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Evolution of fruit and seed characters in the *Diervilla* and *Lonicera* clades (Caprifoliaceae, Dipsacales)

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• *Background and Aims* The *Diervilla* and *Lonicera* clades are members of the family Caprifoliaceae (Dipsacales *sensu* Donoghue *et al.*, 2001, *Harvard Papers in Botany* **6**: 459–479). So far, the intergeneric relationships of the *Lonicera* clade and the systematic position of *Heptacodium* remain equivocal. By studying fruit and seed morphology and anatomy, an attempt is made to clarify these issues. In addition, this study deals with the evolution of fruit and seed characters of the *Diervilla* and *Lonicera* clades with reference to allied taxa.
• *Methods* Light and scanning electron microscopy were used for the morphological and anatomical investigations. Phylogenetic analyses were carried out by applying the parsimony and Bayesian inference optimality criteria. Character evolution was studied by means of parsimony optimization and stochastic character mapping.
• *Key Results Diervilla* and *Weigela* (*Diervilla* clade) are characterized by several unique traits in Dipsacales, including capsules with numerous seeds, seed coats without sclerified outer tangential exotestal cell walls, and dehiscent fruits. Seeds with completely sclerified exotestal cells and fleshy fruits characterize the *Lonicera* clade. *Leycesteria* and *Lonicera* have berries, ovaries without sterile carpels and several seeds per locule, whereas *Symphoricarpos* and *Triosteum* have drupes, ovaries with one or two sterile carpels and a single seed per locule. *Heptacodium* shares several characteristics with members of the Linnina clade, e.g. achenes, single-seeded fruits and a compressed, parenchymatous seed coat.
• *Conclusions* The results confirm the monophyly of the *Diervilla* and *Lonicera* clades and allow us to hypothesize a close relationship between *Leycesteria* and *Lonicera* and between *Symphoricarpos* and *Triosteum*. Fruit and seed morphology and anatomy point to a sister relationship of *Heptacodium* with the Linnina clade, rather than with the *Lonicera* clade.

Key words: *Diervilla*, *Weigela*, *Symphoricarpos*, *Lonicera*, *Triosteum*, *Leycesteria*, *Heptacodium*, Caprifoliaceae, Dipsacales, fruit, seed, evolution.

INTRODUCTION

Dipsacales fall within euasterids II and accommodate Adoxaceae and Caprifoliaceae (Donoghue *et al.*, 1992, 2001, 2003; Pyck *et al.*, 1999; Bell *et al.*, 2001; Bremer *et al.*, 2001; APG II, 2003; Zhang *et al.*, 2003; Winkworth *et al.*, 2008*a*, *b*; see Fig. 1). Recently, Winkworth *et al.* (2008*a*) postulated that Dipsacales are sister to Paracryphiaceae, and Dipsacales and Paracryphiaceae are together sister to Apiales.

Traditionally, Caprifoliaceae include the tribes Caprifolieae, Diervilleae and Linnaeeae, and the genera *Sambucus* and *Viburnum* (Hutchinson, 1967, 1973; Thorne, 1976; Takhtajan, 1980; Cronquist, 1981; Hara, 1983). Recent investigations based on molecular and morphological data (Donoghue *et al.*, 1992, 2001, 2003; Gustafsson *et al.*, 1996; Backlund and Pyck, 1998; Pyck *et al.*, 1999; Bell *et al.*, 2001; Bremer *et al.*, 2001; Zhang *et al.*, 2003; Winkworth *et al.*, 2008*b*) have indicated that in this traditional circumscription Caprifoliaceae are polyphyletic. In 1998, Backlund and Pyck proposed a new classification in which two new families were erected, Diervillaceae and Linnaeaceae, corresponding to the former tribes Diervilleae and Linnaeeae, respectively. Caprifoliaceae corresponded to the former tribe Caprifolieae. In 2001, Donoghue *et al.* proposed a new classification for Caprifoliaceae accommodating the former tribes Caprifolieae, Diervilleae and Linnaeeae, and the former families Dipsacaceae, Morinaceae and Valerianaceae (Table 1). They proposed a rank-free classification in which traditional names of tribes and families were preserved to avoid confusion. In both the classifications of Backlund and Pyck (1998) and Donoghue *et al.* (2001), *Sambucus* and *Viburnum* were included in Adoxaceae with *Adoxa*, *Sinadoxa* and *Tetradoxa*. In this study, we adopt the upcoming classification of APG III (in preparation) in which two families are recognized, Adoxaceae and Caprifoliaceae (cf. Donoghue *et al.*, 2001). We decided not to adopt the rank-free classification of Caprifoliaceae as proposed by Donoghue *et al.* (2001) as the use of informal names based on names of former tribes and families causes confusion. Instead, we have assigned informal names to the major clades of the family (Table 1, Fig. 1).

Several investigations (Backlund, 1996; Backlund and Pyck, 1998; Pyck *et al.*, 1999; Donoghue *et al.*, 2001, 2003; Bell *et al.*, 2001; Zhang *et al.*, 2003; Winkworth *et al.*, 2008*b*) have dealt with the phylogenetic relationships of Caprifoliaceae (*sensu* Donoghue *et al.*, 2001; Fig. 1). There is general consensus with regard to the sister relationship of

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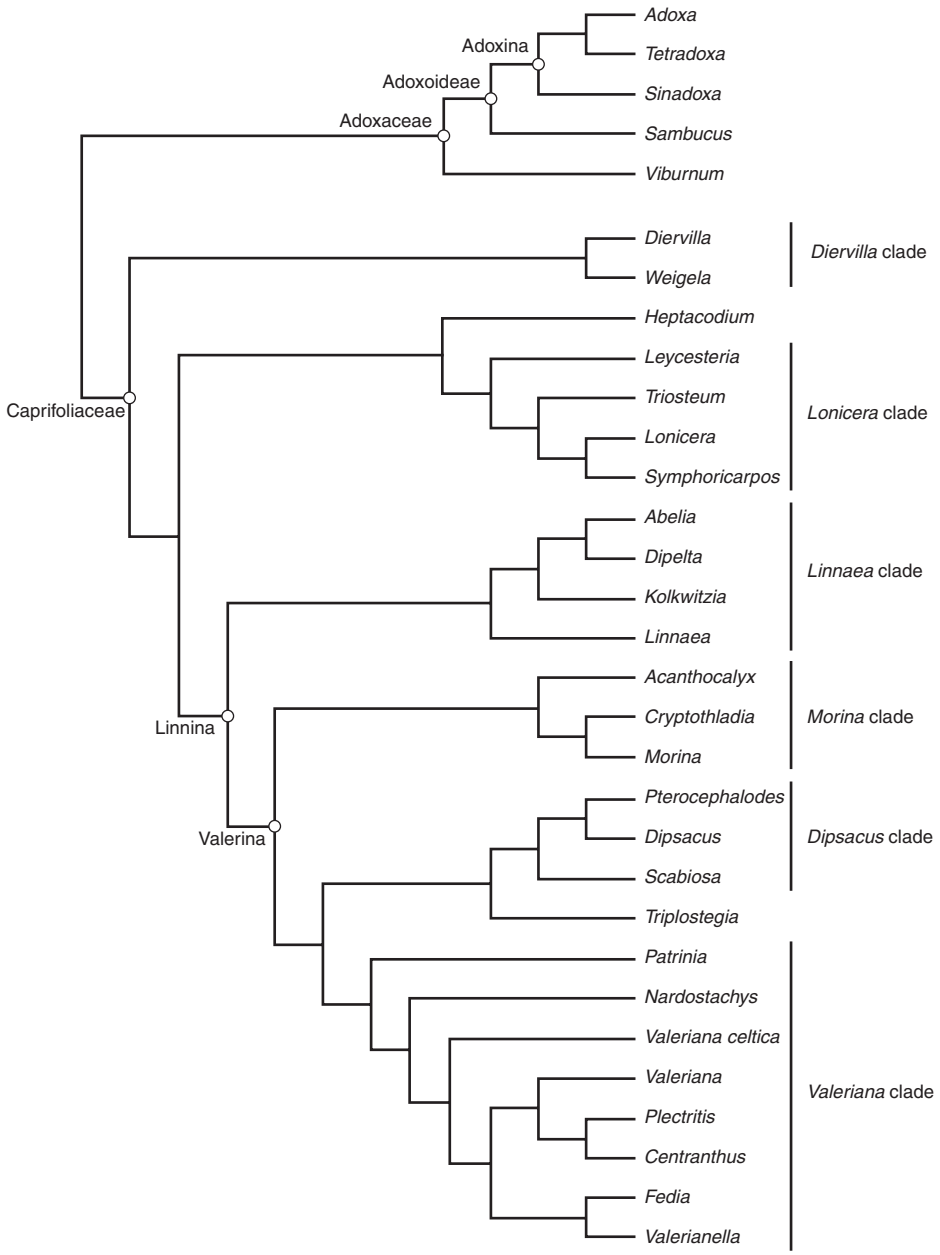

FIG. 1. Phylogenetic tree for the order Dipsacales with indication of families and informal clade names (based on that of Winkworth *et al.*, 2008*b*, fig. 3a).

Adoxaceae and Caprifoliaceae. Furthermore, the clade containing *Diervilla* and *Weigela* is sister to the remainder of Caprifoliaceae, and the *Lonicera* clade is sister to the Linnina clade *sensu* Donoghue *et al.* (2001; including the *Dipsacus*, *Linnaea*, *Morina* and *Valeriana* clades). The *Lonicera* clade comprises the genera *Leycesteria*, *Lonicera*, *Symphoricarpos* and *Triosteum* (Fig. 1). Although the position of *Heptacodium* is still ambiguous, the results of the analyses of Pyck and Smets (2000), Donoghue *et al.* (2003) and Winkworth *et al.* (2008*b*) suggest a position as sister to the *Lonicera* clade (Fig. 1); however, the possibility that *Heptacodium* is sister to the Linnina clade should not be discarded (Winkworth *et al.*, 2008*b*).

The *Diervilla* clade contains deciduous shrubs and small trees with simple, opposite leaves (Hara, 1983). Flowers are organized in a cyme and have a cylindrical, bilocular ovary with numerous fertile ovules (Hara, 1983; Backlund, 1996; Donoghue *et al.*, 2003). The clade comprises the genera *Diervilla*, including three species from eastern North America, and *Weigela*, including twelve species from East Asia (Hara, 1983; Backlund, 1996; Backlund and Pyck, 1998). *Diervilla* differs from *Weigela* by being stoloniferous and bearing capsules that split weakly or do not split at all (Hara, 1983). The seeds of *Diervilla* are lenticular and wingless, whereas those of *Weigela* are mostly cylindroid and morphologically characterized by a more-or-less prominent wing.

TABLE 1. *Overview of classifications of Dipsacales*

Present study		Hara (1983)			Backlund and Pyck (1998)	Donoghue *et al.* (2001)		
Adoxaceae	*Adoxa*, *Sinadoxa*, *Tetradoxa*	Adoxaceae		*Adoxa*, *Sinadoxa*, *Tetradoxa*	Adoxaceae	Adoxaceae	Adoxoideae	*Adoxa*, *Sinadoxa*, *Tetradoxa*
	Sambucus		Sambucoideae	*Sambucus*				*Sambucus*
	Viburnum	Caprifoliaceae	Viburnoideae	*Viburnum*				*Viburnum*
Caprifoliaceae	*Diervilla* clade		Caprifolioideae	Diervilleae	Diervillaceae	Caprifoliaceae	Diervilleae	
	Lonicera clade			Caprifolieae	Caprifoliaceae		Caprifolieae	
				Triosteae				
	Linnaea clade			Linnaeaceae	Linnaeaceae		Linnaeaceae	
	Morina clade	Morinaceae			Morinaceae		Morinaceae	
	Dipsacus clade	Dipsacaceae			Dipsacaceae		Dipsacaceae	
	Valeriana clade	Valerianaceae			Valerianaceae		Valerianaceae	

The intergeneric relationships of the *Lonicera* clade remain ambiguous, most likely due to the rapid diversification of its four genera (Winkworth *et al.*, 2008*b*). Several studies have tackled this problem, starting with work by Backlund (1996) in which he analysed a data set of *rbcL* sequence data and 109 morphological characters. The results hypothesized a sister relationship between *Lonicera* plus *Leycesteria* and a clade of *Heptacodium* and *Triosteum* plus *Symphoricarpos*. In 2001, Donoghue *et al.* performed two analyses based on *rbcL* sequence data. The first analysis (*rbcL* only) placed *Heptacodium* as sister to a clade in which *Leycesteria* was sister to a polytomy of the remainder of the *Lonicera* clade. In the second analysis, in which the *rbcL* sequence data were supplemented with the morphological data of Backlund (1996), the authors suggested a clade consisting of *Leycesteria* and *Lonicera* being sister to a clade comprising *Symphoricarpos* and *Triosteum*. Once again, *Heptacodium* was sister to the *Lonicera* clade. In 2003, Donoghue *et al.* published the results of a maximum likelihood analysis based on nuclear (ITS) and plastid (*matK*, *trnL*) sequence data. The results showed strong support (95 %) for the sister relationship of *Heptacodium* and the *Lonicera* clade and 100 % support for the sister relationship of *Leycesteria* and the remaining three genera of the *Lonicera* clade. Recently, Winkworth *et al.* (2008*b*) carried out a series of analyses based on mitochondrial and plastid sequence data. Two datasets indicated a sister relationship of *Heptacodium* and the *Lonicera* clade with strong support. A third dataset supported a sister relationship of *Heptacodium* and the Linnina clade. The latter hypothesis was weakly supported by both parsimony and maximum likelihood, but strongly corroborated by Bayesian inference. The systematic equivocality surrounding the *Lonicera* clade was confirmed by incongruency between data sets, which largely disappeared when the *Lonicera* clade was excluded from the statistical tests. Theis *et al.* (2008) conducted a phylogenetic study focused on the phylogenetic relationships of the *Lonicera* clade and the genus *Lonicera* in particular. Based on ITS and five plastid markers, they concluded that *Triosteum* was sister to *Symphoricarpos* plus *Leycesteria* and *Lonicera*; however, sampling of the remainder of Dipsacales was rather limited.

Leycesteria, comprising five species distributed in the Himalayas and West China, resembles *Lonicera*, but has been considered more primitive (Horne, 1914) due to the development of a gynoecium with five (rarely four) fertile locules with numerous ovules in each locule. The gynoecium matures into a fleshy berry with numerous seeds. Chemotaxonomically, however, *Leycesteria* has been considered to be rather advanced in comparison with the other three genera of the clade (Bohm and Glennie, 1971). *Lonicera* comprises about 200 species of shrubs, trees and woody vines occurring in temperate and subtropical regions of Europe, North and Central America, North Africa and Asia. The genus is subdivided into two subgenera (Hara, 1983), *Lonicera* (180 species) and *Caprifolium* (22 species). The gynoecium is composed of two or three locules (rarely five), each locule containing three-to-eight ovules (Hara, 1983; Roels and Smets, 1996). The gynoecium matures into a fleshy berry. *Triosteum* comprises 6–7 species of perennial herbs with woody rhizomes found in East Asia and North

America (Hara, 1983; Wilkinson, 1949). The zygomorphic flowers are sessile and located in the leaf axils or in a terminal spike (Hara, 1983). The gynoecium is composed of four carpels, of which one is sterile (Wilkinson, 1949). The fertile carpel holds a single pendent ovule and develops into a drupe containing three pyrenes (Wilkinson, 1949). *Symphoricarpos* contains about 15 species of shrubs mostly distributed in North America, but with one species, *S. sinensis*, in parts of China (Hara, 1983). The flowers are organized in a raceme or spike and have a tetralocular gynoecium, of which two carpels are infertile (Hara, 1983; Roels and Smets, 1996). The gynoecium develops into a drupe with two pyrenes (Hara, 1983; Roels and Smets, 1996).

