

## Paraphyletic *Syringa* (Oleaceae): Evidence from Sequences of Nuclear Ribosomal DNA ITS and ETS Regions

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**ABSTRACT.** Sequences of nuclear ribosomal DNA ITS and ETS regions were used to examine phylogenetic relationships of *Syringa* and *Ligustrum*. Twenty-seven samples were included in parsimony analyses, representing all major groups of these two genera. Two species of *Fraxinus* and one species of *Jasminum* were also included in analyses for rooting purposes. In the resulting phylogenetic hypothesis series *Syringa* (*Syringa*) diverges first and is followed by the monotypic series *Pinnatifoliae* (*Syringa*), which is sister to a clade containing the remaining species of *Syringa* and species of *Ligustrum*. However, this sister relationship is weakly supported. Our results support the recognition of monophyletic groups corresponding to subgenus *Ligustrina*, and series *Syringa*, *Pubescentes*, and *Villosae*, and suggest that *Ligustrum* as a monophyletic group is derived from within *Syringa*, such that *Syringa* as traditionally circumscribed is paraphyletic. Forcing *Syringa* to be monophyletic entails nine extra steps, which is significant, as judged by the Templeton test. *Parasyringa sempervirens* is phylogenetically embedded within the *Ligustrum* clade, supporting its placement in *Ligustrum*. Berries are a synapomorphy of *Ligustrum* species, and the dehiscent berry of *Parasyringa sempervirens* is likely to be an evolutionary reversal towards a capsule, which is characteristic of *Syringa*. The evolution of berries might have contributed to an accelerated rate of speciation in *Ligustrum*.

*Syringa* L., the lilacs, is well known for beautiful and fragrant flowers, and its species have been widely cultivated as ornamentals. Twenty-two to 28 species of *Syringa* have been recognized, and are distributed in southern Europe and East Asia (McKelvey 1928; Fiala 1988; Chang et al. 1996). Recent molecular studies have indicated that *Syringa* belongs to the monophyletic subfamily Oleoideae, and is closely related to *Ligustrum* (Wallander and Albert 2000).

Two subgenera have been recognized in *Syringa* (McKelvey 1928; Rehder 1945; Chang et al. 1996). Subgenus *Ligustrina*, commonly referred to as the tree lilacs, consists of two species and one variety (McKelvey 1928; Li et al. 2001). The remaining *Syringa* species belong to subgenus *Syringa* (Pringle 1981; Chang et al. 1996). Rehder (1945), based on leaf pubescence and shape, and inflorescence development, divided subgenus *Syringa* into four series: *Pinnatifoliae*, *Syringa* (= *Vulgares*), *Pubescentes*, and *Villosae* (Table 1). A recent phylogenetic study based on chloroplast DNA restriction fragment length polymorphisms (RFLPs) supported the monophyly of each of Rehder's series (Kim and Jansen 1998). However, phylogenetic analyses based on DNA sequence data have not been carried out to further test these groups of *Syringa*.

Privets (*Ligustrum* L.) are rarely cultivated as floral ornamentals because their flowers are neither as showy nor as pleasantly scented as lilacs. Nevertheless, some species are commonly used as hedge plants. There are 40–50 species of *Ligustrum*, distributed in Asia, Europe, Malaysia, and Australia (Rehder 1945; Mabblerley 1997). Mansfeld (1924) divided *Ligustrum* into three sections. Section *Sarcocarpion* consists of a single spe-

cies, *L. sempervirens* (Franch.) Lingelsh, and is endemic to southeastern Sichuan and northeastern Yunnan Provinces of China. Section *Baccatae* is also monotypic (*L. vulgare* L.), and is widely distributed in Europe, Asia Minor, and the Caucasus. The remaining species of *Ligustrum* belong to section *Subdrupacea*. Mansfeld (1924) further divided this section into two subsections, *Robustae* and *Sinenses*. Other authors, in contrast, recognize only two sections: *Sarcocarpion* and *Ibota*. The latter is a combination of Mansfeld's sections, *Baccatae* and *Subdrupacea* (Koehe 1904; Chang and Miao 1986; Chang and Qiu 1992; Chang et al. 1996).

