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Molecular systematics of *Gagea* and *Lloydia* (Liliaceae; Liliales): implications of analyses of nuclear ribosomal and plastid DNA sequences for infrageneric classification

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• *Background and Aims Gagea* is a Eurasian genus of petaloid monocots, with a few species in North Africa, comprising between 70 and approximately 275 species depending on the author. *Lloydia* (thought to be the closest relative of *Gagea*) consists of 12–20 species that have a mostly eastern Asian distribution. Delimitation of these genera and their subdivisions are unresolved questions in Liliaceae taxonomy. The objective of this study is to evaluate generic and infrageneric circumscription of *Gagea* and *Lloydia* using DNA sequence data.

• *Methods* A phylogenetic study of *Gagea* and *Lloydia* (Liliaceae) was conducted using sequences of nuclear ribosomal internal transcribed spacer (ITS) and plastid (*rpl16* intron, *trnL* intron, *trnL-F* spacer, *matK* and the *psbA-trnH* spacer) DNA regions. This included 149 accessions (seven as outgroups), with multiple accessions of some taxa; 552 sequences were included, of which 393 were generated as part of this research.

• *Key Results* A close relationship of *Gagea* and *Lloydia* was confirmed in analyses using different datasets, but neither *Gagea* nor *Lloydia* forms a monophyletic group as currently circumscribed; however, the ITS and plastid analyses did not produce congruent results for the placement of *Lloydia* relative to the major groups within *Gagea*. *Gagea* accessions formed five moderately to strongly supported clades in all trees, with most *Lloydia* taxa positioned at the basal nodes; in the strict consensus trees from the combined data a basal polytomy occurs. There is limited congruence between the classical, morphology-derived infrageneric taxonomy in *Gagea* (including *Lloydia*) and clades in the present phylogenetic analyses.

• *Conclusions* The analyses support monophyly of *Gagea*/*Lloydia* collectively, and they clearly comprise a single lineage, as some previous authors have hypothesized. The results provide the basis for a new classification of *Gagea* that has support from some morphological features. Incongruence between plastid and nuclear ITS results is interpreted as potentially due to ancient hybridization and/or paralogy of ITS rDNA.

Key words: *Gagea*, *Lloydia*, Liliaceae, ITS ribosomal DNA sequences, plastid DNA sequences.

INTRODUCTION

Gagea (Liliaceae) is a geophytic, perennial, largely Eurasian genus with a few species in North Africa; it comprises somewhere between 70 and approx. 275 species, depending on the author (Stroh, 1937; Uphof, 1958–1960; Melchior, 1964; Willis, 1980; Hyam and Pankhurst, 1995; Mabberley, 1997; Levichev, 1999; Peruzzi, 2003; Govaerts, 2006; Zarrei *et al.*, 2007; Peterson *et al.*, 2008). At the time of original publication (Salisbury, 1806), the genus contained only seven species; these had long been placed in *Ornithogalum* L. (Hyacinthaceae; e.g. Gerard, 1663; Linnaeus, 1753, 1762; Pallas, 1773, 1776). Salisbury did not take into consideration priority of the names he used, and this was a source of confusion for later authors (Heyn and Dafni, 1971). Many novel species have been added, particularly in the last two decades (Levichev, 1981, 1988, 1991, 2000, 2001, 2006*a*; Dasgupta and Deb, 1983; Rechinger, 1986; Levichev and Navruzshoev, 1997; Tison, 2004; Zhao and Zhao, 2004; Henker, 2005; Zarrei and Zarre, 2005*a*; Ali, 2006; Levichev and Ali, 2006; Zhao and Yang, 2006; Peruzzi *et al.*, 2007; Hamzaoğlu *et al.*, 2008), but a comparative systematic study is needed to elucidate relationships of species and species groups and to clarify taxonomic boundaries.

The first attempt to classify species within *Gagea* was carried out by Koch (1849), who divided the genus into two sections, *Holobolbos* K.Koch and *Didymobolbos* K.Koch, and recognized 17 species; this was followed by the addition of sections *Tribolbos* Boiss. and *Platyspermum* Boiss. (Koch, 1882; Table 1). At the beginning of the 20th Century, Terracciano (1905*a*, *b*, 1906) and Pascher (1904, 1907) made major contributions by revising the Asiatic species. Both authors erected independent classifications nearly simultaneously. Pascher (1904; Table 1) classified the species of *Gagea* into two subgenera, *Gagea* (*Eugagea* Pascher, which is properly subgenus *Gagea*; McNeill *et al.*, 2006) with four sections and *Hornungia* (Bernh.) Pascher with two sections, based mainly on seed shape and bulb characters. Three years later, Pascher (1907) published a more extensive treatment with the addition of new species and complete Latin

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TABLE 1. *Overview of previous infrageneric classifications of* Gagea

Boissier (1882)	Terracciano (1905*a*, *b*)	Pascher (1904, 1907)*	Grossheim (1935)	Davlianidze (1976)	Levichev (1990, 1999b)[†]
Gagea	*Gagea*	*Gagea*	*Gagea*	*Gagea*	*Gagea*
	Subgenus	**Subgenus**	**Subgenus**	**Subgenus**	
	Gagea	*Gagea* (= *Eugagea*)	*Gagea*	*Gagea*	
Section	**Section**	**Section**	**Section**	**Section**	**Section**
Gagea	*Gagea*	*Gagea*	*Gagea*	*Gagea*	*Gagea*
(= *Holobolbos*)	(=Nudiscaposae)	(*Holobolbos*)	(=*Nudiscaposae*)		
Tribolbos		*Tribolbos*			
		Monophyllos			
		Subsection			
		Minimae		*Minimae*	*Minimae*
					Spathaceae
		Fistulosae		*Fistulosae*	*Fistulosae*
Didymobolbos	*Foliatae*	*Didymobolbos*	*Foliatae*	*Didymobolbos*	*Didymobolbos*
	Gageastrum	*Hornungia*			
Platyspermum	*Verticillatae*	*Platyspermum*	*Platyspermum*	*Platyspermum*	*Platyspermum*
					Graminifoliae
					Incrustatae
					Bulbiferae
		Stipitatae		*Stipitatae*	*Stipitatae*
					Dschungaricae
		Plecostigma	*Plecostigma*		*Plecostigma*
	Anthericoides				*Anthericoides*

The classifications are complete only at subgeneric and sectional levels. The only subsections listed are those later raised to section level by Davlianidze (1976).

* Stroh (1937) and Uphof (1958–1960) followed the same classification as Pascher (1904, 1907). See text for details.

[†] Levichev's classification (1990, 1999*b*, 2006*b*) has been updated by Levichev in Peterson *et al.* (2008).

descriptions, but he maintained his previous infrageneric taxa with some additional subsections (Table 1). Terracciano (1905*a*, *b*, 1906) also adopted two subgenera, *Gagea* and *Gageastrum*, with two sections under each (Table 1). Neither author produced a complete revision. However, Pascher's treatment was favoured by several authors, including Stroh (1937), Uphof (1958–1960) and Rechinger (1986). Two major monographic classifications of *Gagea* were produced by Stroh (1937) and Uphof (1958–1960), with 124 and 106 species names, respectively, each representing an updated version of Pascher's (1907) classification. Following the classification of Pascher (1904, 1907), Stroh and Uphof divided *Gagea* into his subgenera, sections and subsections. Because many new descriptions have since appeared, particularly from the Middle East and Central Asia, the species treatments of Stroh (1937) and Uphof (1958–1960) are now substantially out of date. Although there has been no attempt to revise them completely, there are some more recent regional classifications. For example, after studying morphology and karyology, Davlianidze (1976) treated 26 species present in the Caucasus and accepted the two subgenera previously followed by Uphof (1958) and Stroh (1937); he also established six new sections within each of those subgenera (Table 1). Levichev (1990) published a new classification of the western Tien Shan species using general morphological features as well as cross-sections of radical leaves and stem-base characters. Levichev (1990) did not use the subgeneric rank and divided the genus into ten sections, some of which were the same as those of Davlianidze (1976). Apart from Davlianidze (1976) and Levichev (1990), who published new classifications, several authors (Heyn and Dafni, 1971, 1977; Dasgupta and Deb, 1983; Feinburn-Dotham, 1986; Wendelbo and Rechinger, 1990; Federov, 2001; Grubov and Egorova, 2003) have accepted the general outline of Pascher's infrageneric classification (1904, 1907) with few modifications. However, recent papers such as Zarrei and Zarre (2005*a*, *b*), Peruzzi *et al.* (2008*a*, *b*) and Peterson *et al.* (2008) have instead referred to Levichev's classification (Levichev, 1990).

Lloydia Salisb., a small bulbiferous herb from the temperate Northern Hemisphere, has always been considered the closest relative of *Gagea. Lloydia* consists of 12–20 species (Willis 1980; Hyam and Pankhurst, 1995; Mabberley, 1997; Govaerts, 2006) that have a mostly eastern Asian distribution. The only species that occurs in Europe is *L. serotina* (L.) Rchb., which is also distributed in western North America (Phillips and Rix, 1989). It is a protected species in Britain and has some ornamental use, unlike most species of *Gagea. Lloydia* replaces *Gagea* in the Himalayas and adjoining areas, although some species of *Gagea* reach Japan [e.g. *G. lutea* (L.) Ker-Gawl]. The taxonomic status of *Lloydia* has been problematic since its description by Salisbury and validation by Reichenbach in 1830 (Dasgupta and Deb, 1986). Many species have been moved between *Gagea* and *Lloydia* in the last two centuries. For example, *L. libanotica* Hochst. and *L. graeca* (L.) Endl. ex. Kunth are now known as *G. libanotica* (Hochst) Greuter and *G. graeca* (L.) Irmisch, respectively (Greuter, 1970).