Heptacodium holds two species, *H. miconioides* and *H. jasminoides*. Both are shrubs occurring in central China (Hara, 1983; Pyck and Smets, 2000). The flowers have a persistent calyx and a slightly curved, tubular corolla (Hara, 1983; Pyck and Smets, 2000; Zhang *et al.*, 2002). The gynoecium is composed of three locules, of which two are abortive (Pyck and Smets, 2000; Zhang *et al.*, 2002). Several ovules are present in the fertile locule, but only one matures into a long, spindle-shaped seed. The mature fruit is single-seeded and is often described as an achene (Pyck and Smets, 2000; Zhang *et al.*, 2002; Donoghue *et al.*, 2003). As mentioned earlier, the phylogenetic position of *Heptacodium* remains uncertain.

This current study documents the morphology and anatomy of the fruits and seeds of the *Diervilla* and *Lonicera* clades and *Heptacodium*. The impact of fruit and seed characters on the phylogenetic relationships of the *Diervilla* and *Lonicera* clades and the systematic position of *Heptacodium* is studied using sequence data (ITS, *trnK* and *matK*), and using a combined dataset composed of this sequence data and 17 fruit and seed characters plus 12 morphological characters from the study of Backlund (1996). The resulting topologies are used to study the evolution of fruit and seed characters. The decision to carry out original analyses instead of adopting a previously published phylogenetic analyses was motivated by our aim of matching more accurately the sampling of the morphological study. The primary focus of this paper is the study of the evolution of fruit and seed characters. Although our aim is not to present a better-supported or more-resolved phylogenetic analysis of Dipsacales, we try to contribute to a better understanding of the phylogenetic relationships.

MATERIALS AND METHODS

Taxon sampling

Material for morphological and anatomical investigation was collected in the field and botanic gardens or acquired through collaboration with seed banks and herbaria (see Appendix 1). This study includes seven species of the *Diervilla* clade, 25 species of the *Lonicera* clade, one specimen of *Heptacodium miconioides*, 13 species of the Linnina clade and seven species of Adoxaceae. For a complete list of all specimens, please refer to Appendix 1. Leaf material for DNA extraction was collected in the field or in botanic gardens and preserved in silica gel.

Molecular methods

DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Tel-Zur *et al.*, 1999). The tissue was ground and washed three times with extraction buffer (100 mM TrisHCL pH 8, 5 mM EDTA pH 8, 0·35 M sorbitol) to remove secondary metabolites. 700 μL CTAB lysis buffer (as described in Chase and Hills, 1991, with addition of 3% PVP-40) and 30 μL Sarkosyl were added to the samples, after which they were incubated for 1 h at 60°C. The aqueous phase was extracted twice with chloroform-isoamylalcohol (24/1, v/v) and subsequently subjected to an ethanol–salt precipitation (1/10 volume sodium acetate 3 M, 2/3 volume absolute ethanol). After centrifugation, the pellet was washed twice (70% ethanol), air-dried and dissolved in 100 μL TE buffer (10 mM TrisHCl pH 8, 1 mM EDTA pH 8).

Primers used for amplification and sequencing of ITS, *trnK* and *matK* are listed in Table 2, and statistics for the aligned sequence data are given in Table 3. Amplification of double-stranded copies of all three regions was done using standard PCR in 25-μL volume reactions. All reactions included an initial heating at 95 °C for 3 min. For ITS, the initial heating was followed by 30 cycles consisting of 95 °C for 60 s, 50 °C for 30 s and 72 °C for 30 s. For *trnK* and *matK*, the initial heating was followed by 30 cycles consisting of 95 °C for 60 s, 50–52 °C for 60 s and 72 °C for 60 s. All reactions were terminated with a final incubation of 72 °C for 3 min. To prevent the formation of secondary structures, we added 5% dimethylsulphoxide (DMSO) to the reaction mixture for ITS (Geuten *et al.*, 2004). Samples were sequenced by the Macrogen sequencing facilities (Macrogen, Seoul, South Korea). Sequencing files were edited and assembled using Staden for Mac OS X (Staden *et al.*, 1998).

Morphological methods

Rehydration and fixation of the material was done by immersion in glutaraldehyde (2·5%) buffered with sodium cacodylate buffer (0·05 M, 24 h) and a subsequent wash in sodium cacodylate buffer (0·05 M, 24 h). The material was put through an ethanol series for dehydration purposes.

For light microscopy, the material was embedded in a hydroxyethylmethacrylate-based resin (Technovit 7100, Kulzer Histo-Technik, Wehreim, Germany), cut with a rotation microtome (HM360, Microm, Walldorf, Germany) and stained with toluidine blue. Longitudinal and cross-sections (5 μm in thickness) were observed and photographed with a Leitz Dialux 20 (Leitz, Wetzlar, Germany) equipped with a PL-B622CF PixeLINK digital camera and Microscopica v1·3 (Orbicule, Leuven, Belgium).

For scanning electron microscopy, the material was critical-point dried and sputtered with gold (Spi-Supplies, Walldorf, USA) prior to mounting on stubs. A JSM-6360 scanning electron microscope was used to observe and photograph the specimens. Pyrenes of *Sambucus*, *Symphoricarpos*, *Triosteum* and *Viburnum* were subjected to a hydrogen peroxide treatment (35%, 3–4 h, 60 °C) and subsequently cleaned with a toothbrush to remove the mesocarp.

TABLE 2. *Base composition of amplification and sequencing primers*

Locus	Primers	Sequence 5′–3′	Reference
ITS	ITS5	GGA AGT AAA AGT CGT AAC AAG G	White *et al.* (1990)
	ITS4	TCC TCC GCT TAT TGA TAT GC	White *et al.* (1990)
trnK	trnK11	CTC AAC GGT AGA GTA CTC G	Young *et al.* (1999)
	matK510R	GAA GAG TTT GAA CCA AKA YTT CC	Young *et al.* (1999)
matK	matK-53F	CTT GTT TTG RCT NTA TCG CAC TAT G	Young *et al.* (1999)
	matK950R	CCA CAR CGA AAA ATR MCA TTG CC	Young *et al.* (1999)
	matK775F	TCT TGA ACG AAT CTA TTT CTR YGG	Young *et al.* (1999)
	matK1349R	CTT TTG TGT TTC CGA GCY AAA GTT C	Young *et al.* (1999)

TABLE 3. *Statistics for the aligned sequence data*

	ITS	*trnK*	*matK*
Number of taxa	32	25	30
Aligned matrix length (sequence data)	673	776	954
Number of constant characters	313	476	609
Number of variable characters	360	300	345
Number of potentially parsimony-informative characters	265	144	204

A Leica MZ6 stereomicroscope (Leica Microsystems Ltd, Heerbrugg, Switzerland) was used to measure seeds and pyrenes. Seed-coat thickness and endocarp thickness were measured using a Leitz Dialux 20 equipped with a PL-B622CF PixeLINK digital camera and Microscopica v1·3 in combination with Macnification v0·2 (Orbicule, Leuven, Belgium).

Mean and s.d. values of seed and pyrene dimensions are based on five specimens per species; mean and s.d. values of seed coat and endocarp thickness are based on ten measurements on one specimen.

Character state delimitation

Table 4 provides a summary of the fruit and seed characters and their respective character states. Characters 18–29 were adopted from Backlund (1996) and we refer to this publication for a more extensive definition of these characters. The delimitation of four characters is arbitrary and deserves further explanation. We chose to define the second character (maximum number of carpels) as maximum number of carpels instead of number of carpels, because SIMMAP v1·0 beta 2·4 (Bollback, 2006; build 04082008–1·0-B2·4; Intel version) does not allow multiple character states as a single entry (e.g. '3–4' is not a valid entry). The delimitation of character 4 (number of seeds) is based on the presence of clear gaps in the data. Although the bounds of character states 5 and 6 seem arbitrary, seed number for *Lonicera* fruits does not exceed 20 (state 5), whereas seed number for fruits of *Leycesteria*, *Diervilla* and *Weigela* varies from 50 to more than 100 (state 6). The delimitation of character 15 (embryo size) is based on observations and the presence of clear gaps. An unmistakable size and morphological difference is apparent between the

TABLE 4. *Description of morphological characters*

1	Fruit type: (0) drupe, (1) berry, (2) achene, (3) capsule
2*	Maximum number of carpels: (0) 1, (1) 2, (2) 3, (3) 4, (4) 5
3	Number of sterile carpels: (0) no sterile carpels, (1) single sterile carpel, (2) two sterile carpels
4*	Number of seeds: (0) 1, (1) 2, (2) 3, (3) 4, (4) 5, (5) 6–20, (6) more than 20
5	Endocarp sclerification: (0) endocarp not sclerified, (1) endocarp sclerified
6	Number of endocarp layers: (0) 1, (1) 2, (2) 3
7	Endocarp part of diaspore: (0) endocarp not part of diaspore, (1) endocarp part of diaspore
8	Mesocarp anatomy: (0) mesocarp dry, (1) mesocarp fleshy
9	First mechanical layer: (0) epicalyx, (1) pericarp, (2) endocarp, (3) seed coat
10	Presence of epicalyx: (0) epicalyx absent, (1) epicalyx present
11	Exotesta anatomy: (0) exotesta compressed, (1) exotesta parenchymatous, (2) exotesta sclerified
12	Seed coat crystals: (0) crystals absent, (1) crystals present
13	Presence of sclerified endotesta: (0) sclerified endotesta absent, (1) sclerified endotesta present
14	Presence of anti-raphe: (0) anti-raphe absent, (1) anti-raphe present
15*	Embryo size: (0) embryo length less than 3/4 of seed length, (1) embryo length greater than 3/4 of seed length but not occupying entire seed, (2) embryo occupying entire seed and no endosperm present
16*	Seed coat thickness: (0) less than 25 μm, (1) greater than 25 μm and less than 100 μm, (2) greater than 100 μm
17	Winged seeds: (0) wingless seeds, (1) winged seeds
18	Sepal modification for fruit dispersal: (0) none, (1) developing into a plumose seed/fruit, (2) developing to seeds/fruits with awns/bristles, (3) enlarged and leaf-like aiming for wind dispersal
19	Carpel vascularization: (0) free adaxial and abaxial, (1) adaxial bundles only, (2) only free abaxial, adaxial not recessed
20	Ovule vascularization: (0) single bundle, (1) double or compound bundles
21	Ovule position with respect to the central axis: (0) marginal, (1) marginal above, median below, (2) median
22	Ovule reduction: (0) no traces of reductions, (1) sterile ovules, (2) vestigial archespore surrounded by nucellar tissue, (3) vestigial archespore
23	Integument number: (0) unitegmic, (1) bitegmic
24	Nucellus thickness: (0) tenuinucellate, (1) crassinucellate
25	Endothelium: (0) absent, (1) present, feebly differentiated, (2) prominent, with crystal layer
26	Embryo sac development: (0) Polygonum type, (1) Adoxa type, (2) Allium type
27	Embryogeny type: (0) Solanad, (1) Asterad, (2) Piperad, (3) Onagrad
28	Endosperm in seed: (0) absent, (1) scanty, (2) copious
29	Embryo development: (0) leucoembryote, (1) chlorophyllous

* Character state delimitation explained in detail in the Materials and Methods. A more extensive description of characters 18–29 can be found in Backlund (1996).

TABLE 5. *ILD statistics from data set comparison*

	Original aligned matrices			*Lonicera* clade excluded			*Linnaea* clade excluded			*Heptacodium* excluded		
	trnK	*matK*	morphology	*trnK*	*matK*	morphology	*trnK*	*matK*	morphology	*trnK*	*matK*	morphology
ITS	**0·00**	0·45	**0·00**	0·18	1·00	0·97	**0·00**	**0·02**	**0·00**	**0·00**	0·86	**0·02**
trnK	–	**0·00**	**0·00**	–	**0·02**	0·17	–	**0·00**	**0·00**	–	**0·01**	**0·00**
matK	–	–	**0·00**	–	–	0·70	–	–	**0·00**	–	–	**0·01**

Incongruencies are indicated in bold.

large embryos of *Sambucus*, the *Dipsacus* clade and the *Valeriana* clade and the smaller embryos of the rest of the order. Additionally, we made a distinction between the embryos of *Sambucus* and the *Dipsacus* clade and the embryos of the *Valeriana* clade, which occupy the entire seed (no endosperm). Character 16 (seed-coat thickness) is continuous, and instead of using an algorithm we chose to delimit states based on the presence of gaps in the continuous data and the overall anatomy of the seed coat.

Phylogenetic analyses

ITS, *trnK* and *matK* sequences of 32 species were aligned using MUSCLE v3·6 (Edgar, 2004), with default settings applied, after which small adjustments were made in MacClade v4·04 (Maddison and Maddison, 2002) to improve the alignment. The aligned matrices were submitted to TreeBASE (www.treebase.org). Five members of Adoxaceae were assigned to the outgroup (Appendix 2). Maximum parsimony (MP) and Bayesian inference (BI) were chosen to analyse three data sets: (1) molecular sequence data; (2) morphological data (Table 4, and see Table 8); and (3) molecular and morphological data combined. Parsimony analyses were carried out using PAUP* v4·0b10 (Swofford, 2002). Mr. Bayes v3·1·2 (Ronquist and Huelsenbeck, 2003) was used for BI analyses. Parsimony analyses were conducted on 1000 random addition replicates with tree-bisection-reconnection (TBR) branch swapping applied. Five trees were held at each step. Characters were unordered and equally weighted. Support for individual clades in the optimal tree was tested by a bootstrap analysis with 100 pseudoreplicates with settings identical to those of the original analysis except for the number of repetitions (100). Prior to the BI analyses, the molecular and morphological data were placed in separate partitions and a model was assigned to each partition. Mr. Modeltest v2·2 (Nylander, 2004) suggested a General Time Reversible model (GTR) with an invariable gamma-shaped distribution of rates across sites (GTR + I + G) for ITS and a GTR model with a variable gamma-shaped distribution of rates across sites (GTR + G) for *trnK* and *matK*. The standard discrete model was chosen for the analysis of the morphological data set (Ronquist and Huelsenbeck, 2003). The BI analyses were run for one million generations with partitions unlinked and sample frequency and burn-in set to 100 and 2500, respectively.

Comparing matrices and topologies

To search for incongruencies between matrices and topologies, we performed incongruence length difference (ILD; Farris *et al.*, 1995) tests along with approximately unbiased (AU; Shimodaira, 2002) and Simodaira–Hasegawa (SH; Shimodaira and Hasegawa, 1999) tests. For the ILD tests, all data partitions were compared with each other (Table 5) using PAUP* v4·0b10 (Swofford, 2002). Additionally, we performed the same ILD tests with the exclusion of (1) the *Lonicera* clade, (2) the *Linnaea* clade and (3) *Heptacodium* in order to investigate the impact of these taxa on the incongruency of the data sets (Table 5), and we visually inspected the MP and BI topologies of the separate data sets to trace the cause of the incongruence. Furthermore, we used the AU and SH tests to compare three data sets (all sequence data, ITS sequence data, and plastid sequence data; Table 6) with eight alternative hypotheses: (1) the consensus topology of the BI analysis based on combined data; (2) the consensus topology of the BI analysis based on all molecular data; (3) all shortest MP trees based on combined data; (4) all shortest MP trees based on morphological data; and the maximum likelihood (ML) topology of (5) ITS, (6) *trnK*, (7) *matK*, and (8) all molecular data. PAUP* v4·0b10 (Swofford, 2002) was used to calculate the site-wise log-likelihoods, whereafter we used Consel v0·1j (Shimodaira and Hasegawa, 2001) to perform multiscale bootstrap resampling (ten sets of 10 000 replicates with scale parameters between 0·5 and 1·4). The ML topologies were calculated using GARLI v0·951 (Zwickl, 2006).