*Syringa* and *Ligustrum* share similar floral structures but differ in fruit type: berries in *Ligustrum* and capsules in *Syringa*. Curiously, *Parasyringa sempervirens* (Franch.) Smith. (= *L. sempervirens*) produces dehiscent berries, which are fleshy in the fall and become dry, and open in late Spring. This "aberrant" fruit type suggests a close link between *Syringa* and *Ligustrum*. The taxonomic position of *P. sempervirens* has been controversial. It has been treated as a species of *Syringa* (Franchet 1886), or of *Ligustrum* (Mansfeld 1924; Chang and Miao 1986; Green and Fliegner 1991; Chang et al. 1996), or as a monotypic genus (Schneider 1911; Smith 1916; Stapf 1933).

In this study we conducted phylogenetic analyses of *Syringa* and *Ligustrum* using DNA sequence data 1) to evaluate the monophyly of *Syringa*, *Ligustrum*, and their subgroups; and 2) to examine the systematic position of *Parasyringa sempervirens*. We used DNA sequence data of both the internal and external transcribed spacers of nuclear ribosomal DNA (ITS and ETS); both markers have been used successfully in re-

TABLE 1. Species used in this analysis. Rehder's (1945) classification system of *Syringa* is shown in the first column. All voucher specimens are deposited in A at Jamaica Plain, MA.

Species	Vouchers	GenBank accession number	
		ITS	ETS
<i>Syringa</i> subgenus <i>Ligustrina</i>			
<i>S. pekinensis</i> Rupr.	21634A	AF297077	AF297067
<i>S. reticulata</i> (Blume) Hara	1111A	AF297079	AF297069
<i>S. reticulata</i> (Blume) Hara var. <i>amurensis</i> (Rupr.) Pringle	11706A	AF297072	AF297062
<i>Syringa</i> subgenus <i>Syringa</i>			
Series <i>Pinnatifoliae</i>			
<i>S. pinnatifolia</i> Hemsley	745-93A	AF297081	AF297071
Series <i>Syringa</i>			
<i>S. vulgaris</i> L.	949-34B	AF361289	AF361266
<i>S. oblata</i> Lindley	307-78A	AF361288	AF361290
Series <i>Pubescentes</i>			
<i>S. pubescens</i> Turczaninow	1594A	AF277746	AF361263
<i>S. julianae</i> Schneider	657-80mass	AF277749	AF361262
Series <i>Villosae</i>			
<i>S. emodi</i> Wallich.	18804B	AF277761	AF361256
<i>S. villosa</i> Vahl	17362A	AF361283	AF361257
<i>S. wolfii</i> Schneider	184-79F	AF361284	AF361258
<i>S. reflexa</i> Schneider	109-81A	AF361281	AF361254
<i>S. yunnanensis</i> Franch.	1211-76A	AF361285	AF361259
<i>S. komarowii</i> Schneider	1333-38A	AF361286	AF361260
<i>S. tigerstedtii</i> H. Smith	1254-52B	AF361287	AF361261
<i>Parasyringa sempervirens</i> W.W.Smith	Xin Tian 2066	AF361300	AF361277
<i>Ligustrum</i> L.			
<i>L. vulgare</i> L.	901-85B	AF361298	AF361275
<i>L. japonicum</i> Thunb.	61-90A	AF361299	AF361276
<i>L. compactum</i> (Wall. ex G. Don) Hook.f. & Thoms. ex Brandis	2043-77B	AF361292	AF361269
<i>L. lindleyi</i> (Wall. ex G. Don) P. S. Green	1003-77mass	AF361293	AF361270
<i>L. obtusifolium</i> Sieb. & Zucc.	14890D	AF361294	AF361271
<i>L. acutissimum</i> Koehne	1917-80B	AF361295	AF361272
<i>L. ovatifolium</i> Hassk.	499-90C	AF361296	AF361273
<i>L. ibota</i> Sieb. & Zucc.	545-81mass	AF361297	AF361274
<i>Fraxinus</i> L.			
<i>F. excelsior</i> L.	1394-80A	U82866-U82867 Jeandroz et al., 1997	AF361278
<i>F. americana</i> L.	636-88A	U82906-U82907, Jeandroz et al., 1997	AF361279
<i>Jasminum nudiflorum</i> Lindley	603-81mass	AF361301	AF361280

solving interspecific relationships of plant groups (e.g., Baldwin et al. 1995; Baldwin and Markos 1998; Bena et al. 1998; Li et al. 2000, 2001).