The study of core Liliales conducted by Patterson and Givnish (2002) using a combined sequence matrix of plastid *rbcL* and *ndhF* genes (with only one accession each from *Gagea* and *Lloydia*) showed that these two are sister taxa in a highly supported clade [bootstrap percentage (BP) 100]. Rønsted *et al.* (2005) produced the same result for *Gagea* *wilczekii* (= *G. algeriensis*) and *Lloydia serotina* using a different plastid dataset (*matK*, *trnK* intron). Another analysis of *psbA-trnH* and *trnL-F* sequence data (Peterson *et al.*, 2004) confirmed the monophyly of seven species of *Gagea* and *Lloydia serotina* from Germany, with BPs of 99 and 100 using different datasets.

Analysis based on phenotypic (morphological) data (Patterson and Givnish, 2002) resulted in a weakly supported clade (BP 56) containing *Gagea* and *Lloydia*. This low support may be because they included all genera of Liliales and did not include additional characters that are specific to tribe Tulipeae, in which both *Gagea* and *Lloydia* are placed.

A detailed examination of pollen morphology of Iranian representatives of *Gagea* (Zarrei and Zarre, 2005*b*) revealed that sculpturing of the exine provides valuable characters for separation of species, sometimes even closely related ones, and delimitation of natural groups within the genus. Zarrei and Zarre (2005*b*) distinguished four basic pollen types within *Gagea*.

Chromosome counts for 100 taxa have been reported (Peruzzi, 2003, 2008; Peruzzi and Aquaro, 2005). The base chromosome number is $x = 12$ among species of known chromosome number, and 37·8 % of the studied species have this number and are diploid (Peruzzi, 2003). Chromosome studies suggested that asymmetric karyotypes are an ancestral feature, whereas more balanced ones are derived (Peruzzi and Aquaro, 2005), but this hypothesis needs reconsideration within a phylogenetic framework.

Molecular phylogenetic studies (mostly of plastid DNA) that have included single exemplars of *Gagea* [*Gagea wilczekii* Braun-Blanq. & Maire (= *Gagea algeriensis* Chabert)] and *Lloydia* (*L. serotina*) support a close relationship of the genera, but provide no insight into generic circumscription (Kosenko and Levichev, 1988; Kosenko, 1999; Fay and Chase, 2000; Patterson and Givnish, 2002; Rønsted *et al.*, 2005). A molecular phylogenetic study of seven *Gagea* species from Germany was undertaken by Peterson *et al.* (2004), using plastid DNA sequences (*trnL* intron, *trnL-F* spacer and the *psbA-trnH* spacer) and the nuclear the internal transcribed spacer (ITS) ribosomal region. In this analysis, *L. serotina* was used initially as outgroup, but it was placed among the *Gagea* species in all analyses of plastid data. Subsequent analyses that included morphological data also cast doubt on the validity of maintaining *Lloydia* and *Gagea* as distinct genera (Peterson and Peterson, 2005, 2006). Combined analyses of plastid and ITS DNA demonstrated that *G.* section *Didymobolbos* forms a clade with *G.* section *Monophyllos sensu* Pascher (in particular with *G.* section *Minimae* and *G.* section *Euspathaceae sensu* Levichev) and that *G.* section *Gagea* (*Holobolbos sensu* Pascher) forms a clade with *G.* section *Tribolbos*. Indeed, the last section has been merged by recent authors with *G.* section *Gagea* (Levichev, 1990; Peruzzi & Aquaro, 2005; Peruzzi *et al.*, 2007). Molecular and morphological study of *Gagea* and *Lloydia* has been conducted by Peterson *et al.* (2008), revealing a close relationship between these two genera and further undermining the concept of *Lloydia* as a distinct genus. Moreover, these studies broadly supported Levichev's classification (Levichev, 1990). Peruzzi *et al.* (2008*a*) proposed the genus *Lloydia* as a section within *Gagea*. A detailed phylogenetic study of *Gagea* species in Italy has also been conducted by Peruzzi *et al.* (2008*b*).

To further evaluate phylogenetic relationships of *Lloydia* and *Gagea* and the infrageneric classification of *Gagea*, nuclear (ITS) and plastid (*rpl16* intron, *trnL* intron, *trnL-F* spacer, *matK* and the *psbA-trnH* spacer) DNA data are used here. We have included species representing as many morphologically based species-groups of *Lloydia* and *Gagea* as possible and also aimed for broad geographical sampling of species. The phylogenetic analyses include previously published DNA data and address relationships between and within the two genera; the results are also compared with previous classifications of *Gagea*.

MATERIALS AND METHODS

Plant material

Silica gel-preserved samples of leaf tissue from field collections and, in a few cases, herbarium specimens were used for DNA extraction (see Appendix for source information). The ingroup comprised 142 accessions. In all analyses, *Tulipa clusiana* DC., *T. lehmanniana* Merckl., *T. uniflora* (L.) Besser ex Baker, *Amana erythronioides* (Baker) D.Y.Tan & D.Y.Hong, *Erythronium japonicum* Decne., *Fritillaria persica* L. and *Lilium ledebourii* (Baker) Boiss. served as outgroups, based on the results of Rønsted *et al.* (2005).

DNA extraction, marker amplification and sequencing

Genomic DNA extractions were performed using 0·01–0·23 g of silica-dried leaves or 0·01–0·08 g of leaf tissue from herbarium sheets and a modified version of the 2× CTAB method of Doyle and Doyle (1987). Before precipitation, an aliquot of 150 μL was purified using the NucleoSpin Extract II PCR purification kit (Machery-Nagel, GmbH & Co. KG, Düren, Germany) following the manufacturer's protocols; this provided a small amount of DNA that was able to be used the same day for amplification. The remainder of the DNA was precipitated in 2·5 volumes ethanol (for herbarium specimens, 2/3 volume isopropanol was used instead of ethanol). DNA samples were then purified using a caesium chloride/ethidium bromide gradient (1·55 g mL^{-1}) followed by removal of the ethidium bromide with butanol, dialysis and storage at −80 °C in the DNA Bank at the Royal Botanic Gardens, Kew (http://www.data.kew.org/dnabank/homepage.html).

Amplification of the *psbA-trnH* spacer was undertaken using previously published primers for *psbA* (Sang *et al.*, 1997) and *trnH* (Tate and Simpson, 2003). Owing to the small size of this fragment (339–438 bp), only the *psbA* primer was used for sequencing, unless there were ambiguities that needed resolving in the single electropherogram produced. Amplification of the *rpl16* intron was carried out using the primers 71F and 1661R of Jordan *et al.* (1996). In many cases the internal primer 158F, designed originally for palms (5′-AAGAA ACAGTCACTATATGA-3′; C. Asmussen, University of Copenhagen, unpubl. res.), was used to avoid a long region of T/A, which interfered with sequencing at the beginning of the *rpl16* intron. For degraded DNA from herbarium material, two internal primers were designed for this project, 576F (5′-GATGGCGGAATGAACCAAGA-3′) and 657R (5′-GTT TCGCGGGCGAATAT TGACT-3′), and both were used to amplify the *rpl16* intron in two pieces, in this case 71F + 657R and 576F + 1661R. These primers were also used for sequencing.

The *trnL-F* region (including *trnL* intron and *trnL-F* spacer) was amplified with primers *c* and *f* of Taberlet *et al.* (1991). In some older herbarium material, the *trnL-F* region was amplified in two pieces using primers *d* and *e* designed by Taberlet *et al.* (1991; *c* + *d* and *e* + *f*).

In a similar way, *matK* was amplified in two pieces using primers 19F (Molvray *et al.*, 2000) and 1326R (Sun *et al.*, 2001) for the first piece and 390F (Sun *et al.*, 2001) and 1565R (5′-TCACCAGGTCATTGACACGAA-3′), which we designed for this study. In three cases, 2R (Johnson and Soltis, 1994) was used instead for the reverse primer. For sequencing, 19F and 1326R were used for the first fragment, but only 1565R was used for the second piece because of the large degree of overlap of the two fragments.

Amplification of the ITS region of 18S–26S nuclear ribosomal DNA was carried out using primers 17SE and 26SE of Sun *et al.* (1994). Primers ITS2, ITS3, ITS4 and ITS5 (White *et al.*, 1990) were used for herbarium material to amplify ITS in two pieces (ITS5 + ITS2, ITS3 + ITS4). DMSO (2 %; dimethylsulfoxide) was added to reduce secondary structure problems common in ITS (Winship, 1989; Baldwin *et al.*, 1995; Chase *et al.*, 2003). In all cases, amplified products were purified using NucleoSpin PCR purification columns in accordance with the manufacturer's protocols. Cycle sequencing reactions were performed using the BigDye Terminator Kit ver. 3.1 (Applied Biosystems, Inc., ABI, Warrington, UK). Cycle sequencing products were cleaned using Magnesil (Promega product, Southampton, UK) on a Beckman Coulter robot (Biomek NX S8, Buckinghamshire, UK) following the manufacturer's protocols. Cleaned products were then sequenced on an ABI 3730 following the manufacturer's protocols.