Character evolution

Parsimony optimization (PO) and stochastic character mapping (SCM) were used for the study of character evolution. The consensus tree of the combined BI analysis was chosen for the PO analysis in MacClade v4·04 (Maddison and Maddison, 2002) because of higher branch support and resolution. Additionally, a modified phylogenetic tree based on that of Winkworth *et al.* (2008*b*; see Fig. 1) was used for a second PO analysis in order to investigate the impact of an alternative placement of *Heptacodium*. The modified topology is identical to the consensus tree of our combined BI analysis except for the placement of *Heptacodium*, i.e. as sister to the *Lonicera* clade instead of sister to the Linnina clade. Stochastic character mapping was done through SIMMAP v1·0 beta 2·4 (Bollback, 2006; build 04082008–1·0-B2·4; Intel version) and based on the final 5000 trees of the combined BI analysis. The choice ofSCM was motivated by three factors: (1) multiple shifts along a single branch are possible; (2) potential underestimation of variation inherent to the parsimony algorithm is avoided; and (3) BI deals with uncertainty with respect to

TABLE 6. *Tree comparison using likelihoods and approximately unbiased (AU) and Shimodaira–Hasegawa (SH) tests*

	ITS, *trnK*, *matK* ML tree			ITS ML tree			*trnK*, *matK* ML tree		
	Δ ln L	AU	SH	Δ ln L	AU	SH	Δ ln L	AU	SH
BI consensus tree based on combined data	5·2	0·22	0·90	7·0	0·22	0·82	10·2	0·23	0·88
BI consensus tree based on molecular data	15·1	**<0·01**	0·81	17·9	**<0·01**	0·64	12·6	0·02	0·85
Shortest MP trees based on combined data	4·0–5·2	0·44–0·22	0·91–0·90	5·4–7·0	0·52–0·22	0·86–0·82	10·2	0·22–0·21	0·88
Shortest MP trees based on morphological data	403·1–528·5	**<0·01**	0·12	125·7–146·9	**<0·01**	**<0·01**	293·9–420·1	**<0·01**	**<0·01**
ML tree based on ITS data	126·0	**<0·01**	**<0·01**	best	0·70	0·93	139·4	**<0·01**	0·03
ML tree based on *trnK* data	1353·0	**<0·01**	**<0·01**	544·8	**<0·01**	**<0·01**	804·4	**<0·01**	**<0·01**
ML tree based on *matK* data	849·3	**<0·01**	**<0·01**	480·6	**<0·01**	**<0·01**	274·4	**<0·01**	**<0·01**
ML tree based on molecular data	best	0·69	0·97	12·7	0·08	0·72	4·5	0·85	0·98

phylogenetic reconstruction (Bollback, 2006). The results of the SCM analysis are available as a Supplementary Data, online.

RESULTS

Seed shape and size

In the *Diervilla* clade (Fig. 2A–D), *Diervilla* (Fig. 2A, B) and *Weigela* (Fig. 2C, D) differ in several aspects regarding seed shape and size. Generally, seeds of *Diervilla* are slightly smaller than those of *Weigela* (Table 7, Fig. 2A–D). Seeds of *Diervilla* are elliptic in lateral view (Fig. 2A) and ovate to elliptic in cross-section (Fig. 2B). Seeds of *Weigela* differ in shape from those of *Diervilla* as most seeds are angular and slightly more elongated (Fig. 2C, D). Some species of *Weigela* (e.g. *W. hortensis*) are characterized by having seeds with lateral outgrowths, often called wings (Fig. 2C). These outgrowths are absent in *Diervilla* (Fig. 2A, B). The hilum in both genera is terminal to subterminal (Fig. 2A, C).

In the *Lonicera* clade (Fig. 2E–L), seeds of *Leycesteria* (Fig. 2E, F) resemble those of *Diervilla* (Fig. 2A, B) in being bilaterally symmetrical and slightly dorsoventrally compressed. Generally, seed shape in lateral view varies from circular to elliptic to ovate and clavate (Fig. 2E). In cross-section, seed shape ranges from elliptic to ovate and clavate (Fig. 2F). Seed size differs significantly between *Leycesteria formosa* and *L. crocothyrsos* (Table 7). The seeds of *Lonicera* (Fig. 2G, H) are dorsiventrally compressed and irregular in shape (Fig. 2G, H). Seed shape in lateral view ranges from circular to elliptic to ovate (Fig. 2G), whereas seed shape in cross-section (Fig. 2H) is highly variable, even within species, although the seeds are dorsiventrally compressed in most cases. The hilum is terminal to subterminal. In *Triosteum* (Fig. 2I, J), seed shape in lateral view ranges from spindle-shaped (Fig. 2I) to elliptic or ovate, whereas seed shape in cross-section is predominantly elliptic (Fig. 2J). In *Symphoricarpos* (Fig. 2K, L), seed shape in cross-section is elliptic and uniform throughout the genus (Fig. 2L). Seed shape in lateral view is typically spindle-shaped and can be slightly curved (Fig. 2K).

In *Heptacodium* (Fig. 2M–O), seed shape ranges from spindle-shaped to slightly clavate (Fig. 2M, O). Seed shape in cross-section is circular to slightly elliptic (Fig. 2N).

Seed surface

The seed surface of *Diervilla* (Fig. 3A) and *Weigela* (Fig. 3B) is defined by the exotestal cells, which are characterized by a U-shaped sclerification pattern (Figs. 2B, D and 4A, C). The outline of the cells is mostly pentagonal or hexagonal (Fig. 3A, B). Cell shape and size differ considerably within species, and no clear organizational pattern is discernable (Fig. 3A, B). The thin outer tangential cell wall is not sclerified, which often causes it to be concave and easily removable, uncovering the cell lumen (Fig. 3A, B). In *Weigela*, the seed-coat cells located at the lateral edges of the seed typically have uneven radial cell walls, which are taller than those of typical seed-coat cells (Fig. 2C). These cells give rise to the seed wing (Fig. 2C).

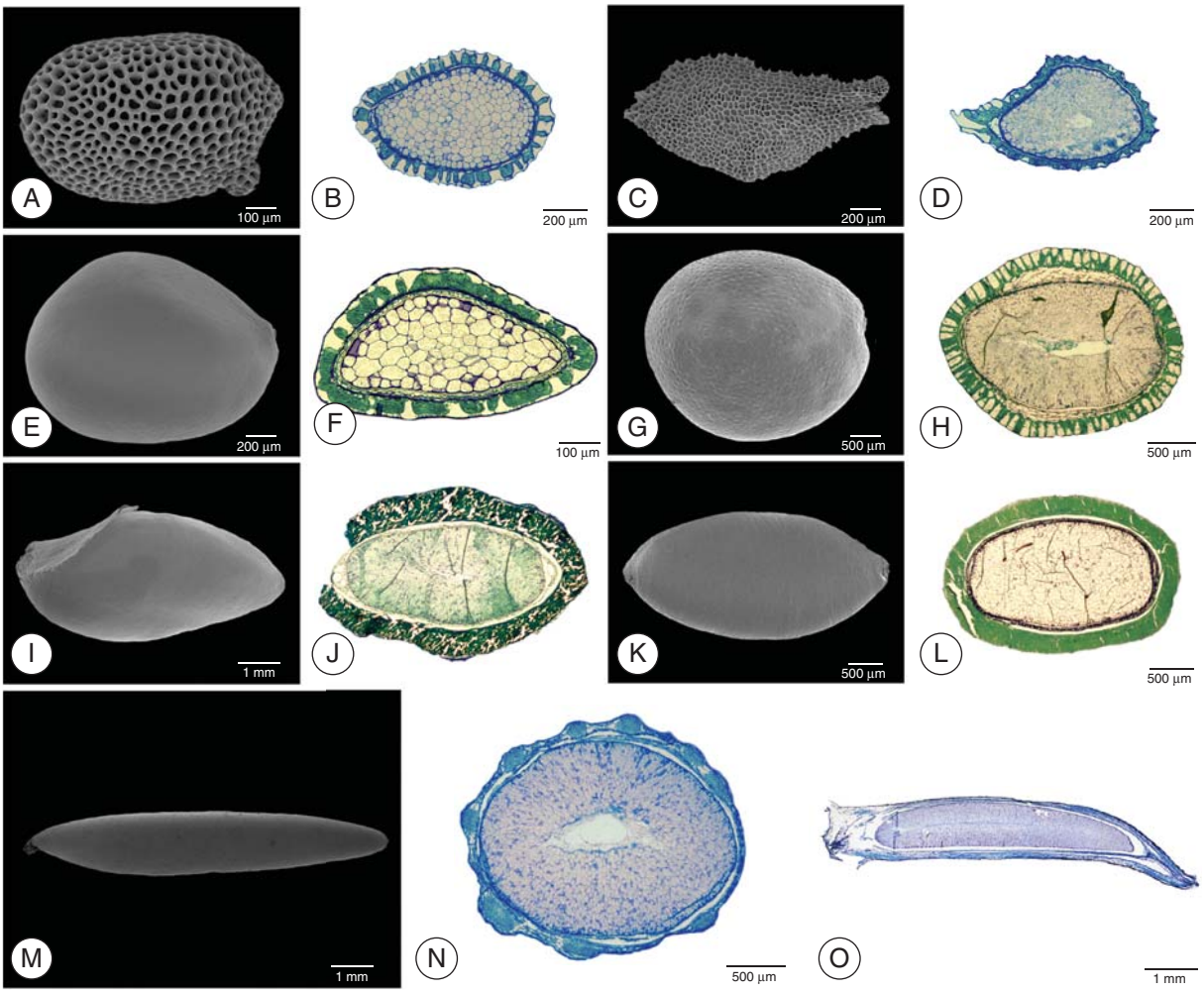

FIG. 2. Seed shape and size. (A, B) *Diervilla sessilifolia*: (A) lateral view; (B) TS, sclerified exo- and endotesta. (C) *Weigela hortensis*, lateral view. (D) *Weigela floribunda*, winged seed shaped by adjacent seeds. (E) *Leycesteria formosa*, lateral view. (F) TS, *Leycesteria crocothyrsos*, dorsiventrally flattened seed with sclerified exo- and endotesta. (G, H) *Lonicera dioica*: (G) lateral view; (H) TS, raphe and anti-raphe. (I) *Triosteum perfoliatum*, lateral view. (J) *Triosteum hirsutum*, TS, sclerified endocarp surrounding seed with sclerified exo- and endotesta, raphe and anti-raphe. (K) *Symphoricarpos mollis*, lateral view. (L) *Symphoricarpos occidentalis*, TS, sclerified endocarp surrounding seed, laterally flattened raphe and anti-raphe. (M–O) *Heptacodium miconioides*: (M) lateral view; (N) TS, pericarp with ribs surrounding seed with compressed, parenchymatous seed coat; (O) LS, fruit with one seed, minute embryo.

In the *Lonicera* clade (Fig. 3C–F), *Leycesteria formosa* has smooth seeds with a subtle sculpturing ascribed to the convex outer tangential walls of the exotestal cells (Fig. 3C). The exotestal cells of *L. crocothyrsos* have a thinner outer tangential cell wall than that in *L. formosa*. This causes the outer tangential cell walls to be concave when dehydrated. Characteristic for both genera is the subtle undulation of the cell outline of the exotestal cells (Fig. 3C). In *Lonicera* (Fig. 3D), the seed surface sculpture is defined by the anatomy of the outer tangential cell wall of the exotestal cells. The cell outline of the exotestal cells varies between hexagonal (Fig. 3D), circular and elliptic. In some species (e.g. *L. canadensis*), the cell outline is slightly undulate. Exotestal cell size varies strongly inter- and intraspecifically. The seed surface in *Triosteum* (Fig. 3E) is mostly characterized by exotestal cells with convex outer tangential cell walls (Fig. 4I) and a strongly undulate cell outline (Fig. 3E). Although anatomical cross-sections look highly similar when compared with other *Triosteum* spp., the undulate pattern is lacking in *T. hirsutum*. The exotestal cell outline of *T. hirsutum* is mostly square or rectangular, although slightly undulate exotestal cells were also observed. Exotestal cells of *Symphoricarpos* have moderately sclerified radial and inner tangential cell walls and a weakly sclerified outer tangential cell wall (Figs 3F and 4K, L). The exotestal cell outline is modestly undulate (Fig. 3F) and varies between rectangular, square and slightly elongated. Observation of the seed surface of *Symphoricarpos* is difficult as exotesta and endocarp are closely associated (Fig. 4K, L) and endocarp removal generally results in a simultaneous removal of the exotesta. In *Heptacodium* (Fig. 3G), the seeds have a smooth surface with a subtle sculpturing due to the outlines of the compressed exotestal cells (Figs 3G and 4M–O).

Anatomy of seed coat

In the *Diervilla* clade, seed-coat anatomy of both genera is highly comparable (Fig. 4A–D). The seed coat consists of

TABLE 7. *Seed and endocarp characteristics of studied species*

Taxon	Tribe	Seed				Endocarp
		Length (mm)	Width (mm)	Thickness (mm)	Coat thickness(μm)	Thickness (μm)*
Diervilla sessilifolia	*Diervilla* clade	1·13 ± 0·05	0·79 ± 0·07	0·50 ± 0·06	79·77 ± 11·24	–
D. rivularis	*Diervilla* clade	0·98 ± 0·06	0·68 ± 0·04	0·50 ± 0·02	71·03 ± 6·72	–
Weigela florida	*Diervilla* clade	1·82 ± 0·22	0·91 ± 0·09	0·54 ± 0·05	74·38 ± 47·11	–
W. subsessilis	*Diervilla* clade	1·96 ± 0·18	1·10 ± 0·08	0·66 ± 0·07	72·58 ± 53·66	–
W. floribunda	*Diervilla* clade	1·52 ± 0·07	0·74 ± 0·10	0·54 ± 0·08	71·81 ± 34·61	–
W. japonica	*Diervilla* clade	1·19 ± 0·19	0·84 ± 0·09	0·58 ± 0·06	78·57 ± 33·08	–
W. hortensis	*Diervilla* clade	1·71 ± 0·16	1·13 ± 0·26	0·62 ± 0·03	54·91 ± 17·06	–
Leycesteria formosa	*Lonicera* clade	1·30 ± 0·05	0·96 ± 0·04	0·59 ± 0·04	56·23 ± 6·63	–
L. crocothyrsos	*Lonicera* clade	0·86 ± 0·04	0·67 ± 0·07	0·40 ± 0·01	66·28 ± 6·28	–
Lonicera dioica	*Lonicera* clade	3·60 ± 0·11	2·85 ± 0·18	1·96 ± 0·09	286·94 ± 55·66	–
L. canadensis	*Lonicera* clade	3·34 ± 0·38	2·13 ± 0·12	1·46 ± 0·09	124·82 ± 12·76	–
L. etrusca	*Lonicera* clade	5·02 ± 0·24	3·33 ± 0·22	1·70 ± 0·09	237·10 ± 24·29	–
L. implexa	*Lonicera* clade	3·93 ± 0·16	2·82 ± 0·11	1·44 ± 0·18	292·50 ± 62·73	–
L. caprifolium	*Lonicera* clade	4·63 ± 0·23	3·45 ± 0·21	1·37 ± 0·47	258·67 ± 40·02	–
L. vesicaria	*Lonicera* clade	5·37 ± 0·23	3·27 ± 0·17	1·26 ± 0·11	215·86 ± 34·00	–
L. alpigena	*Lonicera* clade	6·38 ± 0·22	5·08 ± 0·22	2·12 ± 0·08	432·33 ± 45·45	–
L. muscaviensis	*Lonicera* clade	3·17 ± 0·10	2·65 ± 0·09	1·08 ± 0·07	176·92 ± 31·69	–
L. involucrata	*Lonicera* clade	2·55 ± 0·07	1·87 ± 0·24	1·00 ± 0·08	71·17 ± 12·00	–
L. javanica	*Lonicera* clade	4·33 ± 0·34	2·76 ± 0·14	0·80 ± 0·06	101·64 ± 9·22	–
L. chrysantha	*Lonicera* clade	4·00 ± 0·20	3·38 ± 0·31	1·24 ± 0·10	155·70 ± 19·43	–
L. maximowiczii	*Lonicera* clade	4·02 ± 0·40	3·19 ± 0·43	1·85 ± 0·22	150·66 ± 41·81	–
L. xylosteum	*Lonicera* clade	3·88 ± 0·19	3·12 ± 0·19	0·88 ± 0·15	120·12 ± 21·33	–
L. maackii	*Lonicera* clade	4·63 ± 0·33	2·77 ± 0·25	1·58 ± 0·08	107·48 ± 18·58	–
Symphoricarpos albus	*Lonicera* clade	3·73 ± 0·21	1·90 ± 0·10	1·13 ± 0·03	51·18 ± 10·03	190·67 ± 26·27
S. oreophilus	*Lonicera* clade	3·37 ± 0·06	1·88 ± 0·08	1·07 ± 0·03	59·65 ± 28·71	240·35 ± 27·90
S. occidentalis	*Lonicera* clade	3·30 ± 0·08	2·24 ± 0·05	1·15 ± 0·00	59·54 ± 15·99	211·58 ± 27·12
S. mollis	*Lonicera* clade	3·35 ± 0·13	1·88 ± 0·03	1·05 ± 0·05	68·87 ± 23·52	267·50 ± 48·72
Triosteum perfoliatum	*Lonicera* clade	5·18 ± 0·60	2·50 ± 0·28	1·43 ± 0·25	60·84 ± 11·54	505·27 ± 326·00
T. angustifolium	*Lonicera* clade	5·07 ± 0·06	2·08 ± 0·03	1·27 ± 0·12	39·11 ± 3·96	456·35 ± 306·86
T. hirsutum	*Lonicera* clade	6·50 ± 0·10	3·20 ± 0·00	0·97 ± 0·06	60·99 ± 11·80	355·28 ± 54·89
T. sinuatum	*Lonicera* clade	6·12 ± 0·13	2·80 ± 0·22	1·46 ± 0·05	94·38 ± 28·76	875·42 ± 466·11
Heptacodium miconioides	–	9·17 ± 0·10	1·37 ± 0·10	1·40 ± 0·09	7·33 ± 2·30	26·92 ± 4·05

* Endocarp thickness only applies to fruits containing pyrenes, i.e. *Symphoricarpos* and *Triosteum*.