#### MATERIALS AND METHODS

**Taxa Sampled.** Twenty-seven species were included in our analyses, representing all major groups of *Syringa* and *Ligustrum*, and two species of *Fraxinus* and one species of *Jasminum* were used as outgroups (Table 1). These latter two genera are closely related to *Syringa* and *Ligustrum* (Wallander and Albert 2000). Sequences of both ITS-1 and ITS-2 of two species of *Fraxinus* were obtained from GenBank (U82906-U82907 and U82866-U82867; Jeandroz et al. 1997).

**Molecular Techniques.** DNAs were extracted from silica gel dried leaves using the Qiagen DNeasy plant mini kit (Clarita, CA). Procedures for PCR (polymerase chain reaction) and DNA sequencing are described in detail elsewhere (Li et al. 2001) as are the primers used in the reactions. Sequences were analyzed using an ABI 377 Automated Sequencer and edited using the computer program Sequencher 3.0 (Gene Codes Corp., Inc., Ann Arbor, MI). Limits of the ITS-1 and ITS-2 regions were determined by comparison with published sequences (GenBank accessions, U82906-U82907, U82866-U82867, and U82918-U82919; Table 1).

**Phylogenetic Analyses.** Sequences were imported into PAUP\* (4.0b4a, Swofford 2000) and aligned by eye. Characters were equally weighted and their states were unordered. Parsimony analyses were carried out using PAUP\* with gaps treated as missing data, and heuristic tree search options included random sequence addition for 500 replicates, each of which held 10 trees, TBR branch swapping, MULPARS on, and steepest descent off. Bootstrap analyses of 100 replicates were conducted using PAUP\* to measure relative support for clades (Felsenstein 1985); heuristic tree search options were simple sequence addition, TBR branch swapping, MULPARS on, and steepest descent off. Maximum likelihood analyses were conducted using the HKY'85 model (Hasegawa et al. 1985), with rate heterogeneity ( $\Gamma=0.5$ ) and a transition/transversion ratio of 2. Trees search options included heuristic tree search, as-is sequence addition, TBR tree swapping, MULPARS on, and steepest descent off. Sequences of the outgroups are markedly different from those of *Syringa*+*Ligustrum* in G+C contents (see below). As noticed by Nyffeler and Baum (2000), even slight (~2%) difference in G+C content may cause false tree topologies by pulling some ingroup taxa toward the outgroups. To avoid this problem, as recommended by Nyffeler and Baum (2000), we conducted ML analyses without the outgroups while enforcing the molecular clock option.

**Data Incongruence Test.** The ILD (Incongruence Length Difference) test was carried out to determine whether the ETS and ITS

data sets are incongruent (Farris et al. 1994), and was implemented using the PARTITION HOMOGENEITY option in PAUP\*. Search options were as described in bootstrap analyses.

**Test the Monophyly of *Syringa* and *Ligustrum*.** We used MacClade 3.04 (Maddison and Maddison 1992) to rearrange the combined tree topology so that *Syringa* and *Ligustrum* were monophyletic and sister to each other. The modified phylogeny was then used as a constraint tree in parsimony analyses to determine extra steps entailed to achieve monophyly of *Syringa* and *Ligustrum*. A Wilcoxon sign rank (WSR) test (Templeton 1983) was also conducted using PAUP\* to test whether the constrained tree is significantly longer than the unconstrained trees.

