data are also available from the author.

Phylogenetic Analyses

Data sets were analyzed using PAUP* (Swofford 2003) vers. 4.0b10 with maximum parsimony using heuristic search methods with TBR (tree bisection reconnection) and MULPARS and a simple addition sequence. Random addition with steepest descent was used to check for islands of trees (Maddison 1991). Bootstrap support (Felsenstein 1985) was estimated based on 100,000 FastStep replicates with the same search strategy as simple parsimony. In the initial analyses, gaps were treated as missing. In subsequent analyses, gaps were coded according to Simmons and Ochoterena (2000).

RESULTS

Previous Analyses

In all cases, *Paris* s.l. (tribe Parideae) and *Trillium* (tribe Trillieae) were shown to be sister clades with varying degrees of bootstrap support. Within Parideae, *Paris* and *Daiswa* were placed as sister clades. *Kinugasa* was sometimes sister to *Daiswa* and sometimes outgroup to *Paris* + *Daiswa*. In Trillieae, *T.* subgen. *Phyllantherum* was placed in a well-defined, monophyletic clade, but *T.* subgen. *Trillium* was not monophyletic. In morphological analyses, *Trillidium govanianum* was placed with Parideae, but in molecular analyses, it was placed with Trillieae (Farmer and Schilling 2002). Full details of these analyses are available from the author.

Analysis Using ITS Data Alone

New ITS sequence data were deposited by Y. Li of China with GenBank after the previous analyses were published (Farmer and Schilling 2002). Using these new sequence data, parsimony analysis produced two shortest trees of length 355 with a consistency index (CI) of 0.67; the strict consensus tree is shown in Fig. 10. The data set included 659 characters, 202 variable and 83 informative. As in all other analyses, Trillieae and Parideae were placed as sister clades; the basal split into these two clades was poorly supported, but individual clades typically had bootstrap support over 90%. These new data indicated that with the recognition of the segregate genera *Daiswa* and *Kinugasa*, *Paris* s.s. (Fig. 10) could be restricted to those species with a narrow creeping rhizome and without a seed aril. In addition, *Daiswa* (*Paris*) *polyphylla* and its varieties were found to be non-monophyletic. The topology of Trillieae changed slightly from previous analyses in that the putative group formed by *T. undulatum* and *Trillidium govanianum* was not placed in a basal position in this tree and was less well supported (bootstrap = 69%).

Analysis of Delostylis Group Using Combined ITS and matK Data

Preliminary parsimony analysis of combined ITS and matK sequence data produced 454 shortest trees of length

913 with a CI of 0.59 in multiple islands; the 50% majority rule is shown in Fig. 11. A poorly supported basal dichotomy separated Parideae and Trillieae. Within Parideae, *Paris* s.s. was supported at 98%, *Daiswa* at 100% with *Kinugasa* as basal to *Daiswa* at 89%. Within Trillieae, the most notable result was the placement of *Trillium ovatum* var. *hibbersonii* as not only the most basal taxon in the clade but also not as a diminutive form of *T. ovatum* var. *ovatum* (Taylor and Szczawinski 1974). An unresolved clade comprised sister species *T. undulatum* and *Trillidium govanianum* (supported at 82%), the Erectum Group (supported at 98%), and all other species of *Trillium* as successive sister groups (Fig. 11). The monophyletic group, *T.* subgen. *Phyllantherum* was supported at 100% and is sister to the rest of the pedicellate species of *Trillium*. Within the clade containing the remainder of the pedicellate *Trillium*, the basal *T. ovatum* var. *ovatum* (100% bootstrap support) was followed by paired sister species *T. grandiflorum* and *T. nivale* supported at 61%, with the Delostylis Group most terminal and supported at 99%. Within the Delostylis Group, *T. catesbaei* and *T. persistens* are sister taxa and most basal, leaving the Pusillum Complex most terminal. Within *T. pusillum*, there were successive-sister clades, the most basal of which contains var. *texanum* and the Georgia (var. *indet.* GA) populations. The remainder of the successive sister groups were the wetland sessile-flowered varieties (var. *virginianum*), the montane sessile-flowered variety (var. *monticulum*), populations from North Carolina (var. *indet.*) and Mississippi (var. *indet.* MS), then the Ozarks (var. *ozarkanum*), with the Alabama populations (var. *alabamicum* nom. ined.) comprising the terminal-most clade.