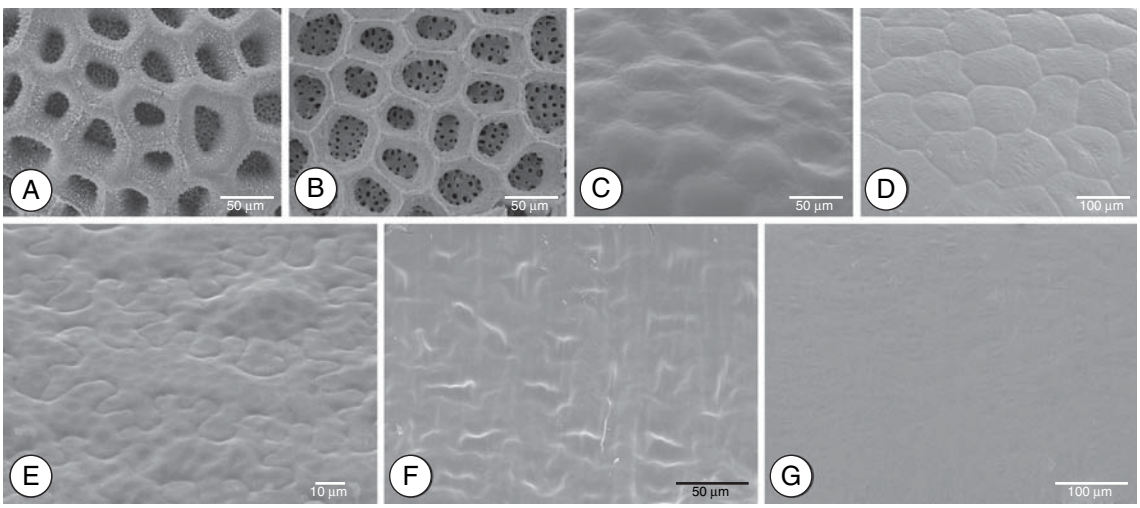

FIG. 3. Seed surface. (A) *Diervilla rivularis*, outer tangential wall removed, exotesta with U-shaped sclerification pattern. (B) *Weigela japonica*, outer tangential wall removed, sclerified exotesta with pits. (C) *Leycesteria crocothyrsos*, sclerified exotesta with concave outer tangential wall and slightly undulating cell outline. (D) *Lonicera dioica*, sclerified, polygonal exotestal cells with distinct cell boundaries. (E) *Triosteum perfoliatum*, sclerified exotestal cells with concave outer tangential cell walls with undulating cell outline. (F) *Symphoricarpos mollis*, sclerified exotesta with polygonal cell outline. (G) *Heptacodium miconioides*, compressed, parenchymatous seed coat without distinct seed sculpture.

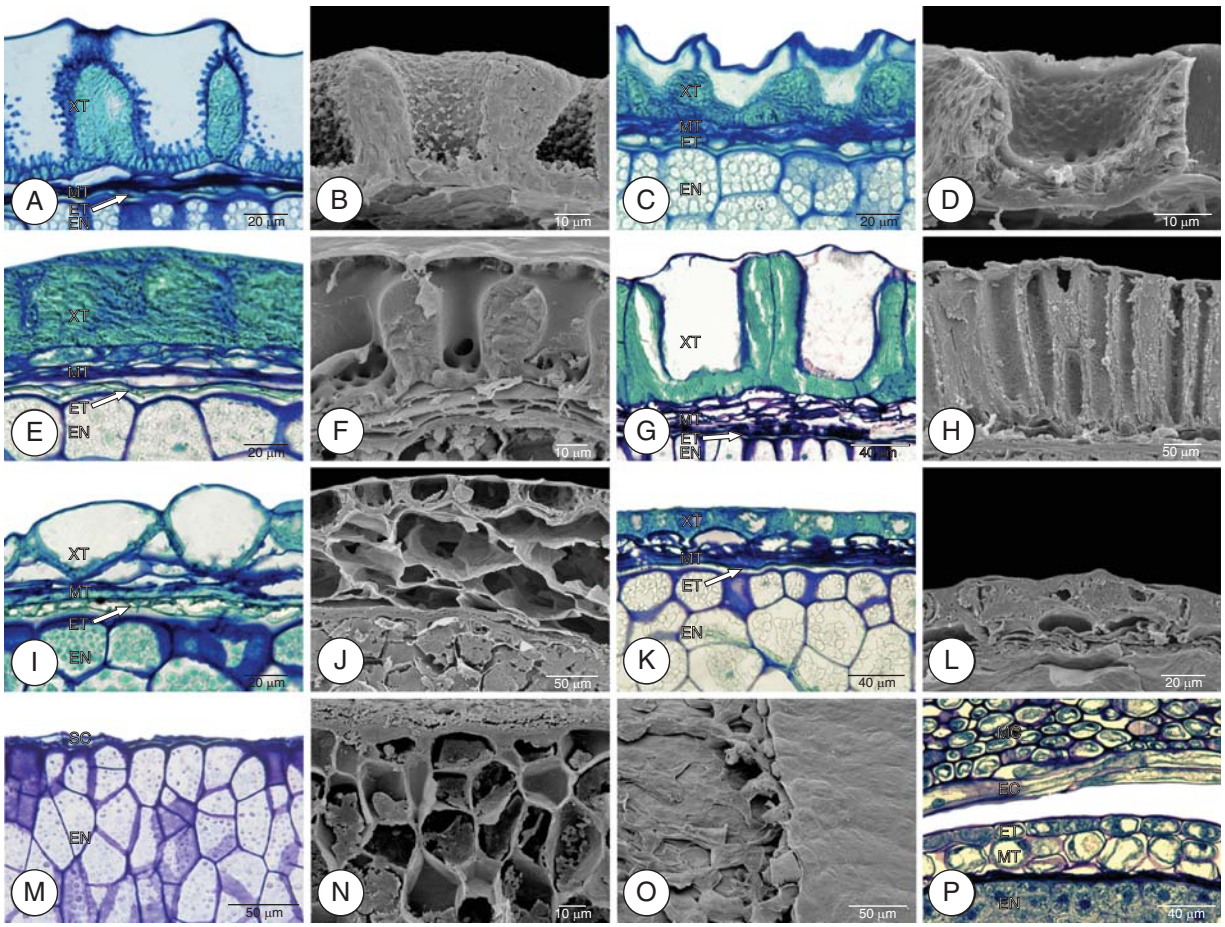

FIG. 4. Seed coat anatomy. (A, B) *Diervilla sessilifolia*: (A) TS, exotesta with U-shaped sclerification pattern, compressed mesotesta, partially compressed, sclerified endotesta; (B) sclerified exotesta, outer tangential wall removed. (C, D) *Weigela florida*: (C) sclerified exotesta, compressed mesotesta, sclerified endotesta and adjacent endosperm with starch granules; (D) sclerified exotesta, outer tangential wall removed. (E) *Leycesteria formosa*, sclerified exotesta, remnants of compressed, parenchymatous mesotesta, sclerified endotesta reduced to thin cell layer. (F) *Leycesteria crocothyrsos*, sclerified exotestal cells with pits. (G) *Lonicera canadensis*, large, sclerified exotesta cells, compressed mesotesta, sclerified endotesta reduced to thin cell layer. (H) *Lonicera alpigena*, tall, sclerified exotestal cells with pits. (I) *Triosteum hirsutum*, moderately sclerified exotestal cells with concave outer tangential walls, compressed mesotesta, small sclerified endotestal cells. (J) *Triosteum sinuatum*, sclerified exotesta, large, parenchymatous mesotestal cells, thin, sclerified endotesta. (K) *Symphoricarpos occidentalis*, sclerified exotesta with inner tangential wall shaped by adjacent mesotesta. (L) *Symphoricarpos oreophilus*, sclerified exotesta, remnants of parenchymatous mesotesta. (M–O) *Heptacodium miconioides*, compressed, parenchymatous seed coat, adjacent endosperm. (P) Immature seed with mesocarp and sclerified endocarp, parenchymatous exotesta and multi-layered mesotesta, endotesta reduced to thin layer. Abbreviations: XT, exotesta; MT, mesotesta; ET, endotesta; EN, endosperm; EC, endocarp; MC, mesocarp; SC, seed coat.

three layers, i.e. an outer, well-developed exotesta, a mesotesta composed of compressed parenchyma cells, and an inner layer of endotesta cells (Fig. 4A, C). The exotesta functions as the mechanical layer and is composed of a single-cell layer (Fig. 4A–D). The radial and inner tangential cell walls are sclerified (Fig. 4A, C). The outermost part of the radial walls and the outer tangential walls are not sclerified, resulting in a U-shaped sclerification pattern (Fig. 4A, C). The adjacent mesotesta is reduced to a fine layer of compressed cells, which is not always visible (Fig. 4C). The inner endotesta is composed of a single layer of small, weakly sclerified cells (Fig. 4A, C). With the exception of *W. japonica*, none of the species studied in the *Diervilla* clade has seed-coat crystals.

In the *Lonicera* clade (Fig. 4E–L), the main difference between *Leycesteria formosa* and *L. crocothyrsos* is the degree of exotestal sclerification. The cell walls of *L. formosa* are heavily sclerified except for the moderately sclerified outer tangential wall (Fig. 4E, F). The exotestal seed-coat cells of *L. crocothyrsos* are moderately sclerified, whereas the outer tangential wall is not sclerified at all. The mesotestal cells in *Leycesteria* are reduced to a compressed layer of parenchyma cells (Fig. 4E, F). At the lateral edges, one or more cell layers of parenchymatous mesotestal cells are discernable, and the raphe is embedded in this mesotestal layer. The inner endotesta is slightly sclerified and consists of small dorsiventrally flattened cells (Fig. 4E, F). In *L. crocothyrsos*, one or two layers of endotestal cells are present, whereas in *L. formosa* only a single-cell layer is present at maturity (Fig. 4E). In *Lonicera* (Fig. 4G, H), the degree of exotestal cell sclerification varies greatly, from weakly sclerified cell walls in *L. implexa* to heavily sclerified cell walls in *L. etrusca*. In the majority of the species examined, the outer tangential wall is not sclerified (Fig. 4G, H). Although several species have cuboid exotestal cells (e.g.

L. canadensis, Fig. 4G), exotestal cells are mostly more tall than wide. The mesotesta is reduced to a layer of compressed cells (Fig. 4G, H) except at the vascular bundles where multiple mesotestal cell layers can be present (cf. *Leycesteria*). In most species, the inner tangential cell walls are shaped by the mesotestal cells during seed maturation (Fig. 4G, H). The expanding endosperm subsequently compresses the mesotesta, resulting in a thin layer of cells. In most *Lonicera* spp., the mesotestal layer is characterized by the presence of druses. The endotesta is composed of small, dorsiventrally compressed cells, which are weakly sclerified (Fig. 4G). The seed-coat anatomy of *Symphoricarpos* and *Triosteum* is similar to that of *Leycesteria* and *Lonicera*, i.e. an outer layer of sclerified exotestal cells, a compressed layer of parenchymatous mesotestal cells, and an inner layer of small, sclerified, dorsiventrally compressed endotestal cells (Fig. 4I–L). In both *Symphoricarpos* and *Triosteum*, the mesotesta is characterized by druses. In some species (e.g. *T. hirsutum*), exotesta and endotesta can be multi-layered at the lateral edges of the seed. With the exception of *Leycesteria*, *T. hirsutum* and two species of *Lonicera* (*L. canadensis* and *L. involucrata*), all species studied in the *Lonicera* clade are characterized by seed-coat crystals (both druses and prismatic crystals were observed). The concave inner tangential exotestal cell wall of both *Symphoricarpos* and *Triosteum* is a clear indication that the mesotesta is composed of large, well-developed parenchymatous cells during seed development (Fig. 4I–K).

The seed coat of *Heptacodium* (Fig. 4M–P) is composed of compressed parenchymatous cells (Fig. 4M–O). During seed maturation, a single-layered exotesta and multilayered mesotesta is present (Fig. 4P). Although not observed, an endotesta is possibly present early in seed development. During seed development, the seed coat contains druses. The crystals, however, are absent at maturity.

Endosperm

In the *Diervilla* clade, endosperm cells of *Diervilla* (Fig. 5A) and *Weigela* (Fig. 5B) are similar in shape and size. The cells have thin cell walls and contain numerous starch grains. Although anatomically identical, cells located at the periphery of the endosperm are slightly smaller in size.

In the *Lonicera* clade, the endosperm of *Leycesteria* is composed of large, isodiametric cells with several, large starch grains (Fig. 5C). No significant differences were observed between the peripheral endosperm layers and the rest of the endosperm. Endosperm of *Lonicera* (Fig. 5D) is the most variable within the *Lonicera* clade. Cell shape and size differ among species, as do number and size of the starch grains. *Lonicera* is the only genus of the tribe in which some species have endosperm cells with slightly thickened cell walls. Endosperm cells of *Triosteum* (Fig. 5E) and *Symphoricarpos* (Fig. 5F) are similar to those of *Leycesteria* (Fig. 5C). Starch grains of the peripheral cells are markedly smaller than those of the rest of the endosperm. The number of starch grains per cell differs from species to species and does not seem to be genus-specific. *Triosteum sinuatum*, for example, has only a few starch grains per cell, whereas endosperm cells of *T. hirsutum* are filled with numerous, smaller starch grains.

The endosperm of *Heptacodium* (Fig. 5G) consists of large, isodiametric cells, which are slightly smaller at the periphery of the endosperm. Endosperm cells are filled with numerous starch grains. Endosperm anatomy is similar to that of *Leycesteria* (Fig. 5C), *Triosteum* (Fig. 5E) and *Symphoricarpos* (Fig. 5F).

Embryo

In the *Diervilla* clade, seeds of both *Diervilla* and *Weigela* (Fig. 5H) have a straight, linear embryo. Embryo length varies between 1/2 to 1/3 of seed length and embryo width is about 1/4 of seed width.

In the *Lonicera* clade, *Leycesteria* is characterized by a straight, linear embryo. Embryo length is about 1/3 of seed length and embryo width is about 1/4 of seed width. The embryos of *Lonicera* (Fig. 5I) and *Symphoricarpos* are $<1/3$ of the length of the seed and shape and size is similar to that of *Leycesteria*. The embryo of *Triosteum* (Fig. 5J) is slender, straight and linear to ovate. The length of the embryo is $<1/4$ of seed length (or smaller), whereas embryo width is about 1/8 of seed width.

Embryo length in *Heptacodium* is about 1/10 of the length of the seed and embryo width is about 1/4 of seed width. The embryo is straight, linear and drop-shaped (Fig. 2O).