## RESULTS

**Sequence Characteristics.** The length of the ITS regions, including the 5.8S gene, ranged from 621 to 626 base pairs (bp), with an average of 623 bp, and the alignment generated a data set of 635 characters, of which 115 were parsimony informative. Sequence divergence ranged from 19–24% between *Ligustrum* plus *Syringa* and the outgroups, with an average of 21%; from 0–7% within *Syringa*, with an average of 3.1%, and from 2.0–7.4% between *Syringa* and *Ligustrum*, with an average of 3.4%. G plus C content ranged from 53.8–56.0%, with an average of 55% in *Syringa*+*Ligustrum*, and from 59.0–64.8% in the outgroups.

The sequenced segment of the ETS ranged from 375–378 bp in length, and the alignment resulted in a data matrix of 384 characters, of which 83 were parsimony informative. Sequence divergence of the ETS region ranged from 15.6–37.6% between *Syringa* plus *Ligustrum* and the outgroups, and from 0–9.4% within *Syringa*, and from 3.2–9.0% between *Ligustrum* and *Syringa*. G plus C content of the ETS region ranged from 48–50% in *Syringa*+*Ligustrum*, and from 53–58% in the outgroups. All sequences have been submitted to GenBank (Table 1) and data matrices and trees are available from TreeBASE (<http://www.harvard.edu/TreeBase>).

**Phylogenetic Relationships.** The ILD test resulted in a probability of 0.88, indicating that the ITS and ETS data sets are not significantly incongruent. On this basis, we conducted parsimony analyses using the combined data set of 1019 sites, resulting in 12 trees of 648 steps (CI=0.79, RI=0.75). A strict consensus tree is shown in Fig. 1. All groups recognized within *Syringa*, except for subgenus *Syringa*, but including subgenus *Ligustrina* (clade J), series *Villosae* (E), *Pubescentes* (I), *Syringa* (H), and *Pinnatifoliae*, are resolved as monophyletic. *Syringa* subgenus *Ligustrina* (J) is sister to series *Pubescentes* (I), but this relationship is only weakly supported (bootstrap <50%). *Syringa* series *Pinnatifoliae* and *Syringa* are basal clades. *Ligustrum* (including *Parasyringa*) (F) is monophyletic and is sister to the clade containing *Syringa* series *Villosae*, *Pubescentes*, and subgenus *Ligustrina*. *Ligustrum japonicum* is sister to a clade containing the remaining species of *Ligustrum*. Constraining all species of *Syringa* to monophyly

resulted in trees that are nine steps longer than the shortest trees; this difference is significant as judged by a WSR test ( $P=0.005$ ).

The ML, molecular clock analysis of the combined data set without the outgroups generated congruent phylogenies with those based on parsimony analyses including the outgroups.

## DISCUSSION

**Phylogenetic Usefulness of ETS.** The usefulness of the ETS region in molecular systematics has been suggested previously (Baldwin and Markos 1998; Bena et al. 1998). In *Syringa* and *Ligustrum*, the sequenced segment of the ETS region is much shorter than the ITS region (384 vs. 635 aligned bp); nevertheless, it produces a slightly higher percentage of informative sites (21.61%) than the ITS region (18.11%). Neither the ITS nor the ETS data set individually provides enough informative characters to resolve phylogenetic relationships of *Ligustrum* and *Syringa* (trees not shown). However, sequences of these two regions are highly congruent, and the combined data set generates well-resolved phylogenies with strong support for most of the clades. This suggests that when combined, sequences of the ETS and ITS regions are useful in resolving phylogenetic relationships at lower taxonomic levels.

**Monophyly of *Syringa*.** *Syringa* and *Ligustrum* are closely related (Wallander and Albert 2000). This is supported by similarities of floral structures of tree lilacs (e.g., *Syringa reticulata* (Blume) Hara and *Ligustrum*, and is seemingly strengthened by the existence of *Parasyringa sempervirens* (= *L. sempervirens*), which shows morphological intermediacy between *Syringa* and *Ligustrum*. Nevertheless, the separate generic identity of both *Syringa* and *Ligustrum* has never been a matter of debate because of their distinctive fruit types: berries in *Ligustrum* and capsules in *Syringa*. On this basis, it is curious that in our ETS+ITS trees, species of *Ligustrum* form a well-supported clade and are derived from within *Syringa*. Constraint parsimony analyses forcing *Syringa* to be monophyletic requires nine additional steps, and the WSR test shows that this difference is statistically significant. Furthermore, the basal position of *Syringa* series *Syringa* is unlikely to be the result of the slight difference of G+C content among species, as judged by the fact that congruent trees are generated when molecular clock analysis is conducted without the outgroups (Nyffeler and Baum 2000). *Syringa*, therefore, as traditionally circumscribed, is probably paraphyletic, and the taxonomy of these two genera may need to be reconsidered.