DISCUSSION

Trilliaceae

Based on all molecular analyses, Trilliaceae can be classified into the monotypic *Pseudotrillium rivale* and two monophyletic tribes: Parideae and Trillieae. The distinctiveness of *Pseudotrillium rivale* from both *Paris* and *Trillium* required erection of a new genus (Farmer and Schilling 2002); the basal position of *Pseudotrillium* is supported from prior molecular studies incorporating outgroups to the family (Fig. 8, 9). Tribe Parideae is composed of *Paris*, *Daiswa*, and *Kinugasa* whereas Trillieae is comprised of *Trillium* and apparently (in these analyses) the monotypic *Trillidium govanianum*. However, there are conflicts with the latter hypothesis. Morphologically, *Pseudotrillium* and *Trillium* have omniaperturate, spherical pollen grains with helobial endosperm development, features tentatively hypothesized as ancestral given the basal position of *Pseudotrillium* in the family. In contrast, *Trillidium* together with Parideae are characterized by ellipsoidal, monosulcate pollen grains and nuclear endosperm development, features apparently apomorphic in the family. The placement of *Trillidium* (near *Trillium undulatum*) within Trillieae in the molecular analyses may be an artifact of long-branch attraction (see later discussion). A new key to the genera of Trilliaceae is provided in Appendix 1.

Tribe Parideae

Parideae is monophyletic in all of the analyses (Fig. 10, 11). In addition to the traditionally used trait of merosity,

Table 3. Taxa included in the previously published *matK* and ITS sequencing analysis. Accession numbers are listed with ITS first then *matK*. Publication notes: (A) published in Kazempour Osaloo and Kawano (1999); (B) unpublished data by X. Tang, L. Yao, and R. Tang; (C) sequences produced by S. Farmer; populations will be vouchered at TENN and sequences will be deposited with GenBank at the completion of the project. VIU = voucher information unknown; LMC = leaf material collected; PV = population vouchered.