Sequence alignment and phylogenetic analysis

For this paper, 393 new sequences were generated; 159 sequences of ITS, *psbA-trnH* and *trnL-F* intergenic spacer (IGS) of some taxa were downloaded from GenBank (http://www.ncbi.nlm.nih.gov). GenBank accession numbers for all sequences are listed in the Appendix. New DNA sequences were edited and assembled using Sequence Navigator ver. 1.0 and Autoassembler ver. 1.4.0 (ABI), respectively. All sequences were easily aligned by eye using PAUP v. 4.0b10 for Macintosh (Swofford, 2002), following the guidelines of Kelchner (2000). The matrices are available as NEXUS files upon email request from M.W.C. or M.Z. Parsimony analyses were undertaken using PAUP v. 4.0b10 for Macintosh (Swofford, 2002). All changes were assessed as unordered and were equally weighted (Fitch parsimony; Fitch, 1971).

The data were analysed in three steps. First (analysis I; results not shown), all data were analysed as separate plastid regions. Species for which sequences were taken from GenBank had significant missing data for these plastid regions (e.g. no sequences were available for *matK* and *rpl16* intron in GenBank). BPs were low, which is why these are not shown; these analyses were performed to determine the degree to which our plastid sequences and those previously published were in agreement, which they generally were. Then, we ran analyses of newly generated sequences only as separate plastid and ITS matrices (i.e. no separate analyses of the individual plastid regions; analysis II). Thirdly, combined analyses were run for all data (ITS plus plastid regions) generated in this paper plus the sequences downloaded from GenBank (analysis III). We were worried that the amount of missing data would make evaluation of incongruence and internal support difficult; missing data might reduce bootstrap support and thus might conceal hard incongruence (Cameron *et al.*, 2001). Thus, we analysed all data produced only by us as combined plastid and ITS matrices on only the sequences generated for this study, which are with few exceptions (Appendix) complete for each of our accessions (analysis IV).

All searches were conducted using 1000 random taxon-addition replicates, tree-bisection-reconnection (TBR) branch swapping and MulTrees on (i.e. keeping multiple, equally parsimonious trees). Ten trees only were saved from each replicate to reduce search time on potentially thousands of trees. All trees collected were then used as starting trees in another search without a tree limit. Support for clades was estimated using 1000 bootstrap replicates (Felsenstein, 1985), with simple taxon addition, and TBR swapping but permitting only ten trees per replicate to be held. Groups were retained with BP $\geq$ 50. Summary data for all analyses are presented in Table 2.

RESULTS

Analysis I

The trees obtained from separate analyses of the plastid regions of all available datasets show limited resolution, and

TABLE 2. *Tree and matrix statistics related to the various datasets and analysis*

Analyses	No. of positions	No. of variable positions	No. of parsimony-informative positions	No. of trees	Length	CI	RI
ITS	706	312 (44 %)	253 (36 %)	8152	850	0·62	0·89
Plastid	3474	727 (21 %)	507 (15 %)	7620	1115	0·75	0·93
Combined total*	4180	1039 (25 %)	760 (18 %)	7276	1993	0·68	0·91
Combined total†	4180	1088 (26 %)	810 (19 %)	6430	2388	0·62	0·90

* Only newly generated datasets.
† Whole datasets including sequences from GenBank.

therefore these trees are not presented. Statistics from these separate plastid analyses are shown in Table 2.

Analysis II, ITS

Analysis of 78 ITS sequences yielded 8152 equally most-parsimonious trees, each of length (L) = 850 steps, consistency index (CI) = 0·62 and retention index (RI) = 0·89. Tree and matrix statistics are presented in Table 2. One of the most-parsimonious trees was randomly selected and is shown in Fig. 1. *Gagea* and *Lloydia* collectively are supported as monophyletic within Liliaceae (BP 100), but neither is monophyletic (Fig. 1). Species of *Lloydia* are dispersed throughout the tree. *Lloydia serotina* and *L. delicatula* Noltie comprise a well-supported clade (BP 95). *Lloydia flavonutans* and *L. oxycarpa* also form a pair (BP 100). Apart from *Gagea graeca*, which is sister to the rest of the ingroup (BP 94), all other members of *Gagea* form four well-supported clades (clades A–D; BPs = 99–100). These five *Gagea* clades (including *G. graeca*) were recovered in all the different analyses presented here. Bootstrap support for each clade is high in every tree. However, there are a small number of soft incongruences regarding the placement of some constituent taxa of those clades, and these are discussed below.

Analysis II, plastid regions

Analyses of the newly generated data for plastid regions included 87 accessions, of which seven were outgroups (tree and matrix statistics are presented in Table 2). The strict consensus tree with BPs is presented in Fig. 2. The ingroup is strongly supported (BP 100). Neither *Gagea* nor *Lloydia* accessions form monophyletic groups. The topology of the tree differs from that obtained from analysis of the combined matrix (see Fig. 4) regarding the relative positions of *Lloydia* taxa. All *Lloydia* accessions and *G. graeca* form a grade within a larger, moderately supported clade (BP 81; Fig. 2) that also includes clade A. Although the major clades A–D are strongly supported (BP = 90–100), they form a polytomy in the strict consensus tree.

Combined matrix of all datasets including sequences from GenBank (analysis III)

The combined matrix included 135 accessions of *Gagea*, seven of *Lloydia* and seven outgroups species. Tree and matrix statistics are presented in Table 2. Figure 3A and B show one of the most-parsimonious trees selected randomly from 6430 trees. In this analysis, *Lloydia* and *Gagea* together comprise a strongly supported clade (BP 100; Fig. 3A, B). All *G. graeca* accessions are collectively sister to all other accessions of *Lloydia* and *Gagea*, but with low support (BP 59).

The spine of the tree is poorly resolved, but multiple accessions of the same species and groups of closely related species form clades, in some cases with moderate to strong bootstrap support. Clades A, B and D are well supported, but apart from within clade A, taxa within these clades mainly form polytomies. These polytomies usually comprise species that morphology indicates are closely related.

Lloydia species occur in three distinct parts of the trees. *Lloydia serotina* and *L. delicatula* are among a weakly supported polytomy towards the basal nodes of the tree. However, *L. yunnanensis* Franch. is sister to other accessions in clade A (BP 79; Fig. 3A). *Lloydia oxycarpa* Franch. and *L. flavonutans* H.Hara form a clade (BP 100).

Combined matrix of newly generated sequences (analysis IV)

Owing to the amount of missing data for taxa obtained from GenBank, separate analyses were performed using only newly generated sequences (tree and matrix statistics presented in Table 2). Bootstrap percentages (BP $\geq$ 50) are shown on the strict consensus tree (Fig. 4). The tree generally has the same topology as that obtained from analysis of the ITS matrix (Fig. 1). However, there is incongruence between them in the positions of *G. bulbifera* (Pall.) Salisb. and *L. yunnanensis.* Clade support is relatively high in comparison with those in Fig. 3A and B; for example, clade C, which is completely unresolved in Fig. 3B, has a BP of 100 in Fig. 4. Moreover, clade membership is not identical in clade C in all the analyses. However, there is weak support (BP 53) for *G. graeca* being sister to all other members of the ingroup.

DISCUSSION

In the present survey, sequence data were generated from both biparentally (nuclear; Álvarez and Wendel, 2003) and maternally (plastid; Bohdanowicz and Lewandowska, 1999) inherited genomes to reconstruct a phylogenetic tree for *Gagea* and *Lloydia*, controversial and difficult taxa within Liliaceae. For several species, multiple accessions were used to assess intraspecific genetic variation and species delimitation. Consideration was also given to morphological variation within species and geographical distribution when developing the taxon sampling strategy. In many cases, multiple accessions of a single species form clades, usually with high bootstrap support. However, in some species such as *G. setifolia* Baker, accessions are nested in separate clades or interdigitated or unresolved in groupings with accessions of other taxa that have previously been considered to be closely related.