**Phylogenetic Relationships in *Syringa*.** Rehder (1945) divided *Syringa* into two subgenera, *Ligustrina* and *Syringa*. Species of *Ligustrina*, the tree lilacs, are characterized by tree-like habit, yellowish white flow-

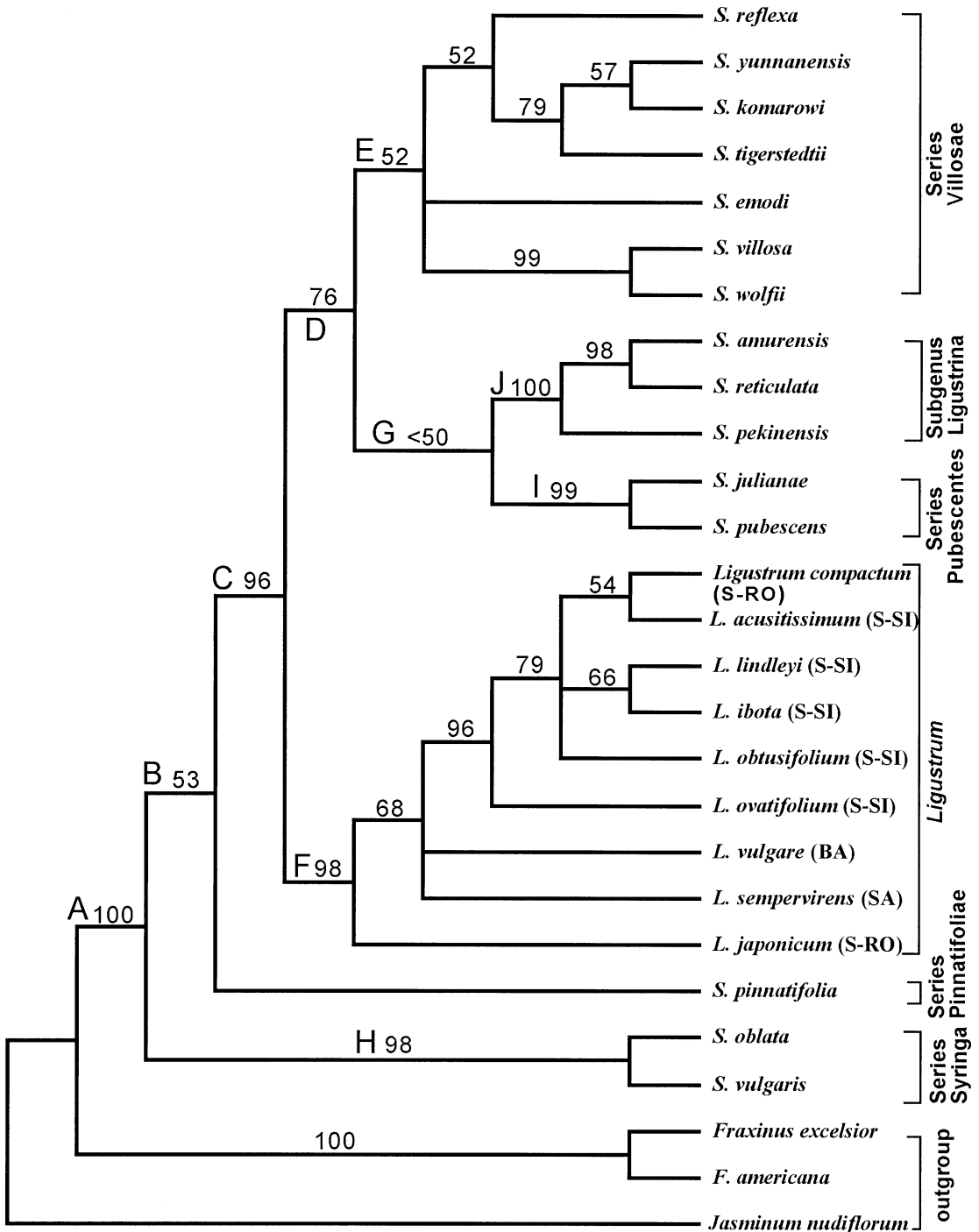


FIG. 1. A strict consensus of 8 trees of 773 steps based on the combined data set of ITS and ETS sequences (CI=0.79, RI=0.79). Numbers above branches represent bootstrap estimates for 100 replicates. Acronyms in parentheses show groupings: SA = *Sarcocarpion*, BA = *Baccatae*, S-SI = *Subdrupacea-Sinenses*, S-RO = *Subdrupacea-Robustae*. Letters A-J represent clades discussed in the text.

ers, short corolla tubes, and long exerted stamens. The remaining species of *Syringa* comprise the subgenus *Syringa*, plants of which are distinguished by the shrubby habit, pinkish flowers, long corolla tubes, and

enclosed stamens. This taxonomic treatment is supported by chloroplast DNA RFLP data (Kim and Jansen 1998). Within subgenus *Syringa*, Rehder (1945) recognized four series based on inflorescence develop-

ment, and leaf shape and pubescence. Series *Pinnatifoliae* is unique in having pinnately compound leaves, and series *Syringa* can be easily distinguished by its simple, glabrous leaves with truncate or subcordate bases. Series *Pubescentes* is characterized by its evident leaf pubescence, whereas series *Villosae* differs from the above three series in having terminal development of the inflorescence. In the ITS+ETS tree (Fig. 1), species of the subgenus *Ligustrina* form a well-supported clade; however, this group is not sister to the remaining *Syringa*. Instead, it is in the clade containing series *Villosae* plus series *Pubescentes*, and is weakly supported (bootstrap < 50%) as sister to series *Pubescentes*. Thus, more data are needed to elucidate phylogenetic relationships of tree lilacs with other groups of *Syringa*. Nevertheless, our results suggest that subgenus *Ligustrina* may be derived from within subgenus *Syringa*, and that the latter therefore is paraphyletic.