Taxon	Locality	Voucher information	Source	GenBank numbers
<i>Kinugasa japonica</i> (Franch. & Sav.) Tatew. & Sutō	Japan: Toyama, Tateyama-machi, Mt. Tateyama	<i>Kato</i> VIU	A	AB018804, AB018831
<i>Paris axialis</i> H. Li		VIU	B	AY192537
<i>P. cronquistii</i> (Takht.) H. Li		VIU	B	AY192025
<i>P. daliensis</i> H. Li & V. G. Soukup		VIU	B	AY192530, AY192529
<i>P. fargesii</i> Franch.	Japan: Setsunan University, cultivated in botanical garden	<i>Murata & Murata</i> VIU	A	AB018800, AB018827
		VIU	B	AY192536
<i>P. incompleta</i> M. Bieb.	UK: Edinburgh, cultivated in Royal Botanic Garden	VIU	A	AB018805, AB018832
<i>P. luquanensis</i> H. Li		VIU	B	AY192534
<i>P. marmorata</i> Stearn		VIU	B	AY192535
<i>P. polyphylla</i> (Smith) Raf.	Thailand: Chiang Mai, Doi Inthanon	<i>Tamura</i> VIU	A	AB018801, AB018828
<i>P. polyphylla</i> var. <i>chinensis</i> (Franch.) H. Hara		VIU	B	AY192531
<i>P. polyphylla</i> var. <i>minora</i> S. F. Wang		VIU	B	AY192540
<i>P. polyphylla</i> var. <i>stenophylla</i> Franch.		VIU	B	AY192538
<i>P. polyphylla</i> var. <i>yunnanensis</i> (Franch.) Hand.-Mazz.		VIU	B	AY192539
<i>P. tetraphylla</i> A. Gray	Japan: Hokkaido, Hakodate City, Mt. Hakodate-yama	<i>Kato</i> VIU	A	AB018806, AB018833
<i>P. thibetica</i> Franch.	UK: Edinburgh, cultivated in Royal Botanic Garden	VIU	A	AB018802, AB018829
		VIU	B	AY192532
<i>P. undulata</i> H. Li & V. G. Soukup		VIU	B	AY192533
<i>P. verticillata</i> M. Bieb.	Japan: Hokkaido, Hakodate City, Mt. Hakodate-yama	<i>Kato</i> VIU	A	AB018807, AB018834
<i>P. violacea</i> Lév.	Japan: Setsunan University, cultivated in botanical garden	<i>Murata & Murata</i> VIU	A	AB018803, AB018830
<i>P. sp.</i>	Purchased, Chen Yi Nursery	<i>Farmer s. n.</i> May 2002 (DNA 787)	C	
<i>P. sp.</i>	Purchased, Chen Yi Nursery	<i>Farmer s. n.</i> May 2002 (DNA 788)	C	
<i>Pseudotrillium rivale</i> (S. Watson) S. B. Farmer	USA: OR, Josephine Co.	<i>Hayashi et al.</i> VIU	A	AB018822, AB017404
	USA: CA, Del Norte Co.	<i>Kawano et al.</i> VIU	A	AB018822, AB017404
	USA: OR, Josephine Co.	<i>Kawano et al.</i> VIU	A	AB017404
	USA: OR, Polk Co., in cultivation	<i>Graham s. n.</i> Apr 2001 (DNA 792)	C	
<i>Trillidium govanianum</i> Wall. ex Royle	Bhutan: Himalayas, Shin-gonpa	<i>Umezawa</i> VIU	A	AB018813, AB017391
<i>Trillium camschatcense</i> Ker Gawl.	Japan: Hokkaido, Samani-cho	<i>Kato</i> VIU	A	AB018808, AB017379
<i>T. catesbaei</i> Elliott	USA: AL, Escambia Co.	<i>Farmer & Searcy s. n.</i> 19 Mar 2002 (DNA 780), <i>Farmer & Searcy s. n.</i> 19 Mar 2002 (DNA 781)	C	
	USA: AL, DeKalb Co.; TN, Knox Co., in cultivation	LMC: <i>Farmer s. n.</i> 14 May 1996 (DNA 807); PV: <i>Sherman</i> 227 10 May 1959 TENN	C	
	USA: GA, Whitfield Co.	<i>Jones s. n.</i> 11 Apr 2001 (DNA 808)	C	
<i>T. chloropetalum</i> (Torr.) Howell	USA: CA, Santa Cruz Co.	<i>Ohara et al.</i> VIU	A	AB018809, AB017382
<i>T. decipiens</i> Freeman	USA: FL, Jackson Co.	<i>Ohara et al.</i> VIU	A	AB018810, AB017385
<i>T. discolor</i> Wray ex Hooker	USA: SC, McCormick Co.	<i>Ohara et al.</i> VIU	A	AB018811, AB017387
<i>T. erectum</i> L.	USA: PA, Westmoreland Co.	<i>Kawano et al.</i> VIU	A	AB018812, AB017388

Table 3. Continued.