Vasculature

In the *Diervilla* clade, seeds of *Diervilla* (Figs 2B and 5K) and *Weigela* (Fig. 2D) have a raphal bundle (no anti-raphal bundle) located in the mesotesta, containing an amphicribral vascular bundle. In the mature seed, the sclerified spiral tracheids are easily noticed and the thin-walled phloem cells are mostly compressed (Fig. 5K). At the location of the raphal bundle, often one or more mesotestal cell layers are present (Fig. 5K). Rexigenous cavities were observed, although not in all species.In the *Lonicera* clade (Figs 2F, H, J, L and 5L, M), all members except for *Leycesteria* are marked by seeds with both a raphal and an anti-raphal vascular bundle located in the mesotesta. The vascular bundles are of the amphicribral type. The raphal bundle of *Leycesteria* (Fig. 2F) is composed of a few spiral tracheids surrounded by one or two layers of phloem cells. In *Lonicera* (Figs 2H and 5L), the raphal bundle is generally larger than the anti-raphal bundle and both vascular bundles are often characterized by a rexigenous cavity (Fig. 5L). The vascular bundles in *Symphoricarpos* (Fig. 2L) are rather small in comparison with *Triosteum* (Figs 2J and 5M) and size and shape differs interspecifically. Rexigenous cavities occur in *Symphoricarpos*, although not in all species. In some species of *Triosteum* (e.g. *T. sinuatum*), the anti-raphal bundle splits in two strands. Large rexigenous cavities are present in most species of *Triosteum* (Fig. 5M).

Seeds of *Heptacodium* lack an anti-raphe. In young stages, the raphal bundle appears to be amphicribral, although no clear phloem cells could be observed (Fig. 5N). The metaxylem surrounds a rexigenous cavity and is itself surrounded by multiple layers of collenchyma (Fig. 5N). At maturity, only the metaxylem of the vascular bundle is visible. The other tissues are compressed as a result of seed growth.

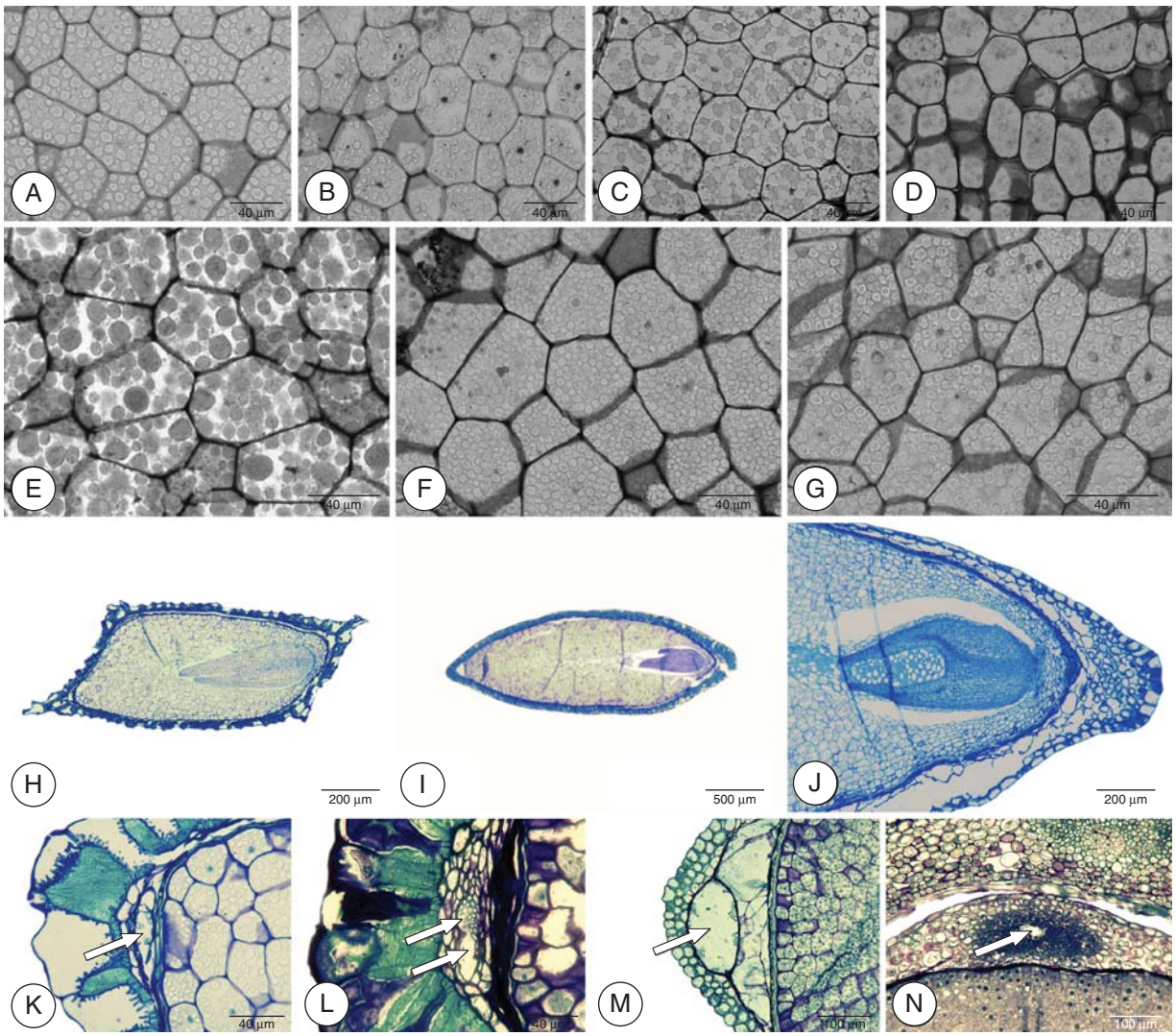

FIG. 5. Endosperm anatomy, embryo morphology and vascular anatomy. (A) *Diervilla sessilifolia*, TS, thin-walled, isodiametric endosperm cells. (B) *Weigela hortensis*, TS, endosperm. (C) *Leycesteria formosa*, TS, endosperm. (D) *Lonicera maackii*, TS, slightly thickened, isodiametric endosperm cells. (E) *Triosteum perfoliatum*, TS, endosperm. (F) *Symphoricarpos albus*, TS, endosperm. (G) *Heptacodium miconioides*, TS, large, isodiametric endosperm cells. (H) *Weigela floribunda*, LS, winged seed with embryo. (I) *Lonicera involucrata*, LS, embryo less than 3/4 of seed length. (J) *Triosteum sinuatum*, small, drop-shaped embryo. (K) *Diervilla sessilifolia*, raphal bundle in mesotesta, rexigenous cavity (arrow) with spiral tracheids. (L) *Lonicera xylosteum*, raphal bundle with rexigenous cavity (arrows) in multi-layered mesotesta. (M) *Triosteum hirsutum*, large raphal bundle, rexigenous cavity (arrow) with spiral tracheids, multi-layered exotesta. (N) *Heptacodium miconioides*, immature seed, raphal bundle in multi-layered mesotesta, rexigenous cavity (arrow) with spiral tracheids surrounded by collenchyma.

Endocarp

Triosteum (Fig. 6A–C, G, H) and *Symphoricarpos* (Fig. 6D–F, I, J) both produce seeds surrounded by a sclerified endocarp, i.e. pyrenes. Pyrene shape differs between the genera as most species of *Triosteum* (except for *T. hirsutum*) have pyrenes with five or six prominent ribs (Fig. 6A–C, G, H), whereas pyrenes of *Symphoricarpos* (Fig. 6D–F, 6I, J) lack such ribs. Another distinct difference is endocarp anatomy. The endocarp of *Symphoricarpos* is composed of three distinct layers (Fig. 6I, J): an inner layer of fibres orientated perpendicular to the pyrene's length axis; a layer of one (rarely more) cell layer of crystal-containing sclereids; and an outer layer of fibres orientated parallel to the length axis of the pyrene. The endocarp of *Triosteum* is composed of two layers (Fig. 6G, H) similar to the fibrous innermost and outermost layers of *Symphoricarpos*. The middle layer is lacking in *Triosteum*, although in *T. angustifolium* and *T. perfoliatum* a small number of crystal-containing sclereids are scattered throughout the endocarp. In *T. angustifolium* the latter sclereids contain druses, whereas the sclereids of *T. perfoliatum* hold prismatic crystals. Due to the presence of ribs in *Triosteum*, the organization of the two layers of fibres (Fig. 6G, H) differs from that of *Symphoricarpos* (Fig. 6I, J). Although the fibres are organized in large strands, a pattern, comparable to that of *Symphoricarpos*, is not discernable. The fibres of the outer layer seem to run dorsiventrally (Fig. 6C, G, H). The endocarp of *Triosteum* is much harder and more robust than the endocarp of *Symphoricarpos*,

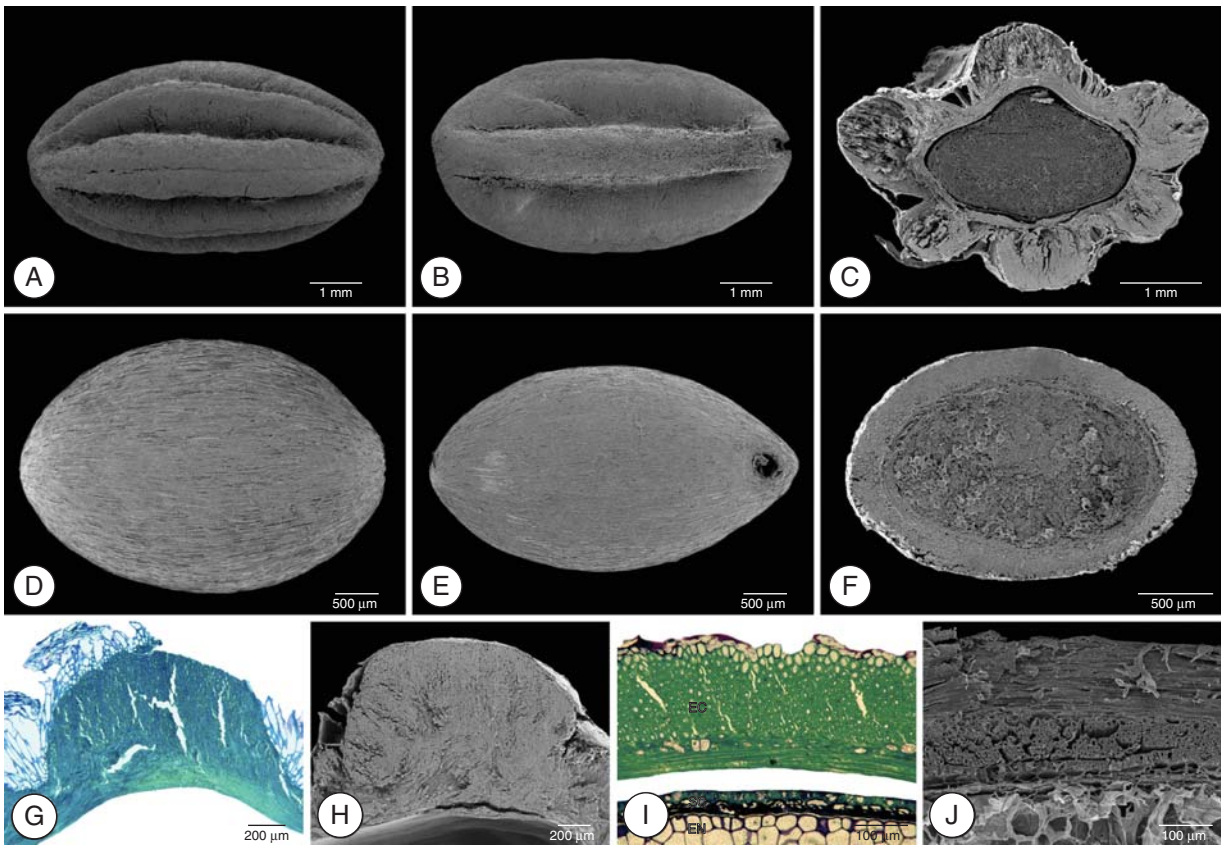

FIG. 6. Endocarp morphology and anatomy. (A, B) *Triosteum angustifolium*: (A) dorsal view; (B) ventral view. (C) *Triosteum perfoliatum*, TS, sclerified endocarp with ribs surrounding seed. (D–F) *Symphoricarpos albus*: (D) dorsal view; (E) ventral view; (F) TS, sclerified endocarp surrounding seed. (G) *Triosteum angustifolium*, TS, endocarp rib with two layers of fibres and adjacent mesocarp. (H) *Triosteum sinuatum*, TS, endocarp rib. (I, J) *Symphoricarpos occidentalis*: (I) endocarp with three distinct layers, i.e. outer layer of fibres, sclereids, inner layer of fibres, seed with sclerified exotesta; (J) endocarp with adjacent seed. Abbreviations: EC, endocarp; SC, seed coat; EN, endosperm.

which is most likely due to the strands of fibres and the different organization of the fibres in *Triosteum* (Fig. 6C, G, H). The endocarp of *Symphoricarpos* tends to be slightly flexible.

The endocarp of *Heptacodium* is composed of a single layer of moderately sclerified fibres (Fig. 4P). Since the fruit of *Heptacodium* does not dehisce, the entire pericarp functions as mechanical layer (Fig. 2O).

Phylogenetic analyses (Figs 7 and 8)

Molecular data. The MP analysis resulted in two most-parsimonious trees of 1904 steps (CI = 0·70; RI = 0·77). The aligned matrix consisted of 2403 characters, of which 613 were potentially parsimony informative. The sister relationship of the *Diervilla* clade and the remainder of Caprifoliaceae gained strong supported (bootstrap, BS = 100; posterior probability, PP = 1·00). Both the MP and BI analysis validate the monophyly of the *Diervilla* (97 BS; 1·00 PP) and *Lonicera* clades (99 BS; 1·00 PP). Intergeneric relationships of the *Lonicera* clade, however, are different. Based on BI, the intergeneric relationships of the four genera remain unclear due to a polytomy that unites all four genera. The MP topology hypothesizes *Triosteum* to be sister to the remainder of the *Lonicera* clade (99 BS).The other intergeneric relationships in the *Lonicera* clade gain low support. The two shortest trees of the MP analysis suggest a sister relationship (no bootstrap support) of *Lonicera* and *Symphoricarpos* plus *Leycesteria* (no bootstrap support). The monophyly of all genera of the *Lonicera* clade, however, gained strong support. In both analyses, the *Lonicera* clade is sister to a clade consisting of *Heptacodium* plus the Linnina clade (94 BS; 1·00 PP). The sister relationship of *Heptacodium* and Linnina gained moderate to strong support (74 BS; 1·00 PP). In Linnina, the relationships between the *Linnaea*, *Morina*, *Dipsacus* and *Valeriana* clades are identical in both analyses, i.e. the *Linnaea* clade is sister (98 BS; 1·00 PP) to Valerina and the *Morina* clade is sister (62 BS; 0·66 PP) to the *Dipsacus* clade plus the *Valeriana* clade (98 BS; 0·99 PP).