Incongruence of plastid and ITS matrices

Owing to the unreliability of the partition homogeneity test in assessing combinability (Farris *et al.*, 1995) as shown by several authors (Reeves *et al.*, 2001; Yoder *et al.*, 2001; Rønsted *et al.*, 2005), incongruence between the plastid and ITS data was investigated by comparing the combined results (Figs 3 and 4) with the those of the separate analyses (Figs 1 and 2) with respect to level of resolution and bootstrap support. Although *G. bulbifera* (Pall.) Salisb. accessions form a strongly supported group with clade C (BP 100) using ITS sequence data (Fig. 1), it does not have support as sister to clade C in analyses of the plastid sequences (Fig. 2). In the combined analysis of all data, this species forms a polytomy with clades B–D (Fig. 4). Thus. its position in the plastid tree is less resolved. We interpreted this as soft rather than a hard incongruence between the ITS rDNA and plastid DNA data, which could be resolved by incorporating more data,

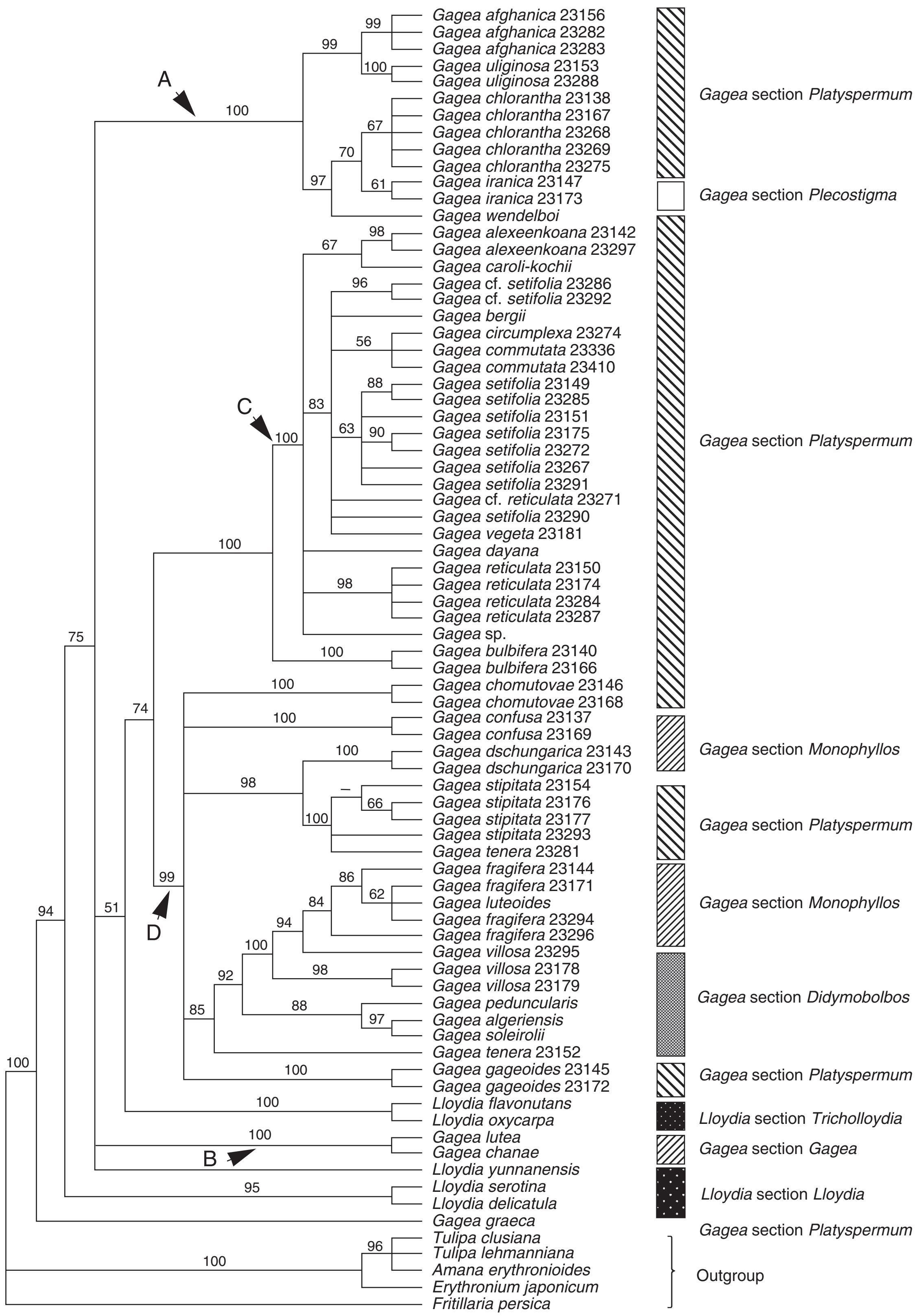

FIG. 1. Bootstrap percentages (≥50) are indicated above branches on the strict consensus tree of 8152 trees obtained from analysis of nuclear ribosomal ITS (sequences from GenBank are excluded). Branches with a hyphen have BP < 50.

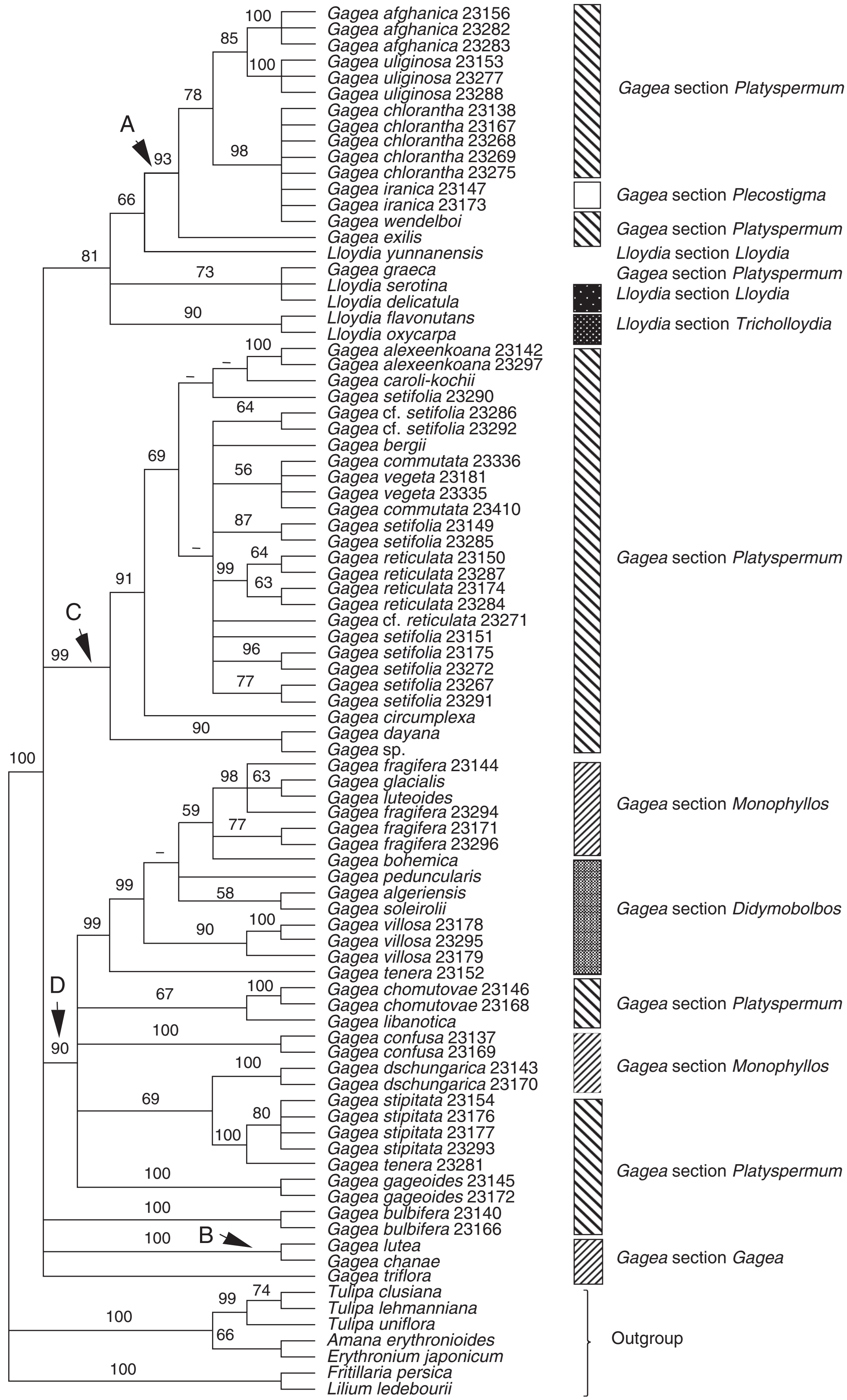

FIG. 2. Bootstrap percentages (≥50) are indicated above branches on the strict consensus tree of 7620 trees obtained from analysis of the combined plastid data matrix (*rpl16* intron, *trnL* intron, *trnL-F* spacer, *matK* and the *psbA-trnH* spacer; sequences from GenBank are excluded). Branches with a hyphen have BP < 50. Pascher's classification (1904, 1907) is shown next to the tree.