Taxon	Locality	Voucher information	Source	GenBank numbers
<i>T. grandiflorum</i> (Michx.) Salisb.	USA: PA, Westmoreland Co.	<i>Kawano et al.</i> VIU	A	AB018814, AB017392
	USA: VA, Wise Co.	<i>Farmer s. n.</i> 2 May 1996 (DNA 812)	C	
<i>T. lancifolium</i> Raf.	USA: SC, McCormick Co.	<i>Ohara et al.</i> VIU	A	AB018815, AB017394
<i>T. maculatum</i> Raf.	USA: GA, Early Co.	<i>Ohara et al.</i> VIU	A	AB018816, AB017397
<i>T. nivale</i> Riddell	USA: IN, Putnam Co.	<i>Steven s. n.</i> 13 Apr 2003 (DNA 789)	C	
<i>T. ovatum</i> Pursh var. <i>ovatum</i>	USA: CA, Del Norte Co.	<i>Kawano et al.</i> VIU	A	AB018817, AB017399
	USA: OR, Washington Co.; TN, Knox Co., in cultivation	<i>Farmer s. n.</i> 7 Apr 2002 (DNA 779)	C	
	Canada: British Columbia, Westbank	<i>Karstad s. n.</i> May 2002 (DNA 799)	C	
	USA: OR, Polk Co., in cultivation	<i>Graham s. n.</i> Apr 2001 (DNA 801)	C	
<i>T. ovatum</i> var. <i>hibbersonii</i> (T. M. C. Taylor & Szczaw.) G. W. Douglas & P. Pojar	Canada: British Columbia, Vancouver, in cultivation	LMC: <i>Gundlandsen s. n.</i> Apr 2001 (DNA 796)	C	
	Canada: British Columbia, Vancouver, in cultivation	LMC: <i>Thimble Farms s. n.</i> May 2001 (DNA 800)	C	
<i>T. persistens</i> Duncan	USA: GA, Habersham Co.	<i>Patrick s. n.</i> Apr 2000 (DNA 806)	C	
<i>T. petiolatum</i> Pursh	USA: WA, Chelan Co.	<i>Ohara et al.</i> VIU	A	AB018818, AB017400
<i>T. pusillum</i> Michx. var. <i>indet.</i>	USA: NC, Sokes Co. (sic.)	<i>Kawano et al.</i> VIU	A	AB018819, AB017401
	USA: MS, Jones Co. (MS)	LMC: <i>Farmer & Wieland s. n.</i> 20 Mar 2002 (DNA 790); PV: <i>Morgan 468</i> 9 May 1978 MNHP		
	USA: GA, Whitfield Co. (GA)	<i>Farmer & Noble s. n.</i> 11 Apr 2001 (DNA 736)		
	USA: GA, Whitfield Co. (GA)	<i>Farmer s. n.</i> 5 Apr 2002 (DNA 809)		
<i>T. pusillum</i> var. <i>alabamicum</i> nom ined.	USA: TN, Lincoln Co.	LMC: <i>Farmer & Simmons s. n.</i> 18 Apr 2001 (DNA 735); PV: <i>Patrick & Simmons 1113</i> 29 Apr 1980 TENN	C	
	USA: AL, Limestone Co.	<i>Farmer et al. s. n.</i> 13 Mar 2002 (DNA 793)	C	
<i>T. pusillum</i> var. <i>monticulum</i> Bodkin & Reveal	USA: VA, Rockingham Co.	<i>Farmer & Clevinger s. n.</i> 27 Apr 2002 (DNA 785)	C	
<i>T. pusillum</i> var. <i>ozarkanum</i> (E. J. Palmer & Steyerl.) Steyerl.	USA: MO, Barry Co.	<i>Hollis s. n.</i> 27 Jun 2001 (DNA 734)	C	
	USA: AR, Montgomery Co.	<i>Farmer & Whitsell s. n.</i> 22 Mar 2002 (DNA 791)	C	
<i>T. pusillum</i> var. <i>texanum</i> (Buckley) Reveal & C. R. Broome	USA: TX, Cass Co.	LMC: <i>Singhurst s. n.</i> 22 Mar 2002 (DNA 786); PV: <i>Correll 36935</i> 9 Apr 1969 NCU	C	
	USA: TX, Smith Co.	<i>Farmer et al. s. n.</i> 22 Mar 2002 (DNA 794)	C	
	USA: LA, Caddo Parish	<i>Reynolds s. n.</i> 16 Apr 2002 (DNA 778)	C	
<i>T. pusillum</i> var. <i>virginianum</i> Fernald	USA: VA, James City Co.	LMC: <i>Ware & Grubbs s. n.</i> 16 May 2002 (DNA 782); PV: <i>Ware & Ware 7322</i> 8 Apr 1979 VPI	C	
	USA: VA, Grayson Co.	<i>Farmer & Wieboldt s. n.</i> 16 May 2002 (DNA 783)	C	
	USA: MD, Worcester Co.	<i>Frye s. n.</i> 26 Apr 2002 (DNA 811)	C	
<i>T. recurvatum</i> Beck	USA: AR, Newton Co.	<i>Ohara et al.</i> VIU	A	AB018820, AB017402
<i>T. reliquum</i> Freeman	USA: GA, Columbia Co.	<i>Ohara et al.</i> VIU	A	AB018821, AB017403
<i>T. rugelii</i> Rendle	Japan: Hokkaido University, cultivated in botanical garden	VIU	A	AB018823, AB017405
<i>T. sessile</i> L.	USA: PA, Westmoreland Co.	<i>Kawano et al.</i> VIU	A	AB018824, AB017406
<i>T. undulatum</i> Willd.	USA: PA, Westmoreland Co.	<i>Kawano et al.</i> VIU	A	AB018825, AB017413
	USA: VA, Rockingham Co.	<i>Farmer & Clevinger s. n.</i> 27 Apr 2002 (DNA 784)	C	
<i>Veratrum maackii</i> Regel	Japan: Nyukawa-mura	<i>Kato</i> VIU	A	AB018826, AB017417