Combined data. The addition of 29 morphological characters to the molecular data matrix generally resulted in an increase in resolution and support in both analyses. The MP analysis resulted in four shortest trees of 2067 steps (CI = 0·71; RI = 0·77). The monophyly of *Diervilla*, *Weigela* and the *Diervilla* and *Lonicera* clades remained strongly supported (100 BS; 1·00 PP). The intergeneric relationships in the *Lonicera* clade differed from the resultant topologies based on molecular data. The BI analysis based on combined data suggested that *Leycesteria* plus *Lonicera* (0·89 PP) and *Triosteum* plus *Symphoricarpos* (0·87 PP) are sister groups

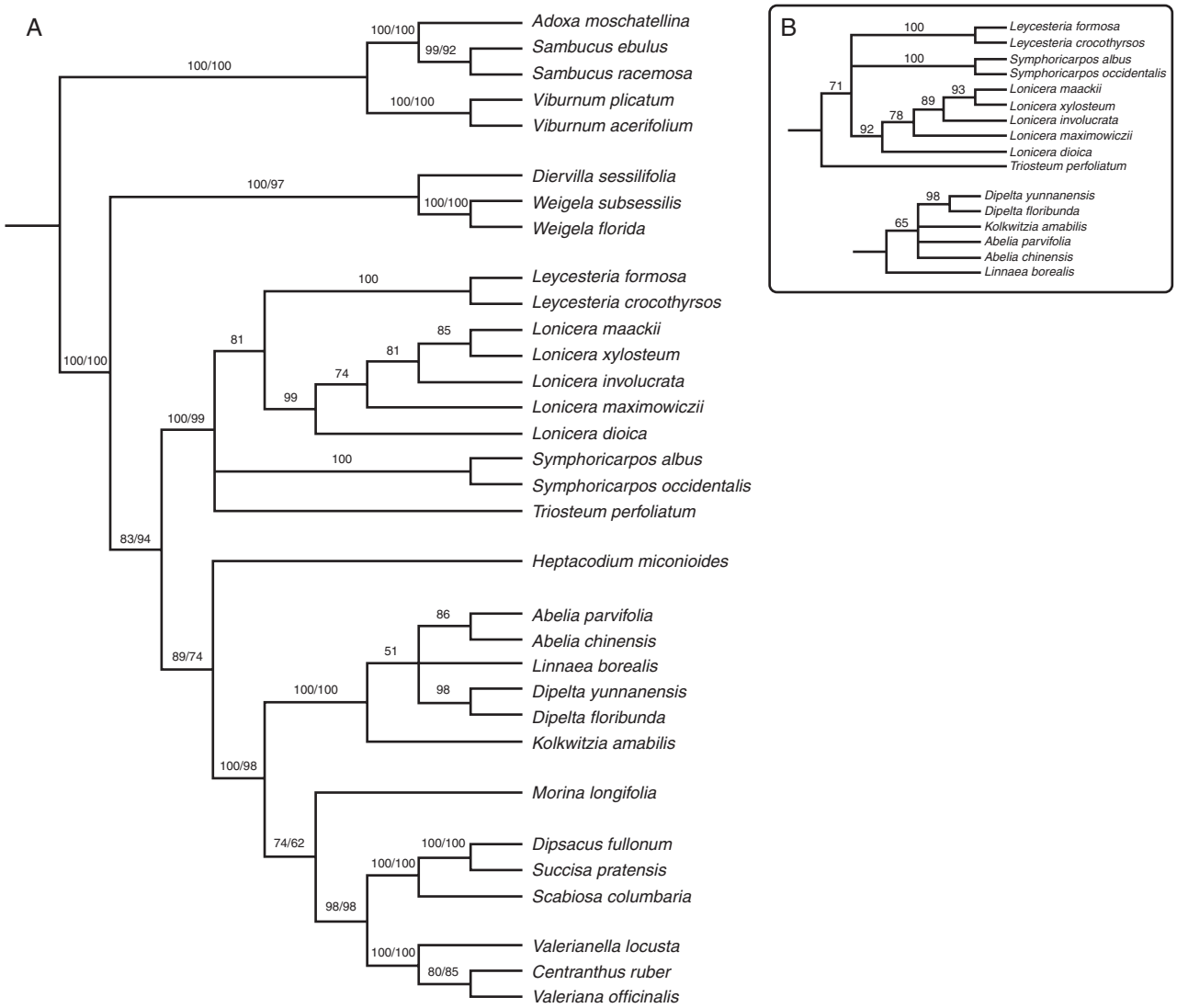

FIG. 7. Consensus trees from maximum parsimony (MP) analyses with bootstrap support values (>50). As the MP topologies based on sequence data (B) and combined data only differed in the *Lonicera* and the *Linnaea* clades, (B) only shows the latter two clades and the respective bootstrap values. (A) Topology based on combined data with bootstrap values of MP analysis of sequence data (left value) and bootstrap values of combined data (right value); bootstrap values with an asterisk (*Lonicera* and *Linnaea* clades) are based on combined data. (B) Topology of *Lonicera* and *Linnaea* clades based on sequence data with bootstrap values.

(1·00 PP). Three out of four of the shortest MP trees are congruent with the BI hypothesis. The other shortest tree is congruent with the shortest MP trees based on molecular data (see above). The sister relationship of the *Lonicera* clade and the clade consisting of *Heptacodium* and Linnina gained lower support than in the analyses based on sequencing data only (83 BS; 1·00 PP). The sister relationship of *Heptacodium* and Linnina, however, gained support (89 BS; 1·00 PP). Support and resolution of the intergeneric relationships of the *Linnaea* clade increased slightly. Based on BI, *Abelia* and *Linnaea borealis* are hypothesized to be sisters (0·75 PP) with *Dipelta* being sister to the pair (0·99 PP). *Kolkwitzia amabilis* is sister to the remainder of the the *Linnaea* clade (1·00 PP). The four shortest MP trees are congruent with the BI topology. Bootstrap support, however, is low except for the sister relationship of *K. amabilis* and the remainder of the *Linnaea* clade (100 BS).

Incongruence between data partitions and topologies

ILD testing (Table 5) shows significant incongruencies are present between ITS and *trnK*, *trnK* and *matK*, and between the individual molecular data sets and the morphological data set. The tests indicate that the exclusion of *Heptacodium* or the *Linnaea* clade has little to no impact on the incongruence of the data sets. The exclusion of the *Lonicera* clade, however, resulted in the disappearance of all significant incongruence between the data sets except for the incongruence between *trnK* and *matK*. The AU and SH tests (Table 6) largely confirm the ILD test results (Table 5). As indicated by the ILD tests, all morphological topologies (MP and BI) differ significantly from the ML topologies based on the three combinations of sequence data. The ML topologies of the individual molecular data sets (ITS, *trnK* and *matK*) differ significantly from the ML topology of the combined

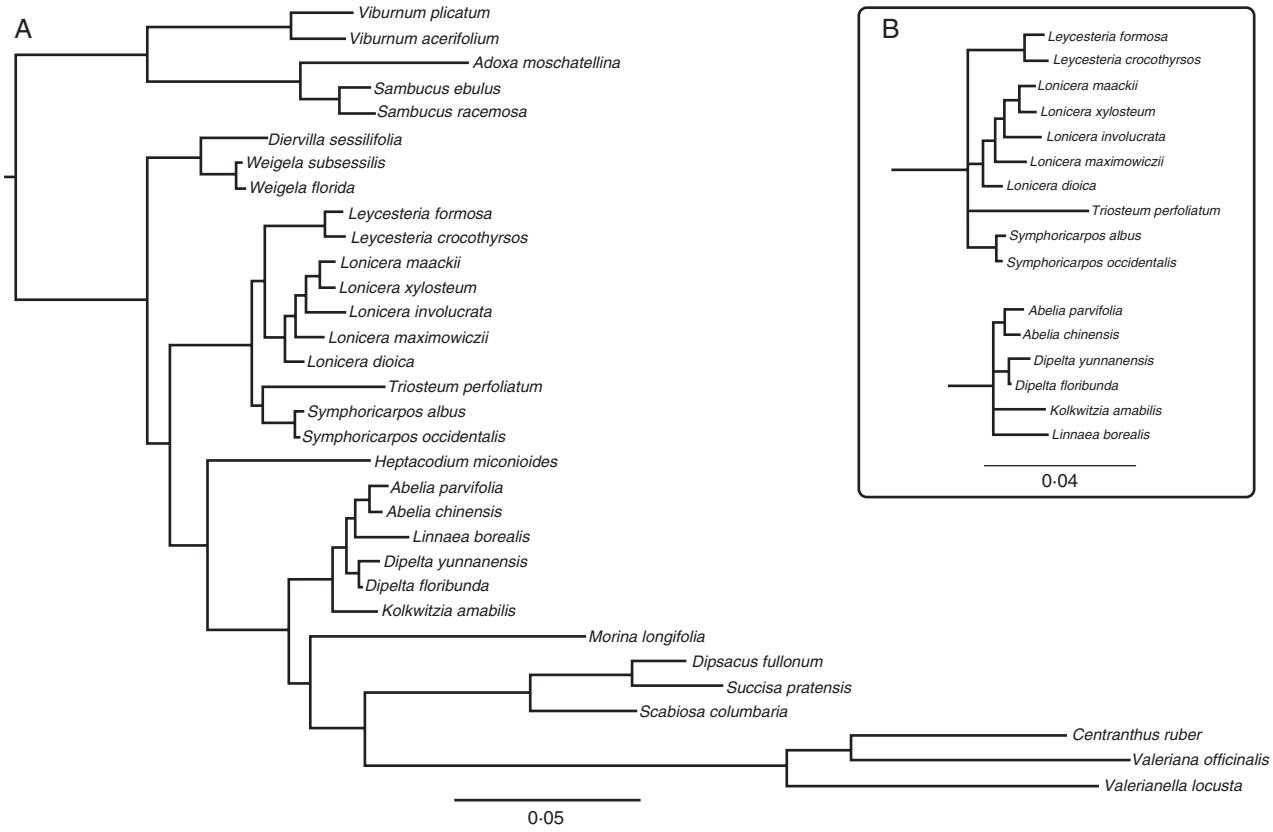

FIG. 8. Majority rule trees of Bayesian inference (BI) analyses. Thicker branches have siginficant posterior probability scores (>0·95). As BI topologies based on sequence data (B) and combined data only differed in the *Lonicera* and *Linnaea* clades, (B) only shows the latter two clades. (A) Topology based on combined data. (B) Topology based on sequence data.

molecular data set. Comparison of topologies and ILD, AU and SH tests indicate significant incongruencies are present between the data sets. Visual inspection of the topologies, however, clearly indicates the incongruencies are primarily related to uncertainty regarding the intergeneric relationships of the *Lonicera* and *Linnaea* clades and the placement of *Heptacodium*. The incongruence of the molecular and morphological data is primarily due to the relatively weak phylogenetic signal of the latter data set.

DISCUSSION

Diervilla *clade*

The composition and monophyly of the *Diervilla* clade, *Diervilla* and *Weigela* are strongly supported in our analyses (Figs 7 and 8). Fruit and seed morphology and anatomy of both genera are quite different from what is encountered in the remainder of the order. *Diervilla* and *Weigela* have bicarpellate, dehiscent capsules with numerous seeds, whereas the remainder of the order is characterized by drupes, berries or achenes. Like the genera of the *Lonicera* clade, *Diervilla* and *Weigela* have seeds with a sclerified exo- and endotesta. The degree of sclerification of the outer tangential exotestal cell wall, however, differs from that of the *Lonicera* clade. The mesotesta of both genera is reduced to a layer of compressed cells, in which a vascular bundle or raphe is located. Abortive carpels are absent in the *Diervilla* clade (Backlund and Pyck, 1998), although a fruit-developmental study is required to address this question confidently. The fruits and seeds of *Diervilla* and *Weigela* are much alike in overall anatomy. Several morphological differences, however, are apparent. Seeds of *Weigela* are in close contact with each other, which causes the shape of the seeds to be partially determined by adjacent seeds, resulting in flattened lateral sides and seed wings (Fig. 2C, D). Seeds of *Diervilla* are less angular. It is still unclear whether this morphological difference is due to abortive ovules or an initial smaller number of ovules. Capsules of *Weigela* are long, slender and cylindrical, whereas those of *Diervilla* are shorter and broader at the base, resulting in bottle-shaped fruits.

Lonicera *clade*

Whereas the monophyly of all four genera is well supported, intergeneric relationships are less clear (Figs 7 and 8). Our results support a close affinity between *Leycesteria* and *Lonicera* and between *Symphoricarpos* and *Triosteum*. Although the latter hypothesis gained only moderate to poor support in our combined analyses, fruit and seed morphology and anatomy provide strong evidence. *Leycesteria* and *Lonicera* lack sterile carpels and a sclerified endocarp, whereas *Symphoricarpos* and *Triosteum* have ovaries with one (*Triosteum*) or two (*Symphoricarpos*) sterile carpels and

fruits with a sclerified endocarp. In *Leycesteria* and *Lonicera*, each carpel contains several fertile ovules, whereas carpels of *Symphoricarpos* and *Triosteum* contain a single pendent ovule. Consequently, fruits of *Leycesteria* and *Lonicera* contain numerous seeds, whereas those of *Symphoricarpos* and *Triosteum* contain two and three seeds, respectively. Furthermore, *Leycesteria* and *Lonicera* have berries, whereas *Symphoricarpos* and *Triosteum* have drupes.

Several studies have dealt with the intergeneric relationships of the *Lonicera* clade (e.g. Backlund, 1996; Pyck *et al.*, 1999; Donoghue *et al.*, 2001; Zhang *et al.*, 2003). Recently, Winkworth *et al.* (2008*b*) tackled the question by analysing mitochondrial and plastid sequence data using several strategies, i.e. separately analysing coding and non-coding data and a total evidence approach, but found the results to be conflicting. The underlying cause for the lack of a stable phylogenetic hypothesis for the *Lonicera* and *Linnaea* clades might be the rapid diversification of the taxa. This hypothesis is supported by a dating study of Dipsacales by Bell and Donoghue (2005). The authors found that the genera of the *Lonicera* clade diverged within a time frame of 3–4 million years, whereas other major clades of the order diverged within a period of 15–60 million years. The branch lengths of the BI topologies confirm this hypothesis (Fig. 8).

Within Dipsacales, the fruits and seeds of the *Lonicera* clade are unique in several ways. Members of the tribe have a sclerified exotesta and a weakly sclerified endotesta as in the *Diervilla* clade, but the outer tangential exotestal cell wall is weakly to moderately sclerified in the *Lonicera* clade, whereas it is not sclerified in the *Diervilla* clade. The *Lonicera* clade is the only clade in the family characterized by the occurrence of fruits with a fleshy mesocarp, i.e. berries and drupes. Seeds of *Diervilla* and *Weigela* show some resemblance to those of *Leycesteria* and *Lonicera*. All four genera have multiple fertile ovules per carpel, produce fruits with numerous seeds and have seeds with a similar seed-coat anatomy (see above).

Heptacodium

Zhang *et al.* (2002) hypothesized that *Heptacodium* might have evolved as a hybrid from ancestors of the *Lonicera* and *Linnaea* clades. This hypothesis provided an explanation for the morphological similarities that *Heptacodium* shares with both tribes. Winkworth *et al.* (2008*b*), however, commented that it is less likely that differences between functional partitions in uniparentally inherited plastid or mitochondrial genomes could be explained by hybridization. Nevertheless, hybridization could explain why our analyses suggest a sister relationship of *Heptacodium* and the Linnina clade. *Heptacodium* shares specific floral characteristics with the *Lonicera* clade and ovary-related characteristics with the *Linnaea* clade (Donoghue *et al.*, 2003; Winkworth *et al.*, 2008*b*). Our results indicate a sister relationship between *Heptacodium* and the Linnina clade.

Transfer of function

The broad fruit diversity in Caprifoliaceae is linked to several functional shifts of the mechanical layer, which is the tissue that protects the embryo and endosperm from the environment. *Diervilla*, *Leycesteria*, *Lonicera* and *Weigela* have seeds with a sclerified exotesta acting as a mechanical layer. In *Symphoricarpos* and *Triosteum*, a first transfer of function occurred with the sclerification of the endocarp and the resulting development of pyrenes. In spite of the presence of a sclerified endocarp acting as a mechanical layer, the exo- and endotesta are also sclerified in *Symphoricarpos* and *Triosteum*. We believe this might be a strong argument for hypothesizing that the ancestor of the *Lonicera* clade had fruits with a sclerified exo- and endotesta and an unsclerified endocarp. Sclerification of the exo- and endotesta should be regarded as a plesiomorphic condition in the *Lonicera* clade and possibly in the order as a whole (see below). In the clade including *Heptacodium* and Linnina, three independent evolutionary shifts are apparent. A first shift toward the development of a protective pericarp and the simultaneous development of a parenchymatous seed coat occurred in the *Linnaea* and *Valeriana* clades and *Heptacodium*. In the *Morina* and *Dipsacus* clades, independent second and third evolutionary shifts resulted in the functional transfer of the mechanical layer to the epicalyx. In the *Dipsacus* clade, for example, the pericarp is reduced to a thin, papery layer surrounding the single seed, whereas epicalyx morphology has diversified into a broad range of shapes and sizes. Finally, in Adoxaceae, the sclerified endocarp functions as a mechanical layer except in *Sinadoxa*. The fruits of *Sinadoxa* have been described as achene-like (Wu *et al.*, 1981), which means the entire pericarp protects the seed. Thus, in Dipsacales a general trend is apparent in which the function of a mechanical layer is transferred to outer tissues in more derived clades.