In Figure 1, each of the four series forms its own clade, supporting the monophyly of Rehder's (1945) series. However, series *Villosae* is weakly supported (bootstrap < 50%). A phylogenetic analysis based on RFLP data suggests that *S. pinnatifolia* is the most basal taxon (Kim and Jansen 1998). In our ITS+ETS tree, series *Syringa* diverges first and is sister to a clade containing series *Pinnatifoliae*, *Pubescentes*, and *Villosae*. However, the sister relationship of *S. pinnatifolia* with the clade containing *Pubescentes*, *Villosae*, subg. *Ligustrina*, and *Ligustrum* is weakly supported (bootstrap = 53%, Fig. 1). Additional data, therefore, are needed to resolve inter-series relationships of *Syringa*.

**Phylogenetic Relationships of *Ligustrum*.** The monophyly of *Ligustrum* has never been in dispute (Kohne 1904; Mansfeld 1924; Chang and Miao 1986), and has recently been supported by sequences of chloroplast genes (Wallander and Albert 2000). In the ITS+ETS tree (Fig. 1) species of *Ligustrum* form a well-supported clade, providing further support for the monophyly of this genus.

Recent authors have generally adopted Kohne's (1904) taxonomic treatment of *Ligustrum*, combining Mansfeld's (1924) sections *Baccatae* and *Subdrupacea* (Rehder 1927; Chang and Miao 1986; Chang et al. 1996). In our tree (Fig. 1), *L. japonicum* is basal and sister to a clade containing the remaining species, and none of the proposed sections and subsections (*Ibota*, *Robustae*, *Sinenses*) are monophyletic, suggesting that previous groupings in *Ligustrum* might need to be reconsidered.