Fig. 8.—Strict consensus tree from principal analysis of Trilliaceae with *Veratrum maackii* as the outgroup using *matK* data (1326 trees, length 285).

Fig. 9.—Strict consensus tree from principal analysis of Trilliaceae with *Veratrum maackii* as the outgroup using ITS data (6 trees, length 748). (From Farmer and Schilling 2002).

Parideae can be distinguished by synapomorphies such as filiform petals, elliptical, monosulcate pollen, and nuclear endosperm (Farmer and Schilling 2002).

The molecular analyses supported the separation of Parideae into two sister clades, *Paris* and *Kinugasa* + *Daiswa* (Fig. 10, 11). The support for the monophyly of *Kinugasa* + *Daiswa* was provided primarily by the molecular analyses rather than traditional morphology. In addition, the problematic placements of *D. fargesii* and *D. thibetica* are now resolved because of apparent character-state reversions. Both species exhibit the thick rhizome characteristic of *Daiswa*;

but *D. fargesii* has an indehiscent rather than a dehiscent berry, and *D. thibetica* has a partial rather than a complete aril (Li and Noltie 1997). Character-mapped cladograms are available from author.

In traditional, morphological classifications, *Kinugasa* has always been aligned with *Paris* subgen. *Paris*, but morphology now interpreted in light of the current analyses suggests a closer relationship between *Kinugasa* and *Daiswa*, with both exhibiting the thick rhizome and arillate seeds in addition to many of the same insertions/deletions (indels) and base-pair changes. One alternative is to lump *Kinugasa*

Table 4. PCR and sequencing primers used in the new sequencing analyses of Trilliaceae. Amplification primers are denoted PCR; sequencing primers SEQ.

Region	Reference	Primer	5'-Primer sequence-3'	Use
ITS	White et al. (1990)	ITS5	GGA AGT AAA AGT CGT AAC AAG G	PCR, SEQ
		ITS4	TCC TCC GCT TAT TGA TAT GC	PCR
<i>matK</i>	Sun et al. (1994)	17SE	ACG AAT TCA TGG TCC GGT GAA GTG TTC G	SEQ
	Fuse and Tamura (2000)	trnK-710F	GTA TCG CAC TAT GTA TCA TTT GA	PCR, SEQ
		matK-1470R	AAG ATG TTG ATC GTA AAT GA	SEQ
		matK-1470F	TCA TTT ACG ATC AAC ATC TT	SEQ
		matK-8R	AAA GTT CTA GCA CAA GAA AGT CGA	PCR, SEQ

Fig. 10.—Strict consensus of two trees from the ITS analysis of 38 accessions of Trilliaceae taxa. Length = 355 steps; consistency index = 0.67. Percentages below the branches are bootstrap values.

Fig. 11.—50% majority rule consensus of 454 trees in 57 islands from the ITS-matK analysis of 54 accessions of Trillieae taxa. Length = 913 steps; consistency index = 0.86. Percentages below the branches are bootstrap values. Numbers refer to DNA samples in Table 3.

with *Daiswa*. However, because of the unusual morphology of the species (i.e., the octoploid chromosome count, and showy, white sepals), the segregate genus *Kinugasa* should be retained.

Because of the recognition of *Kinugasa* as a genus, and because *Paris* and *Daiswa* were separated on a molecular level as well as morphologically, *Daiswa* as distinct from *Paris* should be recognized. Nomenclatural consequences will be addressed at a later date.