Character evolution

Due to the morphological differences that separate Adoxaceae and Caprifoliaceae, the uncertain systematic position of several key taxa and the equivocal intergeneric relationships within the *Linnaea* and *Lonicera* clades, the morphology and anatomy of the ancestral Dipsacales is difficult to infer (Donoghue *et al.*, 2003). We can be rather confident, however, that the ancestor of Dipsacales was woody and had simple, opposite leaves without stipules (Donoghue *et al.*, 2003). Floral morphology of the first Dipsacales is more difficult to infer due to the contrasting flower morphology of Adoxaceae and Caprifoliaceae. The first Adoxaceae most likely had actinomorphic flowers with small calyx lobes, rotate corollas, short styles and lobed stigmas, but lacked distinct nectaries, whereas the first Caprifoliaceae had zygomorphic flowers with larger calyx lobes, tubular corollas, elongate styles, capitate stigmas and nectaries composed of unicellular hairs at the base of the corolla (Donoghue *et al.*, 2003). We can also be fairly confident that the ancestor of Dipsacales had perfect, fertile flowers with five corolla lobes and five stamens (Donoghue *et al.*, 2003). A biogeographical study indicated that the first Dipsacales most probably originated in East Asia, where they inhabited the understory of temperate forests (Bell and Donoghue, 2005).

In the following paragraphs, we discuss the evolution of several key fruit and seed characters (Tables 4 and 8) based on parsimony optimization and stochastic character mapping

TABLE 8. *Morphological data set*

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Abelia chinensis	2	3	2	1	?	?	1	0	0	0	?	?	?	?	?	?	0	3	2	1	2	1	0	0	0	0	1	2	0
A. parvifolia	2	3	2	1	?	?	1	0	0	0	?	?	?	?	?	?	0	3	2	1	2	1	0	0	0	0	1	2	0
Adoxa moschatellina	0	4	0	4	1	2	1	1	2	0	1	0	0	1	0	0	0	0	0	0	2	3	0	0	1	1	1	2	0
Centranthus ruber	2	2	2	0	1	2	1	0	1	0	0	?	0	0	2	0	0	1	2	1	2	1	0	0	2	0	1	0	1
Dipelta floribunda	2	3	2	1	1	0	1	0	0	0	0	?	0	0	0	0	0	0	2	1	2	1	0	0	0	0	1	2	0
D. yunnanensis	2	3	2	1	1	0	1	0	0	0	0	?	0	0	0	0	0	0	2	1	2	1	0	0	0	0	1	2	0
Diervilla sessilifolia	3	1	0	6	1	1	0	0	3	0	2	0	1	0	0	1	0	0	2	0	0	1	0	0	0	0	1	2	0
Dipsacus fullonum	2	0	0	0	?	0	1	0	0	1	0	?	0	0	1	0	0	2	2	1	2	1	0	0	2	0	2	2	1
Heptacodium miconioides	2	2	2	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	2	?	?	1	0	0	0	0	1	2	0
Kolkwitzia amabilis	2	2	2	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	2	1	2	1	0	0	0	0	1	2	0
Leycesteria crocothyrsos	1	4	0	5	0	0	1	1	3	0	2	0	1	1	0	1	0	0	2	0	0	1	0	0	0	0	1	2	0
L. formosa	1	4	0	5	0	0	1	1	3	0	2	0	1	1	0	1	0	0	2	0	0	1	0	0	0	0	1	2	0
Linnaea borealis	2	3	2	1	?	?	1	0	0	0	?	?	?	?	?	?	0	0	2	1	2	1	0	0	0	0	1	2	0
Lonicera dioica	1	2	0	5	0	0	1	1	3	0	2	1	1	1	0	2	0	0	2	0	0	1	0	0	0	2	1	2	0
L. involucrata	1	2	0	5	0	0	1	1	3	0	2	0	1	1	0	1	0	0	2	0	0	1	0	0	0	2	1	2	0
L. maximowiczii	1	2	0	5	0	0	1	1	3	0	2	1	1	1	0	2	0	0	2	0	0	1	0	0	0	2	1	2	0
L. xylosteum	1	2	0	5	0	0	1	1	3	0	2	1	1	1	0	2	0	0	2	0	0	1	0	0	0	2	1	2	0
L. maackii	1	2	0	5	0	0	1	1	3	0	2	1	1	1	0	2	0	0	2	0	0	1	0	0	0	2	1	2	0
Morina longifolia	2	2	2	0	1	0	1	0	0	1	0	?	0	1	1	0	0	0	2	1	2	1	0	0	2	0	0	2	0
Symphoricarpos albus	0	3	2	1	1	2	1	1	2	0	2	1	1	1	0	1	0	0	2	1	2	1	0	0	0	0	1	2	0
S. occidentalis	0	3	2	1	1	2	1	1	2	0	2	1	1	1	0	1	0	0	2	1	2	1	0	0	0	0	1	2	0
S. ebulus	0	4	0	4	1	2	1	1	2	0	0	0	0	0	1	0	0	0	0	1	2	3	0	0	0	1	1	2	3
S. racemosa	0	4	0	4	1	2	1	1	2	0	0	0	0	0	1	0	0	0	0	1	2	3	0	0	0	1	1	2	3
Scabiosa columbaria	2	0	0	0	0	0	1	0	0	1	0	?	0	0	1	0	0	2	2	1	2	1	0	0	2	0	2	2	1
Succisa pratensis	2	0	0	0	?	0	1	0	0	1	0	?	0	0	1	0	0	2	2	1	2	1	0	0	2	0	2	2	1
Triosteum perfoliatum	0	3	1	2	1	1	1	1	2	0	2	1	1	1	0	1	0	0	2	1	2	1	0	0	0	0	1	2	0
Viburnum acerifolium	0	2	2	0	1	1	1	1	2	0	1	0	0	1	0	1	0	0	0	1	2	2	0	1	0	2	1	2	1
V. plicatum	0	2	2	0	1	1	1	1	2	0	1	0	0	1	0	1	0	0	0	1	2	2	0	1	0	2	1	2	1
Valeriana officinalis	2	2	2	0	1	2	1	0	1	0	0	0	0	0	2	0	0	1	2	1	2	1	0	0	2	0	1	0	1
Valerianella locusta	2	2	2	0	1	2	1	0	1	0	0	?	0	0	2	0	0	0	2	1	2	1	0	0	2	0	1	0	1
Weigela florida	3	1	0	6	1	1	0	0	3	0	2	0	1	0	0	1	1	0	2	0	0	1	0	0	0	0	1	2	0
W. subsessilis	3	1	0	6	1	1	0	0	3	0	2	0	1	0	0	1	1	0	2	0	0	1	0	0	0	0	1	2	0

Charcters 18–20 (Table 2) were obtained from the study of Backlund (1996).

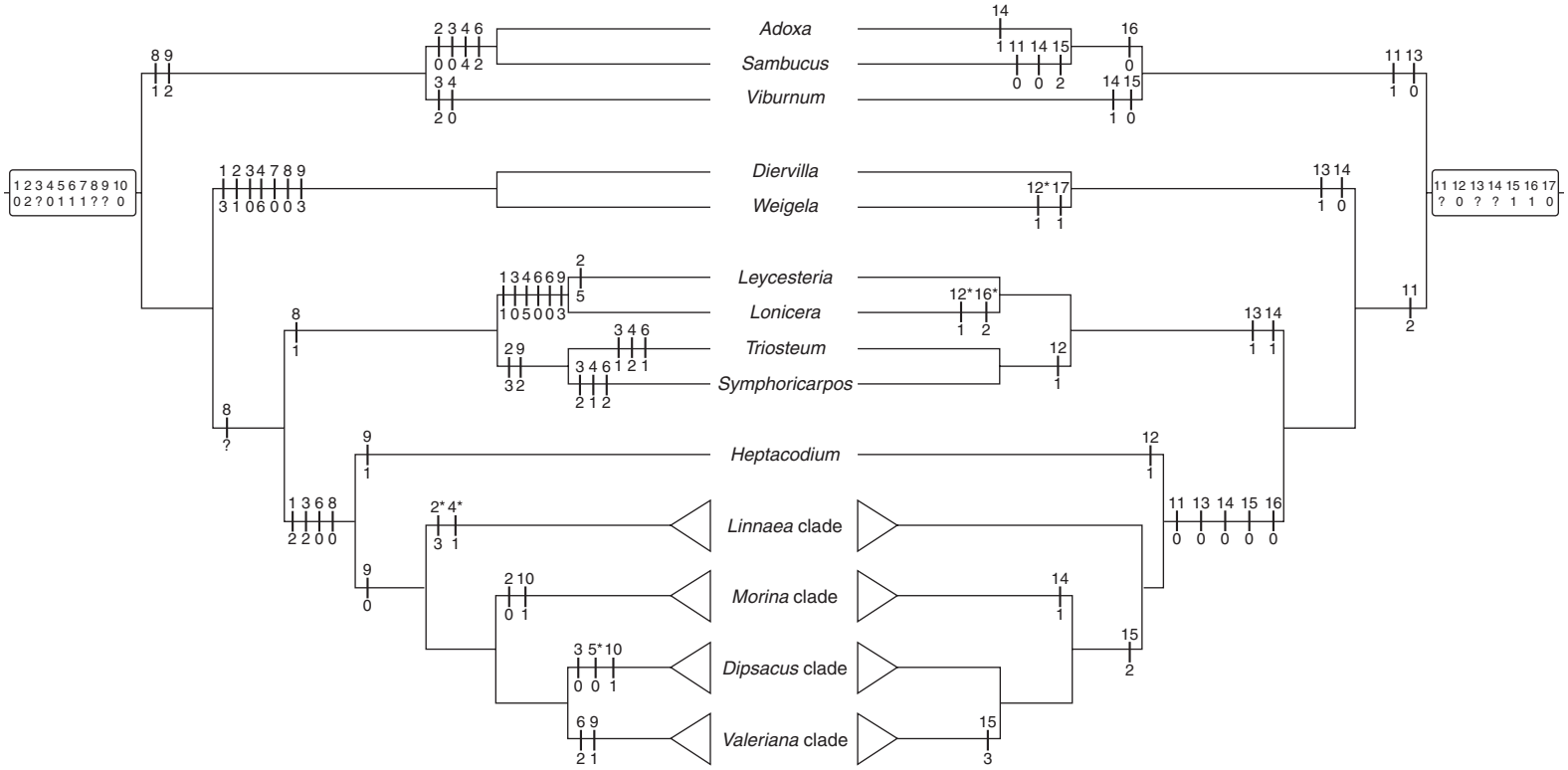

FIG. 9. Parsimony optimization results for ten fruit characters (left) and seven seed characters (right). The upper number indicates the respective character, whilst the bottom number indicates the character state (Table 4).

(Fig. 9). If appropriate, we indicate the posterior probability (PP) scores of the SCM analysis. The results of the SCM analysis are available as Supplementary Data, online.

Fruit type

The plesiomorphic fruit type for Dipsacales (PP 0·99) is a drupe. Based on this hypothesis, several shifts have occurred: (1) a shift to capsules along the branch leading to the *Diervilla* clade; (2) a shift to berries at the origin of the clade including *Leycesteria* and *Lonicera*; (3) a first shift to achenes toward the lineage comprising *Heptacodium* and Linnina; and (4) a second shift to achenes after the split of *Sinadoxa* and the clade containing the drupe-bearing genera *Adoxa* and *Tetradoxa*.

Carpel number and carpel sterility

The PO and SCM analyses generate conflicting results with respect to carpel number of the frist Dipsacales. Ovaries with three carpels are hypothesized to be the plesiomorphic condition for Dipsacales based on PO. Based on this hypothesis, three reductions took place: (1) a reduction to two carpels along the branch leading to *Dipelta*; (2) a first reduction to a single carpel at the origin of the *Dipsacus* clade; and (3) a second reduction to a single carpel after the split of *Sinadoxa* plus the pentacarpellate genera *Adoxa* and *Tetradoxa*. Besides these reductions, three increases occurred: (1) an increase to four carpels along the branch leading to *Symphoricarpos* and *Triosteum*; (2) a first increase to five carpels at the origin of *Leycesteria*; and (3) a second increase to five carpels at the base of subfamily Adoxoideae. The number of carpels in *Lonicera* varies intraspecifically, ranging from two to three per ovary. However, SCM hypothesizes that the first Dipsacales had pentacarpellate ovaries as observed in *Adoxa*, *Leycesteria* and *Sambucus* (and possibly *Tetradoxa*). Six reductions took place: (1) a reduction to two carpels along the branch leading to the *Diervilla* clade; (2) a reduction at the origin of the clade comprising *Symphoricarpos* and *Triosteum*; (3) a reduction along the branch leading to *Lonicera*; (4) a reduction to three carpels at the origin of the Linnina clade; (5) a reduction along the branches leading to *Dipelta* (bicarpellate); and (6) a reduction in the *Dipsacus* clade (monocarpellate). In Adoxaceae, *Viburnum* is characterized by monocarpellate ovaries (Wilkinson, 1948) as is *Sinadoxa* (Wu *et al.*, 1981).

Sterile carpels are absent in the *Diervilla* and *Dipsacus* clades, Adoxoideae (with the exception of *Sinadoxa*), *Leycesteria* and *Lonicera*. The plesiomorphic condition for both Dipsacales and the *Lonicera* clade is equivocal based on PO. *Triosteum* has ovaries with a single sterile carpel, whereas *Symphoricarpos*, *Viburnum* and the clade including *Heptacodium* and Linnina are characterized by ovaries with two sterile carpels. Based on the modified phylogenetic tree (based on that of Winkworth *et al.*, 2008*b*; see Fig. 1), PO indicates that the ancestor giving rise to Caprifoliaceae minus the *Diervilla* clade had two sterile carpels. We believe that the first Dipsacales had flowers lacking sterile carpels and that the presence of sterile carpels is a derived feature in the order. This hypothesis is strongly confirmed by our SCM analysis (PP 0·99). The only Adoxaceae with sterile carpels are *Sinadoxa* and *Viburnum*, and the shift toward the occurrence of sterile carpels most likely took place along the branches leading to these taxa. Moreover, the *Diervilla* clade lacks sterile carpels, as do *Leycesteria* and *Lonicera*, which are often considered as the most 'primitive' genera of the *Lonicera* clade (e.g. Wilkinson, 1949). A pentacarpellate dipsacalean ancestor without sterile carpels as hypothesized by our SCM analysis seems likely.

Seed number

SCM assigns a PP score of 0·99 to the hypothesis of fruits with five seeds. Our PO analysis, however, hypothesizes single-seeded fruits to have characterized the first Dipsacales. However, we believe the latter result is an artefact as *Heptacodium*, *Viburnum* and the entire Linnina clade (excluding *Dipelta*) produce single-seeded fruits. Ovary development in *Viburnum* is characteristic for the genus (e.g. Wilkinson, 1948, 1949; Jacobs *et al.*, 2008; see above) and must therefore have occurred along the evolutionary path leading to *Viburnum*. An evolutionary link with the single-seeded fruits of *Heptacodium* and Linnina is therefore most unlikely as ovary development differs dramatically from ovary development in *Viburnum* (Wilkinson, 1948). The evolution of seed number within the *Lonicera* clade is equivocal. We believe, however, that the ancestor of Caprifoliaceae (and possibly the dipsacalean ancestor) had fruits with numerous seeds, as fruits with fewer (or a single) seed are predominantly found in derived clades (e.g. Linnina and *Viburnum*). Additional support for this hypothesis is provided by the many ovules present in all three carpels of *Heptacodium*, of which only a single ovule in a single carpel matures into a fertile seed. A first shift to carpels with a single fertile ovule occurred at the origin of the clade comprising *Heptacodium* and Linnina, and a second similar shift took place along the branch leading to *Symphoricarpos* and *Triosteum*. These shifts led to fruits with fewer seeds, a single seed in *Heptacodium* and Linnina, two seeds in *Symphoricarpos* and three seeds in *Triosteum*.