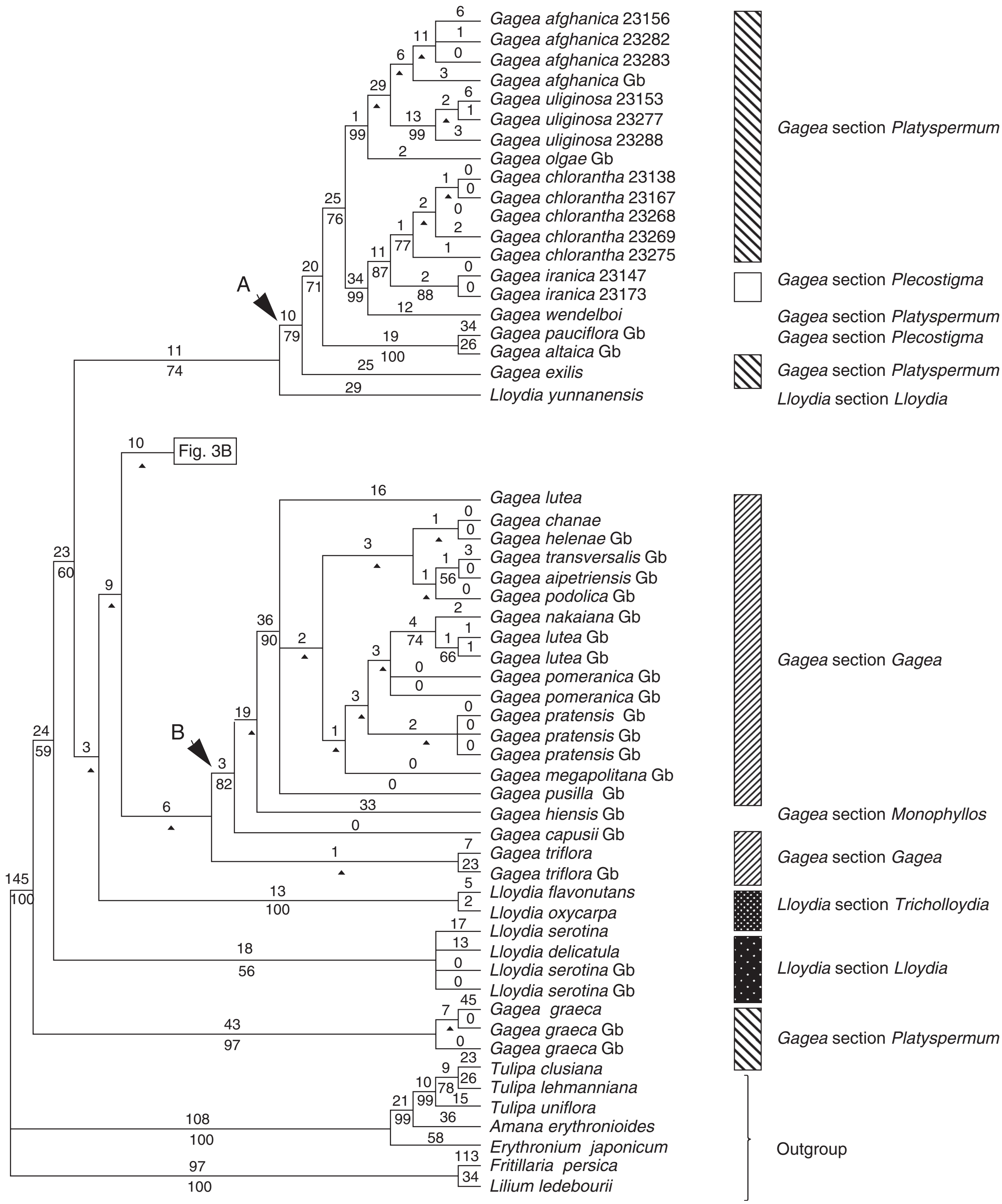

FIG. 3. One of the most-parsimonious trees, randomly selected from 6430 trees, obtained from analysis of the combined data matrix (nuclear ribosomal ITS, and plastid *rpl16* intron, *trnL* intron, *trnL-F* spacer, *matK* and the *psbA-trnH* spacer; sequences from GenBank are included). Tree length = 2388, CI = 0·62, RI = 0·90. Branch lengths (DELTRAN optimization) are indicated above branches and bootstrap percentages below. An arrowhead indicates nodes collapsing in the strict consensus of all most-parsimonious trees. Branches with a hyphen have BP < 50. Gb after the species names indicates those taken from GenBank. For cases in which more than one accession of a species were analysed, numbers after the species names are RBG, Kew, DNA Bank accession numbers (see Appendix).

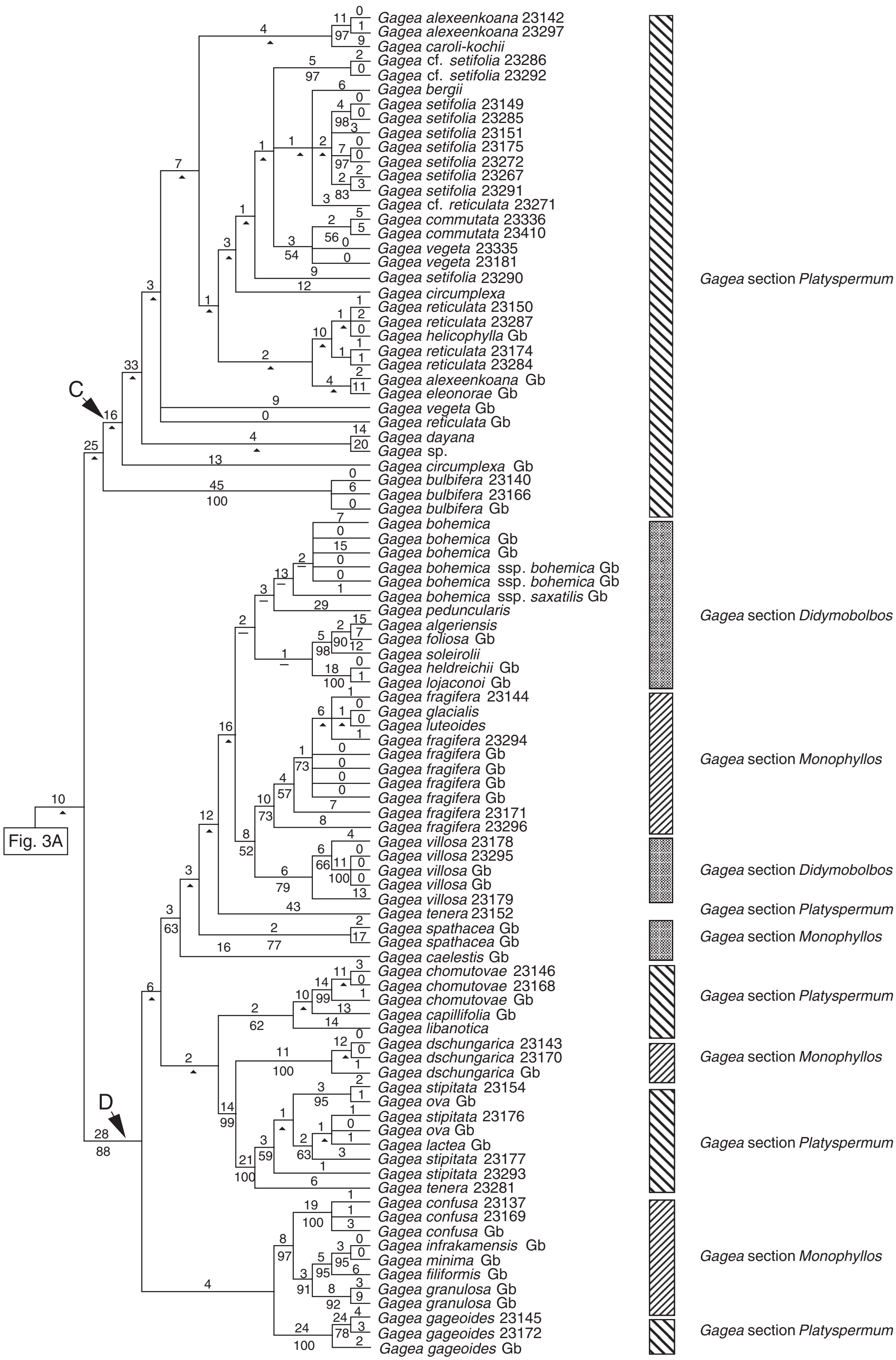

FIG. 3. Continued.