Tribe Trillieae

Trillium ovatum var. *hibbersonii* and *T. ovatum* var. *ovatum* do not together comprise a monophyletic group and occur in separate clades in the combined ITS–*matK* analysis (Fig. 11). *Trillium ovatum* var. *hibbersonii* is placed at a more basal position in the tree than *T. ovatum* var. *ovatum*. There are approximately 29 absolute base pair changes and one indel of five base pairs in length that separate these two taxa in the ITS data set; in the *matK* data set, there are 13 absolute base pair changes within the coding region and nine base pair changes and three indels of length five, six, and 11 base pairs in the 5' spacer region that separate varieties *hibbersonii* and *ovatum*, but only two that unite them as distinct from all other *Trillium* species. There are several morphological characters that separate them as well, most notably the extremely small stature of *T. ovatum* var. *hibbersonii*, but also phenology, gynoeceal and androeceal morphology, as well as a difference in petal coloration (*T. ovatum* var. *hibbersonii* flowers pink before fading to white whereas *T. ovatum* var. *ovatum* flowers white and fades to pink [Wiley 1969]). Thus, it is suggested that *Trillium ovatum* var. *hibbersonii* be elevated to species rank (Farmer in prep.).

With fewer species of Parideae in the analysis shown in Fig. 11, the grouping of *T. undulatum* and *Trillidium gowanianum* is better supported at 82% (Fig. 11) than in Fig. 10. Due to insufficient sampling (Graybeal 1998), it is possible that the problematic placement of *Trillidium* (e.g., as sister species to *Trillium undulatum*, see Fig. 10, 11) is caused by long-branch attraction or lineage sorting. The best solution for eliminating long-branch attraction is to increase taxon sampling (Graybeal 1998). This hypothesis is supported not only by the drop in bootstrap support from 82% (Fig. 11) with only 10 members of Parideae to 69% (Fig. 10) when 20 species of Parideae are included in the analysis, but also the fact that if *Trillium undulatum* is removed from the analysis, *Trillidium* is placed as the basal ingroup species (i.e., sister to *Trillium* and Parideae combined).

The grouping of *Trillium nivale* and *T. grandiflorum* together is poorly supported in the analysis shown in Fig. 11 at 61%; however, results are available from only one accession of *T. nivale*. Traditionally, *T. grandiflorum* and *T. ovatum* have been placed with members of the Delostylis Group because of their anthocyanin chemistry and petal texture (Gleason 1906; Barksdale 1938; Ihara and Ihara 1978, 1982; Patrick 1984); however, they do not share the character states of a fused style and sub-petiolate to petiolate leaves. This analysis does not support a close relationship between *T. grandiflorum* and *T. ovatum*, but rather between *T. grandiflorum* and *T. nivale*.

The Delostylis Group s.s. (i.e., Rafinesque's 1819 definition of *Delostylis* minus *Trillium nivale*) makes up the remainder of the tree shown in Fig. 11. *Trillium persistens* and *T. catesbaei* are sister taxa that form the base of this clade with *T. pusillum* and its varieties monophyletic. In previous analyses, the placement of *T. pusillum* varied depending on the data set. In the large morphological analysis (Farmer and Schilling 2002), *T. pusillum* was part of a clade basal to all other taxa. In addition, its placement in the ITS analysis (Fig. 10) was different from that produced by the combined ITS–*matK* tree (Fig. 11). In the analysis of the relationships among the varieties of *T. pusillum*, the strict consensus tree of 454 most parsimonious trees (Fig. 11) shows several well-defined and well-represented groups. However, in the FastStep Bootstrap of 100,000 reps, separation into distinct varietal units with bootstrap support was not observed—only a polytomy was produced.

Trillium pusillum var. *texanum* and the Georgia populations are more closely related to each other than they are to the rest of the *pusillum* complex. This relationship is supported by the synapomorphy of stomates on the upper surface of the leaves. Based on sampling of other non-coding plastid regions (Shaw et al. 2005), the varieties of *T. pusillum* are separable on a molecular level.