**Systematic Position of *P. sempervirens* (= *L. sempervirens*).** *Parasyringa sempervirens* (= *Ligustrum sempervirens*) was first described as a species of *Syringa* based on a fruiting specimen collected from Yunnan, China (Franchet 1886). Schneider (1911) and Smith (1916), based on its morphological intermediacy between *Syringa* and *Ligustrum*, recognized it as a mono-

typic genus, *Parasyringa*. Lingelsheim (1920), however, considered *P. sempervirens* (= *L. sempervirens*) as a species of *Ligustrum*. This treatment has been generally followed (Mansfeld 1924; Chang and Miao 1986; Green and Fliegner 1991; Chang et al. 1996). Nevertheless, because of its unique fruit morphology, it has long been treated as a separate lineage from the remaining species of *Ligustrum* (Kohne 1904; Mansfeld 1924; Chang and Miao 1986; Chang et al. 1996). On this basis, Stapf (1933) stated that "whilst it [*P. sempervirens* (= *L. sempervirens*)] can evidently not retain its original place in *Syringa*, its status within or without *Ligustrum* is debatable indeed and in the end merely a matter of opinion and convenience." In a phylogenetic sense, this would be true if *P. sempervirens* (= *L. sempervirens*) turned out to be sister to a clade containing the remaining species of *Ligustrum*. However, in the ITS+ETS tree (Fig. 1) *L. japonicum* instead of *P. sempervirens* (= *L. sempervirens*) is the basal taxon in the *Ligustrum* clade (F). Our results thus support neither *Parasyringa* nor section *Sarcocarpion*, and instead, suggest that *P. sempervirens* (= *L. sempervirens*) is a species of *Ligustrum* with a dehiscent berry. This agrees with Green and Fliegner (1991), who concluded that *P. sempervirens* (= *L. sempervirens*) resembles a typical *Ligustrum* in habit, floral structure, and foliar morphology.

**Fruit Evolution and Species Diversity.** The most important difference between *Syringa* and *Ligustrum* is their fruit types: capsules in the former versus berries in the latter. In early development, the fruits of *Ligustrum sempervirens* have a fleshy mesocarp, as do fruits of all other species of *Ligustrum*. However, the fleshiness of the mesocarp disappears later and the fruits split open. In our ITS+ETS tree (Fig. 1), it is equally parsimonious to assume that the fruit dehiscence in *L. sempervirens* is either a maintenance of the ancestral state or a progressing reversal shift from berry to capsule. A comparative developmental study of fruits in *Syringa* and *Ligustrum* (including *L. sempervirens*) shows that the orientation of endocarp fibers in *L. sempervirens* shifts from the latitudinal arrangement (the *Ligustrum* type) to the longitudinal (the *Syringa* type) in the dehiscing region of the endocarp (Lawrence and Green 1994). It, therefore, is more likely that dehiscent berries in *L. sempervirens* are an evolutionary reversal towards capsules.

Our ITS+ETS phylogeny suggests that *Ligustrum* is derived from within *Syringa*, and that the fleshy fruit is a synapomorphy for *Ligustrum* species. Notably, there are more than twice as many species in *Ligustrum* as in its sister clade containing *Syringa* minus series *Syringa* and *Pinnatifoliae*. Fleshy berries attract birds for seed dispersal to diverse habitats, and thus help expand distribution range, which may in turn increase chances of speciation and thus species diversity. Therefore, the evolution of fleshy fruit might be caus-

ally related to the increase of species diversity in *Ligustrum*. It is conceivable that active bird dispersal may increase gene flow among populations, counteracting species diversification. Therefore, only when all relevant factors are analyzed in detail can we know whether or not there is a positive correlation between fleshy berries and species diversification in the *Syringa-Ligustrum* complex. Nevertheless, this relation has been observed in sister clades of other plant taxa (James Smith of Boise State University, pers. comm., and Chuck Davis, Harvard University Herbaria, pers. comm.).

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