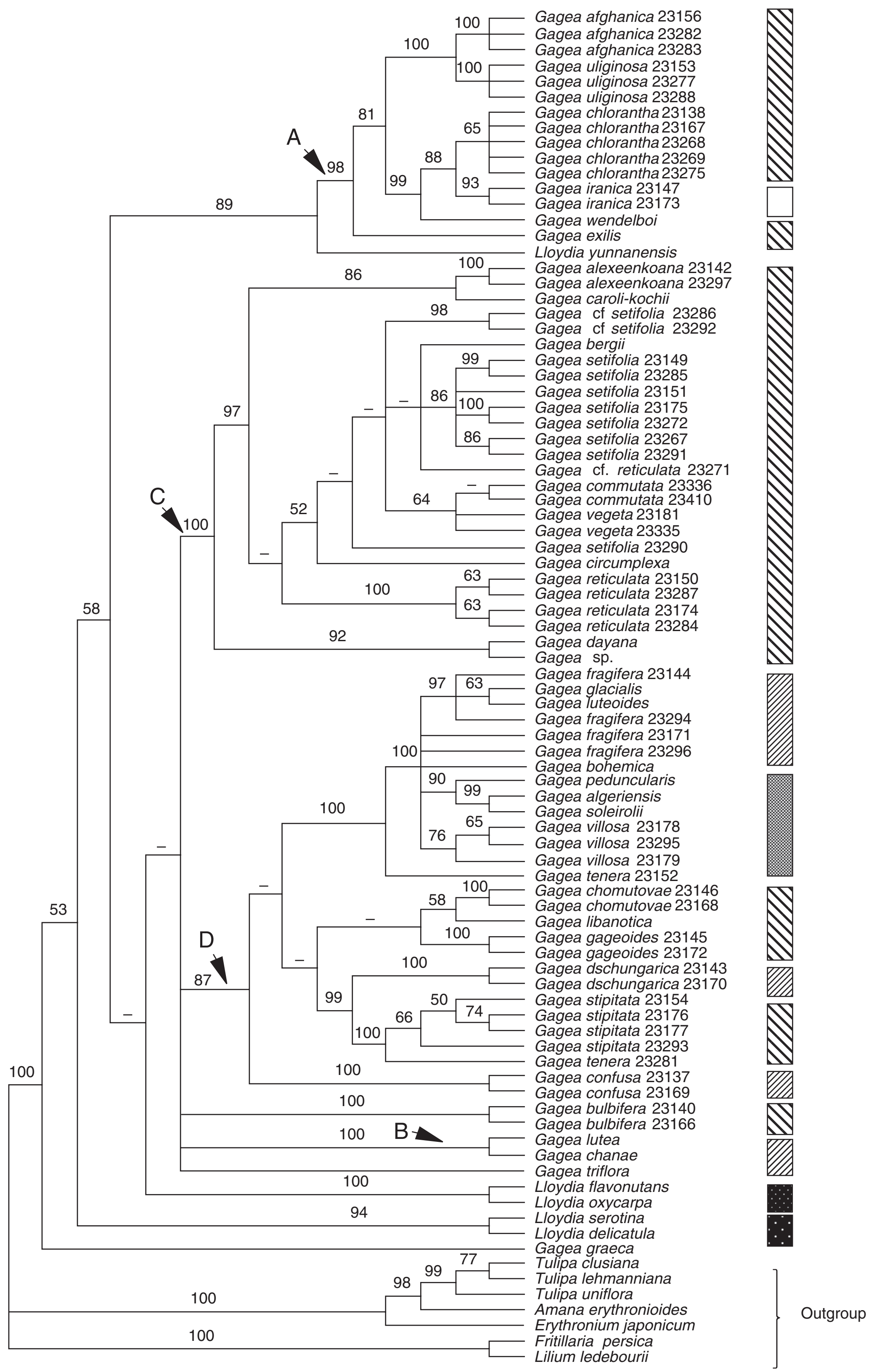

FIG. 4. The strict consensus of 7276 trees obtained from analysis of the combined data matrix (nuclear ITS rDNA and plastid sequences; sequences from GenBank are excluded). Bootstrap percentages ($\geq$50) are indicated above branches. Branches with a hyphen have BP $<$ 50.

particularly more rapidly evolving DNA regions than those used in this research.

Although all *Lloydia* species are part of a moderately supported group (BP 81) with clade A in the tree resulting from analyses of combined plastid data (Fig. 2), there are three distinct clades that include *Lloydia* species based on combined ITS and plastid sequences and also the ITS dataset alone (Figs 1, 3 and 4).

We do not have any clear hypotheses regarding the causes of the incongruence. Both ancient hybridization (with *Lloydia* species perhaps sharing a maternal lineage) and paralogy of ITS sequences (Doyle, 1992; Baldwin *et al.*, 1995; Wendel *et al.*, 1995) are possibilities. Differences in tree topologies may also be due to sampling error (too few data, in the case of *Lloydia* species) (see Hulsenbeck *et al.*, 1996; Whitten *et al.*, 2000; Rønsted *et al.*, 2005), but these hypotheses are difficult to distinguish. We are currently sequencing several low-copy, protein-coding nuclear regions and hope that the results of these analyses can shed some light on the phenomena that may have generated the incongruence detected in our analyses. It is clear in all cases that *Lloydia* and *Gagea* are a single entity and cannot be separately recognized. Incongruence within this clade did not prevent the necessary taxonomic transfers. Peruzzi *et al.* (2008*a*) suggested the transfer of *L. serotina* and *Lloydia delicatula* to *G.* section *Lloydia*.

Monophyly of Gagea *plus* Lloydia

A close relationship of *Gagea* and *Lloydia* was confirmed in all of the analyses derived from the independent datasets presented here. All six species of *Lloydia* and all *Gagea* accessions form a highly supported clade (BP 100; Figs 1–4). These results are consistent with those from previous phylogenetic analyses (Patterson and Givnish, 2002; Peterson *et al.*, 2004, 2008; Rønsted *et al.*, 2005; Peruzzi *et al.*, 2008).

Gagea *and subgenera*

The widely accepted subgeneric classification first published by Pascher (1904, 1907) and subsequently used by other botanists, e.g. Stroh (1937) and Uphof (1958–1960), is superimposed on all figures presented here with the difference that *Gagea* section *Tribolbos* has been merged with *G.* section *Gagea* in this study as in Davlianidze (1976) and Levichev (1990). According to Pascher (1904, 1907), there are two subgenera, i.e. *Gagea* subgenus *Gagea* and *Hornungia* (Table 1). The former is characterized by having globose, angular or edged seeds whereas the latter possesses flat, thin seeds. Neither of these subgenera is monophyletic in the present study, and sections belonging to these taxa are dispersed throughout the tree (Figs 1–4). This indicates that either or both of the two seed forms, which may have specific adaptive roles, have arisen several times during the evolution of the genus, and that these characters are not suitable for classification within *Gagea* due to parallelism. Most species belonging to *G.* subgenus *Gagea*, which possess thick seeds, are adapted to relatively more humid areas. They are generally found in the Euro-Siberian floristic region (Takhtajan, 1986) and less in the Irano-Turanian region; these species are restricted to higher elevations where there is more precipitation, particularly in the form of snow. The other subgenus, *G.* subgenus *Hornungia*, with flattened seeds, is adapted to drier conditions and can usually be found in the Irano-Turanian and Saharo-Arabian regions. There are a few species of this group distributed in Europe, but they are mainly restricted to southern and southeastern areas where the climate is drier.

The placement of species in clades A–D described below indicates that these subgenera are polyphyletic.

Phylogenetic relationships within Gagea *and* Lloydia *and infrageneric classification*

Although the monophyly of *Lloydia/Gagea* is strongly supported, with few exceptions relationships within this clade are not well resolved. *Lloydia* species are mostly positioned towards the base of trees and in strict consensus trees form a polytomy. This may be due to missing data in the various accessions of *Lloydia* (mostly obtained from herbarium material).

As was the case for *Gagea*, there is no consistency between classical infrageneric taxa in *Lloydia* and the results of our phylogenetic analyses. *Lloydia* section *Lloydia* (*L. yunnanensis*, *L. delicatula* and *L. serotina* included in the present study) and *Lloydia* section *Tricholloydia* Engl. (with *L. oxycarpa* and *L. flavonutans* in the present study) are the two recognized infrageneric taxa of *Lloydia* (Dasgupta and Deb, 1986). These species are dispersed throughout the trees without a recognizable pattern, although closely related species such as *L. oxycarpa* and *L. flavonutans* form well-supported clades (BPs 90–100; Figs 1–4). *Lloydia yunnanensis* is usually sister to clade A in trees from analyses of plastid sequences alone and those from combined plastid and nuclear datasets (Figs 2, 3A and 4). However, nuclear data (ITS) did not resolve it in this position.

In some analyses, *L. serotina* and *L. delicatula* are in a clade with high support (BPs 94–95; Figs 1 and 4). In analysis of the combined plastid dataset (Fig. 2), five species, i.e. *G. graeca*, *L. serotina*, *L. delicatula*, *L. flavonutans* and *L. oxycarpa*, form two clades that are moderately to highly supported but for which relationships to each other and clade A are not resolved. These belong to the two sections of *Lloydia*.

Five major monophyletic, moderately to strongly supported groups are revealed for *Gagea* accessions (Figs 1–4); the first clade includes only *G. graeca*, and the other four are referred to as clades A–D. *Gagea graeca* has an eastern Mediterranean distribution and is one of the few *Gagea* species possessing white to pale pink flowers, which resemble those of some species of *Lloydia. Gagea graeca* was placed under *G.* section *Anthericoides* by Terracciano (1905*b*) and Peterson *et al.* (2008). Stroh (1937) classified this species under *G.* section *Platyspermum*. The present results do not support Stroh's (1937) treatment of the species, and it reveals an isolated position for the species as suggested by Terracciano (1905*b*), Peruzzi *et al.* (2008*a*) and Peterson *et al.* (2008).

The second group comprises clade A (Figs 1–4), which includes species of *G.* subgenus *Hornungia* [excluding *G. pauciflora* (Turcz. ex Trautv.) Turcz. ex Ledeb., *G. altaica* Schischk. & Sumnev.]. All species included in clade A belong to *G.* section *Platyspermum* except *G. iranica* and *G. pauciflora*, which belong to *G.* section *Plecostigma* [according to Pascher's (1904, 1907) classification]. According to Levichev (1990), all species in this clade, apart from *G. iranica* and *G. pauciflora*, *G. bulbifera*, *G. chlorantha* and *G. exilis* (Figs 2–4), belong to *G.* section *Plecostigma*. Possession of a cymose inflorescence is a potential morphological synapomorphy for clade A.

In the analysis of the combined matrix of all datasets (Fig 3A), in addition to the grouping of *G. pauciflora* (Turcz. ex Trautv.) Turcz. ex Ledeb. and *G. altaica* Schischk. & Sumnev., which form a well-supported clade (BP 100; Fig. 3A), there are two well-defined subclades (BP 99) within clade A. The first subclade includes *G. uliginosa*, *G. afghanica* and *G. olgae*. All species of this subclade belong to *G.* section *Platyspermum* (*sensu* Pascher, 1907; Stroh, 1937; Uphof, 1958–1960) and *G.* section *Plecostigma* Pascher (*sensu* Levichev, 1990). There is little morphological similarity between the first two species; *G. uliginosa* has a single-flowered inflorescence (rarely with two flowers) and grows in moist meadows in alpine areas of north-western Iran, eastern Turkey and north-western Iraq; *G. afghanica* is a multi-flowered, cymose plant, mostly growing throughout the eastern part of Iran to Central Asia and preferring a drier habitat than *G. uliginosa. Gagea olgae* is morphologically similar to *G. afghanica*, with smaller tepals (6–9 mm). Cord-like roots around the bulb are a synapomorphy for *G. afghanica* and *G. olgae*. However, there is no resolution between *G. olgae* and *G. afghanica* and relatives in analyses using both nuclear and plastid sequence data, and the four accessions of *G. afghanica* and the single accession of *G. olgae* are unresolved in the strict consensus (Fig. 3A).