Biogeography

Distribution and endemism patterns in Trilliaceae.—Each of the Arcto-Tertiary areas of refuge has its own center of diversity. In eastern Asia, which has the highest diversity of this modern Tertiary flora (Tiffney 1985a), the center of diversity is in south central China in the Qinling Mountains (Bufford 1998). In eastern North America the highest diversity is in the southern Appalachians (Bufford 1998; Wen 1999). The Klamath range of northern California and southern Oregon has the greatest diversity in western North America (Wood 1971). In the Europe-Asia Minor refuge, the center of diversity appears to be in the Caucasus Mountains (Wood 1971). This pattern is similar in Trilliaceae with Asia having the greatest diversity (34 species and five known hybrids in five genera). North America is next with 30 species of *Trillium* in eastern North America while western North America has at least eight species in two genera (*Trillium* and *Pseudotrillium*). Europe has the least diversity with only two species of *Paris*.

In addition to the traditional Arcto-Tertiary distribution patterns, genetics provide another distribution pattern; polyploidy is restricted to the Old World. All North American *Trillium* are diploid; although there are scattered reports of triploid specimens (Haga 1942; Sparrow and Pond 1950; Kozuka et al. 1964). Of the nine *Trillium* in Asia, only one is diploid; the rest are triploid, tetraploid, or hexaploid (Samejima and Samejima 1987). Within *Paris*, the primary European taxon, *P. quadrifolia*, is tetraploid. *Paris incompleta* from the Caucasus Mountains is diploid as are most of the other Asian taxa including all members of *Daiswa* (Li 1998). *Kinugasa japonica* is octoploid (Tatewaki and Sutô 1935). Of the Asian polyploid *Trillium*, five are recognized as hybrids. Hybridization is believed to occur in North America in the Erectum Group as well as in the Phyllanthum Group

(Freeman 1969), but has not been proven (Case and Case 1997).

Phylogenetic distribution patterns.—If the phylogeny based on a morphological analysis of the family (Farmer 2000; Farmer and Schilling 2002) is an accurate representation of the evolutionary history of the various species, then the oldest extant lineage is in the Klamath Mountains of the Pacific Northwest, represented by *Pseudotrillium rivale*. Given that Parideae and Trillieae are sister groups occupying largely different geographical areas (Eurasia vs. Asia–North America), a taxon-area cladogram based on phylogeny would not be informative; however, some patterns may be noted. Based on the phylogeny, the basal taxa in the two tribes are either Asian or western North American. From the center of origin, the *Paris*-like species dispersed to Asia and from Asia into Europe. The Erectum Group dispersed to Asia and eastern North America. The American species may have diverged from the Asian species before or after dispersal, and the Asian taxa are likely to be ancestral in this group. The Grandiflorum Group diverged with *Trillium ovatum* persisting in the Pacific Northwest and with *Trillium grandiflorum* migrating (or surviving) in the southern Appalachians (Griffin and Barrett 2004). The Phyllantherum Group diverged and also survives in western North America and eastern North America with the ancestral taxa in the Pacific Northwest.

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APPENDIX 1. KEY TO THE GENERA OF TRILLIACEAE

1. Inflorescence composed of tepals (if outer perianth segments are green, shape and size of inner and outer segments similar); phyllotaxy trimerous *TRILLIDIUM*
1. Inflorescence composed of sepals and petals (shape and size of inner and outer segments dissimilar); phyllotaxy trimerous to numerous 2
2. Sepals petaloid, showy, white; petals filiform (to 1[–2] mm wide) or absent *KINUGASA*
2. Sepals herbaceous, green or purplish; petals filiform to broad (0.1–6 cm wide), or absent 3
3. Phyllotaxy mostly trimerous with leaves (0.8–) 5–15 (–25) cm wide; petals (2–) 7–15 (–60) cm wide (if narrower, petals either white or pink, or plants sessile-flowered) 5
3. Phyllotaxy mostly 4- to 11-merous; leaves (0.8–) 2–5 (–7) cm wide (rarely to 60 cm with fewer leaves and height to 1 m or more); petals filiform 1–2 (–3) mm (rarely 5–6 mm) 4
4. Rhizome slender, running; seeds without aril *PARIS*
4. Rhizome thick; seeds with enclosing red or orange sarcotesta, or partial green aril *DAISWA*
5. Petals generally spotted, ovate, frequently appearing clawed; leaves cordate to rounded, coriaceous . . . *PSEUDOTRILLIUM*
5. Petals not spotted, from ovate to obovate; leaves ovate to obovate, “herbaceous” or not coriaceous *TRILLIUM*