Number of endocarp layers

Based on SCM, the plesiomorphic condition for Dipsacales is the presence of three endocarp layers (PP 0·99), whereas PO hypothesizes two endocarp layers as the plesiomorphic condition for the order. With SCM in mind, a reduction to a single (unsclerified) layer occurred along the branch leading to *Leycesteria* and *Lonicera*. A similar shift took place at the origin of the clade containing *Heptacodium* and Linnina. *Viburnum* and the *Diervilla* clade are marked by fruits with two endocarp layers. When *Heptacodium* is considered sister to the the *Lonicera* clade, the shift to a single layer took place after the *Diervilla* clade separated from the rest of Caprifoliaceae.

Fleshy fruits

Based on PO, it is unclear whether the ancestor of Dipsacales had fleshy fruits or not. However, SCM provides

strong support for the ancestral condition for the order being a fleshy fruit (PP 1·00). Adoxaceae (except for *Sinadoxa*) and the *Lonicera* clade have fleshy fruits. Dry fruits are found in the *Diervilla* clade, Linnina and *Heptacodium*. When the shift toward dry fruits took place is unclear, and it is likely that multiple shifts took place.

Anatomy of seed coat

Although both PO and SCM are unclear about the plesiomorphic condition for Dipsacales, an evolutionary pattern within the order is obvious. The ancestor of Caprifoliaceae is hypothesized to have had seeds with a sclerified exotesta and endotesta (PP 0·99), whereas the branch leading to *Heptacodium* and Linnina is marked by a shift to a compressed, parenchymatous seed coat (PP 0·99). In Adoxaceae, *Adoxa* and *Viburnum* have uncompressed, parenchymatous seed coats, whereas seeds of *Sambucus* have a compressed, parenchymatous seed coat (Jacobs *et al.*, 2008). If we assume *Heptacodium* is sister to the *Lonicera* clade, the plesiomorphic condition for Caprifoliaceae and the clade holding the *Lonicera* clade and *Heptacodium* is equivocal.

Embryo size

In Dipsacales, a trend toward a larger embryo is apparent. Although not significantly supported by SCM (PP 0·84), based on PO the plesiomorphic condition for the order is an embryo less than 3/4 of seed length. Three shifts toward a larger embryo took place: (1) a first shift to an embryo larger than 3/4, but not occupying the entire seed (endosperm present) occurred at the origin of *Sambucus*; (2) a similar shift happened at the origin of the *Dipsacus* clade; and (3) a third shift toward an embryo occupying the entire seed (no endosperm present) characterizes the *Valeriana* clade. Although the character state of *Sambucus* and the *Dipsacus* clade is coded identically, embryo morphology is quite different. *Sambucus* has a long, slender, cylindrical embryo, whereas members of the *Dipsacus* clade have a comparatively larger embryo, which is slightly flattened dorsiventrally.

Conclusions

The *Diervilla* clade is formed of two genera, as confirmed by several features including the presence of capsules, a sclerified exo- and endotesta, and dehiscent fruits. Fruit and seed morphology and anatomy support a sister relationship between *Leycesteria* and *Lonicera* and between *Symphoricarpos* and *Triosteum*. The monophyly of the *Lonicera* clade is supported by several features including a sclerified exo- and endotesta and fleshy fruits. Our results also support the hypothesis of *Heptacodium* being sister to the Linnina clade, rather than to the *Lonicera* clade.

We believe the first Dipsacales had pentacarpellate, fleshy fruits with numerous (>20) seeds, characterized by sclerified seed coats (whether both exo- and endotesta were sclerified is unclear) and small embryos (less than 3/4 of seed length). A shift to bicarpellate capsules with numerous seeds took place along the branch leading to the *Diervilla* clade, which coincided with a shift to dry, dehiscent fruits. This hypothesis implies that the fruit of *Leycesteria* has all the characteristic features of a primitive dipsacalean fruit (Wilkinson, 1949). The main difference with *Lonicera* is the occurrence of sterile ovules in the latter. Within the *Lonicera* clade, a shift to one or two sterile carpels occurred along the branch leading to *Symphoricarpos* and *Triosteum*. This coincided with the sclerification of the endocarp, which means this sclerification occurred independently of the sclerification at the origin of Adoxaceae. Finally, a second shift to dry fruits together with a shift to three carpels (of which two are sterile) took place at the origin of the clade comprising *Heptacodium* and Linnina. This evolution occurred simultaneously with the maturing of a single ovule in the only fertile carpel.

Future prospects

A future study will deal with the evolution of fruits and seeds of the order Dipsacales in more detail. Such a study will need to include members of the sister group of Dipsacales in order to address more accurately questions related to the plesiomorphic character states of the order. Furthermore, an expanded sampling of the Linnina clade is required for the construction of a more highly resolved evolutionary map.

SUPPLEMENTARY DATA

Supplememtary data are available online at www.aob.oxfordjournals.org and consists of a figure with the constraints used in the analysis, and the raw results of the stochastic character-mapping analysis.

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APPENDIX 1

Classification and collection details of sampled species

Taxon	Classification	Herbarium, seed bank or locality[1]	Collector	Accession	Type[2]
Abelia chinensis R. Br.	*Linnaea* clade	National Botanic Garden of Belgium, Belgium	Jacobs B.	19881652	F
Abelia parvifolia Hemsl.	*Linnaea* clade	National Botanic Garden of Belgium, Belgium	Jacobs B.	19850252	F
Adoxa moschatellina L.	Adoxaceae	Heverlee, Belgium	Jacobs B.	290	F
Centranthus ruber (L.) DC.	*Valeriana* clade	Botanical Garden of Jena, Germany	NA	5471	S
Diervilla rivularis Gatt.	*Diervilla* clade	Botanical Garden of Jena, Germany	NA	2079	S
Diervilla sessilifolia Buckley	*Diervilla* clade	L	Boom B.K.	6117	H
Dipelta floribunda Maxim.	*Linnaea* clade	Shangai Botanical Garden, Shangai, China	NA	79	S
Dipelta yunnanensis Franch.	*Linnaea* clade	National Botanic Garden of Belgium, Belgium	Jacobs B.	19921864-04	F
Dipsacus fullonum L.	*Dipsacus* clade	Utrecht University Botanic Garden, Utrecht, The Netherlands	NA	1991ZE00259	S
Heptacodium miconioides Rehder	–	Arboretum Kalmthout, Belgium	Jacobs B.	19990134	F
Kolkwitzia amabilis Graebn.	*Linnaea* clade	Institute of Ecology and Botany of the Hungarian Academy of Sciences, Hungary	NA	562	S
Leycesteria crocothyrsos Airy/Shaw	*Lonicera* clade	L	–	C1123A	H
Leycesteria formosa Wall.	*Lonicera* clade	L	Boom B.K.	956062836	H
Linnaea borealis L.	*Linnaea* clade	Linnaeus Garden, Uppsala University, Uppsala, Sweden	Hansson L.	HL20080001	F
Lonicera alpigena L.	*Lonicera* clade	Botanical Garden of Jena, Germany	NA	2081	S
Lonicera canadensis Bartr. ex Marshall	*Lonicera* clade	L	Senn H.A. & Zinck M.N.	420	H
Lonicera caprifolium L.	*Lonicera* clade	L	Sotiaux P.	811162	H
Lonicera chrysantha Turcz.	*Lonicera* clade	Institute of Ecology and Botany of the Hungarian Academy of Sciences, Hungary	NA	505	S
Lonicera dioica L.	*Lonicera* clade	L	Moldenke H.N.	17763	H
Lonicera etrusca Santi	*Lonicera* clade	L	–	9991	H
Lonicera implexa Aiton	*Lonicera* clade	L	De Langhe J.E.	973727	H
Lonicera involucrata Banks ex Spreng.	*Lonicera* clade	L	Grant J.M.	502710	H
Lonicera japonica Thunb.	*Lonicera* clade	L	Koyama H.	2906	H
Lonicera javanica DC.	*Lonicera* clade	L	Afriastini J.J.	–	H
Lonicera maackii (Rupr.) Herder	*Lonicera* clade	Botanical Garden of Jena, Germany	NA	2083	S
Lonicera maximowiczii Maxim.	*Lonicera* clade	St. Andrews Botanic Garden, Great Britain	NA	80	S
Lonicera muscaviensis Rehder	*Lonicera* clade	L	Boom B.K.	902710	H
Lonicera vesicaria Kom.	*Lonicera* clade	Institute of Ecology and Botany of the Hungarian Academy of Sciences, Hungary	NA	571	S
Lonicera xylosteum L.	*Lonicera* clade	Botanischer Garten Krefeld, Germany	NA	80	S
Morina longifolia Wall.	*Morina* clade	Cruickshank Botanic Garden, Great Britain	NA	52	S
Morina persica L.	*Morina* clade	St. Andrews Botanic Garden, Great Britain	NA	265	S
Sambucus ebulus L.	Adoxaceae	Institute of Ecology and Botany of the Hungarian Academy of Sciences, Hungary	NA	2001-228	S
Sambucus racemosa L.	Adoxaceae	Botanische Garten der Universität Hamburg, Germany	NA	297	S
Scabiosa columbaria L.	*Dipsacus* clade	Institute of Ecology and Botany of the Hungarian Academy of Sciences, Hungary	NA	862	S
Succisa pratensis Moench.	*Dipsacus* clade	Botanical Garden of Nantes, France	NA	187	S

Continued

APPENDIX 1 *Continued*

Taxon	Classification	Herbarium, seed bank or locality[1]	Collector	Accession	Type[2]
Symphoricarpos albus (L.) S.F. Blake *var. laevigatus* (Fernald) G.N. Jones	*Lonicera* clade	Botanical Garden of Ljubljana, Slovenia	NA	191	S
Symphoricarpos mollis Nutt.	*Lonicera* clade	St. Andrews Botanic Garden, Great Britain	NA	190/1964	S
Symphoricarpos occidentalis Hook.	*Lonicera* clade	St. Andrews Botanic Garden, Great Britain	NA	191/1964	S
Symphoricarpos oreophilus Gray	*Lonicera* clade	St. Andrews Botanic Garden, Great Britain	NA	192/1964	S
Triosteum angustifolium L.	*Lonicera* clade	L	Wilhelm N. Suksdorf	899	H
Triosteum hirsutum Roxb.	*Lonicera* clade	L	J. D. H.	202710	H
Triosteum perfoliatum L.	*Lonicera* clade	L	–	845875	H
Triosteum sinuatum Maxim.	*Lonicera* clade	L	Shimizu & N. Fukuoka	499	H
Valeriana officinalis L.	*Valeriana* clade	National Botanic Garden of Belgium, Belgium	NA	19721065	S
Valerianella locusta (L.) Betcke	*Valeriana* clade	National Botanic Garden of Belgium, Belgium	NA	19922077-23	S
Viburnum acerifolium L.	Adoxaceae	St. Andrews Botanic Garden, Great Britain	NA	84	S
Viburnum plicatum var. tomentosum (Thunb.) Miquel	Adoxaceae	L	Fukuoka N.	972060502	H
Weigela floribunda C. A. Mey.	*Diervilla* clade	Botanical Garden of Jena, Germany	NA	9262	S
Weigela florida DC.	*Diervilla* clade	National Botanic Garden of Belgium, Belgium	Jacobs B.	19392515	F
Weigela hortensis C. A. Mey.	*Diervilla* clade	Botanical Garden of Ljubljana, Slovenia	NA	192	S
Weigela japonica Thunb.	*Diervilla* clade	Botanical Garden of Jena, Germany	NA	6240	S
Weigela subsessilis L. H. Bailey	*Diervilla* clade	National Botanic Garden of Belgium, Belgium	Jacobs B.	19931547-84	F

[1] For herbarium material the particular herbarium (acronym) is indicated; for seed bank material the particular institute is indicated; for fresh material the particular locality or botanic garden is indicated.

[2] H, herbarium; S, seed bank; F, fresh material.

na, not applicable; –, missing data.

APPENDIX 2

Voucher and accession details of sampled species

Taxon	Collection and voucher information	*ITS*	*matK*	*trnK*
Abelia chinensis	Sir Harold Hillier Gardens and Arboretum, N. Pyck 1989-2220	**FJ745388**	AY310461	–
Abelia parvifolia	Botanical Garden of Copenhagen, N. Pyck 1943-5025	**FJ745387**	**FJ745398**	–
Adoxa moschatellina	NA	U88194	EF490235	EF490235
Centranthus ruber	Institute of Botany and Microbiology, N. Pyck 001	**FJ745391**	AF446926	AY794313
Diervilla sessilifolia	National Botanical Garden of Belgium, N. Pyck 82-6494	AY236177	AF446907	**FJ745402**
Dipelta floribunda	Sir Harold Hillier Gardens and Arboretum, N. Pyck 1978-4099	**FJ745389**	**FJ745399**	–
Dipelta yunnanensis	NA	AY236180	AF446910	AY290042
Dipsacus fullonum	National Botanical Garden of Belgium, N. Pyck 80-1959	**FJ745390**	**FJ745400**	–
Heptacodium miconioides	National Botanical Garden of Belgium, N. Pyck 92-0130-16	AY236176	AF446906	**FJ745412**
Kolkwitzia amabilis	National Botanical Garden of Belgium, DDM/88/ 0215FB/R67	AY236182	AF446912	**FJ745413**
Leycesteria crocothyrsos	Sir Harold Hillier Gardens and Arboretum, N. Pyck 1992-1691	AF265277	**FJ745393**	**FJ745406**
Leycesteria formosa	National Botanical Garden of Belgium, N. Pyck 82-6395	AF265276	AF446902	**FJ745405**
Linnaea borealis	NA	AY236181	AF446911	AY290040
Lonicera dioica	National Botanical Garden of Belgium, N. Pyck 51-3590	EU240713	**FJ745395**	–

Continued

APPENDIX 2 *Continued*

Taxon	Collection and voucher information	*ITS*	*matK*	*trnK*
Lonicera involucrata	National Botanical Garden of Belgium, N. Pyck 53-6481	**FJ745386**	**FJ745397**	**FJ745408**
Lonicera maackii	National Botanical Garden of Belgium, N. Pyck 88-1731	FJ217883	**FJ745394**	**FJ745407**
Lonicera maximowiczii	National Botanical Garden of Belgium, N. Pyck 81-1860	**FJ745385**	**FJ745396**	–
Lonicera xylosteum	NA	EU240714	AM503819	–
Morina longifolia	NA	AY236185	AF446915	AY290020
Sambucus ebulus	NA	DQ521256	EF490239	EF490239
Scabiosa columbaria	NA	AY236188	AF446918	AY290032
Succisa pratensis	National Botanical Garden of Belgium, N. Pyck 1975-2365	AY290018	**FJ745401**	AY290033
Symphoricarpos albus	Kasteelpark Arenberg, P. Roels 004	AF265282	AY310459	**FJ745410**
Symphoricarpos occidentalis	National Botanical Garden of Belgium, N. Pyck 90-1416	FJ217824	–	**FJ745411**
Sambucus racemosa	NA	AY236171	AY265204	AY265204
Triosteum perfoliatum	Botanical Garden Uppsala University, N. Pyck 1963-1028	AY236175	AF446905	**FJ745409**
Valeriana officinalis	NA	DQ180745	AY310467	AY794362
Valerianella locusta	NA	DQ354168	AF446922	AY794398
Viburnum acerifolium	NA	AY265114	AF446897	AY265160
Viburnum plicatum	NA	AY265143	AY265189	AY265189
Weigela florida	National Botanical Garden of Belgium, N. Pyck 51-0632	AF078711	**FJ745392**	**FJ745404**
Weigela subsessilis	National Botanical Garden of Belgium, N. Pyck 93-1547-84	AF078706	–	**FJ745403**

Boldface accessions refer to sequences obtained for this study.
na, not applicable; –, missing data.