The second subclade comprises *G. chlorantha*, *G. iranica* and *G. wendelboi*. These species are morphologically similar. *Gagea iranica* and *G. wendelboi* are endemic species to northern and north-eastern Iran, whereas *G. chlorantha* is widely distributed through western Iran and other countries of the Middle East. Fewer taxa are included in the other analyses, but to the extent that they overlap in sampling, the same subclades are recovered in all analyses of clade A (Figs 1–4).

The next clade, B (BP 82, in Fig. 3A and BP 100, in Figs 1, 2 and 4), is moderately to strongly supported, although only two taxa are included in Figs 1, 2 and 4. An umbellate inflorescence plus a leathery bulb tunic are potential morphological synapomorphies for this clade. The species forming this clade all grow in humid areas. Relationships between members of clade B are poorly resolved in the analysis conducted using all datasets including sequences from GenBank (Fig. 3A). Clade B accessions are members of two sections, *G.* sections *Gagea* and *Monophyllos*, *sensu* Pascher (1907) Stroh (1937) and Uphof (1958–1960), compared with only one section, *G.* section *Gagea* of Davlianidze (1972), Levichev (1990) and Peterson *et al.* (2008).

Clade C is well supported in all but the combined analysis of all datasets (BPs 99–100 in Figs 1, 2 and 4). All taxa included in clade C are characterized by having a multi-flowered, umbellate inflorescence. Possession of acute to long-acuminate tepal apices is another potential synapomorphy for this group. All taxa in clade C belong to *G.* section *Platyspermum* subsection *Reticulatae* Pascher of *G.* subgenus *Hornungia* (*sensu* Pascher, 1907; Stroh, 1937; Uphof, 1958–1960). Grossheim (1935) and Davlianidze (1976) also treated taxa of clade C as belonging to *G.* section *Platyspermum*. In contrast, Levichev (1990) classified taxa of clade C in three sections, *G.* section *Platyspermum*, *G.* section *Graminifoliae* Levichev and *G.* section *Incrustatae* Levichev, but support for this in the combined analysis of all data is weak (BP < 50), and all taxa collapse in the strict consensus tree (Fig. 3B). *Gagea* section *Platyspermum* (*sensu* Pascher, 1907) is characterized by having a weakly trilobed stigma and flattened seeds.

Gagea bulbifera, sister to the rest of clade C in the analysis of the ITS alone (BP 100, Fig. 1), is usually a single-flowered species. However, in some cases multi-flowered stems have been observed in the field (M. Zarrei, pers. obs.). It was placed in *Gagea* section *Plecostigma* by Levichev (1990). Peterson *et al.* (2008) referred *G. bulbifera* to a new unpublished *G.* section *Bulbiferae* based on possession of a few-flowered, paniculate inflorescence.

Although the accessions are mostly unresolved in the combined analysis of all data (Fig 3B), some well-supported monophyletic groups, usually including multiple accessions of one species, are recognizable within clade C. As shown in Fig. 4, *G. alexeenkoana* Miscz. and *G. caroli-kochii* Grossh. form a moderately supported clade (BP 86), and they are morphologically similar taxa. *Gagea caroli-kochii* is more slender and smaller than *G. alexeenkoana* and possesses a narrower basal leaf and shorter, narrower tepals. In contrast to our accessions of *G. alexeenkoana*, *G. alexeenkoana* from GenBank formed a clade with accessions of *G. reticulata sensu lato* (*s.l.*) (Fig. 3B), and this accession might be just a robust form of *G. reticulata* (Pall.) Schult. & Schult.f., a morphologically polymorphic species. *Gagea reticulata s.l.* accessions form a clade with strong BP support (Figs 1, 2 and 4; BP $>$ 97). This species can be recognized by its long, reticulate, multi-layered neck, single to multi-flowered umbellate inflorescence and tepals with long-acuminate apices. However, it is a polymorphic species with regards to morphological features, and different forms have been designated as distinct species by many authors. Clumped forms with a circinate, narrowly linear basal leaf are recognized as *G. tenuifolia* (Boiss.) Fomin (our accessions 23287, 23174 and 23284), solitary plants with a straight, linear basal leaf as *G. reticulata sensu stricto* (*s.s.*) (23150), and forms with a shorter bulb neck, broader basal leaf and tepals are recognized as *G. tehranica* Gand. (not included in the present study). Although lacking support, an accession of *Gagea helicophylla* Levichev from GenBank is also positioned in the *G. reticulata* group (Fig. 3B). Although *G. reticulata s.l.* accessions fall into two weakly supported groups (BP approx. 64; Figs 2 and 4), these clades do not appear to us to be referable to any named taxa.

Gagea setifolia s.l. accessions are dispersed throughout clade C. This species, like *G. reticulata*, shows considerable morphological variation (Peruzzi and Zarrei, 2007), and many forms have been designated as species by some authors (e.g. *G. anonyma* Rech f. and *G. perpusilla* Pascher). In analyses of the *G. setifolia* complex, there is thus support for recognition of some forms that grow in similar ecological conditions. *Gagea setifolia* 23272 (from the *locus classicus* of *G. anonyma*) and *G. setifolia* 23175 collected from fine sand habitats form a strongly supported group in all analyses (BPs 90–100); this group corresponds to *G. anonyma. Gagea setifolia* 23291 and 23267 share the same habit as 23272, but they are slightly more robust and grow in soils of coarse sand. These two samples, determined as *G. setifolia s.s.*, also form a monophyletic group in analyses including new plastid data and combined datasets (BPs 77 and 86 in Figs 2 and 4, respectively). However, there is no support for this group in the ITS analysis (Fig. 1). The two accessions of *G.* cf. *setifolia* (23286 and 23292) that are morphologically similar to *G. setifolia s.s.* but differ from it in having broader bracts and a longer-necked tunic fall outside the main *G. setifolia* clade (Fig. 4). *Gagea bergii* Litv. also groups with *G. setifolia s.l.*, but it is morphologically distinct from it. It has a short peduncle and long pedicels with a long-villous indumentum. *Gagea vegeta* and *G. commutata* K.Koch are morphologically similar and form a clade in the plastid and plastid plus ITS analyses (BPs 56 and 64 in Figs 2 and 4, respectively). *Gagea vegeta* is the only species of *G.* section *Graminifoliae* (*sensu* Levichev, 1990) included in this analysis. The next species nested in clade C is *G. circumplexa* Vved. Levichev (1990) placed this species in a separate section, *G.* section *Incrustatae*, the only species of this section included in the present analyses, but other authors have classified it in *G.* section *Platyspermum* of *G.* subgenus *Hornungia. Gagea circumplexa* falls towards the basal nodes of clade C (Figs 2 and 4), but its position is not well supported.

The last monophyletic group within *Gagea* is clade D (BPs 87–99; Figs 1–4). The presence of a leathery, dark-grey to dark-brown tunic is a potential morphological synapomorphy for this clade. Although clade D includes several moderately to well-supported clades, the deeper nodes are unresolved. Species included in clade D belong to *G.* sections *Didymobolbos* and *Monophyllos* of *G.* subgenus *Gagea* and *G.* section *Platyspermum* of *G.* subgenus *Hornungia* (Fig. 3B). Three subclades include members of *G.* subgenus *Hornungia* (labelled as section *Platyspermum* in Figs 1–5), i.e. *G. gageoides* (Zucc.) Vved., the clade from *G. libanotica* to *G. chomutovae* and the grouping from *G. tenera* 23281 to *G. stipitata* 23154. Clade D also includes a well-supported grouping of *G. granulosa* Gb to *G. confusa* Gb 23137, which are morphologically similar, sharing a flattened basal leaf and umbellate to subumbellate inflorescences (Fig. 3B). All of these species belong to *G.* section *Monophyllos* subsection *Minimae* Pascher, later promoted to sectional rank by Davlianidze (1976; *G.* section *Minimae* (Pascher) Davlianidze).

The grouping from *Gagea tenera* 23281 to *G. dschungarica* 23143 is a well-supported group (BP 99) within clade D based on combined analysis of all datasets (Fig. 3B). To the extent that there is overlap in sampling, this group is recovered in all analyses (Figs 1–4). *Gagea dschungarica* Regel (*G.* section *Monophyllos* subsection *Minimae*) is morphologically distinct relative to the rest of the members of this group, which are all members of *G.* section *Platyspermum* subsection *Stipitatae* Pascher (*G.* section *Stipitatae sensu* Davlianidze, 1976; Levichev, 1990). *Gagea dschungarica* has recently been placed in a new *G.* section *Dschungaricae* Levichev (Peterson *et al.*, 2008). However, we do not believe that creating a new section for a taxon that is sister to the rest of the clade enhances systematic understanding. The next group within clade D includes *Gagea caelestis* GB–*Gagea bohemica*, which is weakly supported (BP 63; Fig. 3B). This group includes representatives from three sections, *G.* section *Platyspermum*, *Monophyllos* and *Didymobolbos*, and is recovered in all analyses. The production of bulbils in the axil of the lower cauline leaf is a potential synapomorphy for a group comprising *G. tenera* and species such as *G. villosa* and *G. fragifera* (Vill.) E. Bayer & G. López.

All accessions of *G. bohemica* form a group with $<50\,\%$ bootstrap support. All accessions of *G. villosa* form a moderately supported clade (BP 79; Fig. 3B). *Gagea fragifera*, *G. luteoides* Stapf and *G. glacialis* K.Koch (all belong to *G.* section *Monophyllos*) form a clade with moderate support (BP 73; Fig. 3B).

In the analysis conducted using only newly generated sequences, *G. fragifera*, *G. luteoides* and *G. glacialis*, together with *G. villosa* accessions and other morphologically similar species, form a strongly supported group (BPs 85–100; see Figs 1, 2 and 4). These species share a hollow basal leaf and umbellate to sub-umbellate inflorescence; they usually grow in heavy clay soils. The differences between them are so slight that it is difficult to separate them, particularly in the case of herbarium material. *Gagea glacialis* and *G. fragifera*, for example, are distinguished on the basis of tepal length (less than or more than 12 mm, respectively) and number of flowers per inflorescence. *Gagea glacialis* usually has fewer flowers (mostly one and rarely up to three), whereas *G. fragifera* has more than three flowers. The monotypic subsect. *Luteoides* (*G. luteoides*) of Pascher, which was not recognized by later authors, needs to be included in *G.* section *Monophyllos*. The species composition of clade D is compatible with neither Pascher's (1907) nor Levichev's (1990 and in Peterson *et al.*, 2008) classification.

CONCLUSIONS

The present analyses support the collective monophyly of *Gagea* and *Lloydia* – they are clearly a single taxon. They provide a basis for a new classification of *Gagea* that is supported by some previously unused morphological features. Incongruence between the plastid and nuclear ITS results is interpreted as potentially due to ancient hybridization and/or paralogy of ITS rDNA. To resolve the trees, particularly along the spine of the tree and also within closely related species complexes, we will need to conduct additional analyses using more variable, low-copy nuclear genes. Such genes are not subject to concerted evolution and generally show higher evolutionary rates, which make them better tools to understand species relationships when levels of variation in plastid markers and nuclear ribosomal ITS are too low to resolve relationships and hybridization/paralogy prevent clear assessments of patterns of species evolution.

SUPPLEMENTARY DATA

The original version of this manuscript as submitted by the authors to *Annals of Botany* on 3 January 2007 is available as Supplementary Data online at www.aob.oxfordjournals.org.

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APPENDIX

Sources of DNA for taxa included in this study (RBG Kew DNA Bank numbers are in parentheses after voucher information) and GenBank accession numbers for sequences generated in this research and by others

Taxa	Voucher information	ITS	*matK*	*psbA-trnH*	*rpl16*	*trnL-trnF* region
Gagea subgenus *Gagea*:*Gagea* sect. *Didymobolbos* K. Koch						
Gagea bohemica (Zauschn.) Schult. & Schult. f.	Andy Jones s.n. (Kew 7952)	–	EU912103	–	EU912175	EU912253
Gagea bohemica (Zauschn.) Schult. & Schult. f.	Germany: Saxony-Anhalt	–	–	–	–	AJ437197
Gagea bohemica (Zauschn.) Schult. & Schult. f.	Levichev 50 (LE)	AM162672	–	AM085142	–	AJ969117
Gagea bohemica subsp. *bohemica*	Czech Republic: Moravia	AJ427549	–	AJ416370	–	AJ419161
Gagea bohemica subsp. *bohemica*	Germany: Saxony-Anhalt	AJ427548	–	–	–	AJ419160
Gagea bohemica subsp. *saxatilis* (Mert. & W.D.J.Koch) Asch. & Graebn	Germany: Saxony-Anhalt	AJ427547	–	AJ416371	–	AJ419159
Gagea algeriensis Chabert	Chase 748 (K)(Kew 748)	EU912088	AY624470	EU939280	EU912232	EU912311
Gagea foliosa (J.Presl & C.Presl) Schult. & Schult.f	Italy: Sardegna 34697 (Z)	AM162676	–	AM049258	–	AJ969124
Gagea heldreichii (A.Terracc.) Stroh	Levichev 8 (LE)	AM265534	–	AM161464	–	AM180467
Gagea lojaconoi Peruzzi	Italy 9256 (CLU)	AM287272	–	AM282997	–	AM283106
Gagea peduncularis (J.Presl & C.Presl) Pascher	Davis 40349 (K)(Kew 23333)	EU912054	EU912127	EU939252	EU912204	EU912283
Gagea soleirolii F.W.Schultz	Montserrat *et al.* s.n. (Kew 20651)	–	EU912166	EU939297	EU912244	EU912330
Gagea villosa (M.Bieb.) Sweet	Zarrei & Kamrani 35273 (TUH)(Kew 23178)	EU912084	EU912151	EU939276	EU912228	EU912307
Gagea villosa (M.Bieb.) Sweet	Zarrei & Golzarian 35247 (TUH)(Kew 23179)	EU912085	EU912152	EU939277	EU912229	EU912308
Gagea villosa (M.Bieb.) Sweet	Zarrei & Golzarian 35253 (TUH)(Kew 23295)	EU912087	EU912154	EU939279	EU912231	EU912310
Gagea villosa (M.Bieb.) Sweet	Levichev 7 (LE)	AM180453	–	AJ973170	–	AM238538
Gagea villosa (M.Bieb.) Sweet	Germany: Saxony-Anhalt	AJ427545	–	AJ416373	–	AJ419163
Gagea sect. *Monophyllos* Pascher						
Gagea caelestis Levichev	Levichev 44 (LE)	AM180456	–	AJ973165	–	AJ969118
Gagea confusa A.Terracc.	TUH-E BOT.EXP. 35712 (TUH)(Kew 23169)	EU912041	EU912117	EU939239	EU912189	EU912268
Gagea confusa A.Terracc.	Zarrei & Zarrei 35266 (TUH)(Kew 23137)	EU912040	EU912116	EU939238	EU912188	EU912267
Gagea confusa A.Terracc.	Levichev 13 (LE)	AM087949	–	AJ973173	–	AJ890369
Gagea dschungarica Regel	Zarrei 35815 (TUH)(Kew 23143)	EU912043	EU912118	EU939240	EU912191	EU912270
Gagea dschungarica Regel	Zarrei 35290 (TUH)(Kew 23170)	EU912044	EU912119	EU939241	EU912192	EU912271
Gagea dschungarica Regel	Levichev 14 (LE)	AM087952	–	AJ973164	–	AJ970175
Gagea filiformis (Ledeb.) Kunth	Levichev 12 (LE)	AM180457	–	AM161459	–	AM084904
Gagea fragifera (Vill.) E.Bayer & G.López	Zarrei 35820 (TUH)(Kew 23144)	EU912045	EU912120	EU939243	EU912194	EU912273
Gagea fragifera (Vill.) E.Bayer & G.López	TUH-E BOT.EXP. 35711 (TUH)(Kew 23171)	EU912046	EU912121	EU939244	EU912195	EU912274
Gagea fragifera (Vill.) E.Bayer & G.López	TUH-E BOT.EXP. 35307 (TUH)(Kew 23294)	EU912086	EU912153	EU939278	EU912230	EU912309
Gagea fragifera (Vill.) E.Bayer & G.López	Zarrei & Zarrei 35265 (K, TUH)(Kew 23296)	EU912047	EU912122	EU939245	EU912196	EU912275
Gagea fragifera (Vill.) E.Bayer & G.López	Italy: 12692 (CLU)	AM287285	–	AM282995	–	AM283102
Gagea fragifera (Vill.) E.Bayer & G.López	Switzerland: Canton Graubuenden 10726 (ZT)	–	–	AM238531	–	AJ890375
Gagea fragifera (Vill.) E.Bayer & G.López	Bulgaria: Pirin-mountains 070407 (HAL)	AM162677	–	AJ973158	–	AJ890368
Gagea fragifera (Vill.) E.Bayer & G.López	Levichev 29b (LE)	AM180455	–	AM238521	–	AM161467
Gagea glacialis K.Koch	Marais 1565 (K)(Kew 23279)	–	–	–	EU912199	EU912278
Gagea granulosa Turcz.	Levichev 11b (LE)	AM287278	–	AM238517	–	AM180463
Gagea granulosa Turcz.	Levichev 11a (LE)	AM265533	–	AM238518	–	AM180462
Gagea hiensis Pascher	Mongolia: Bogd-Ul Mountains 070426 (HAL)	AM287279	–	AJ973169	–	AJ890367
Gagea infrakamensis Levichev	Levichev 10 (LE)	AM180459	–	AM238519	–	AM180471
Gagea luteoides Stapf	Baytor, T. ISTE 44270 (K)(Kew 23280)	EU912053	EU912126	EU939251	EU912203	EU912282
Gagea minima (L.) Ker-Gawl.	Germany: Saxony-Anhalt	AJ427546	–	AJ416374	–	AJ419164
Gagea spathacea (Hayne) Salisb.	Levichev 37 (LE)	–		AJ973174		AJ969126
Gagea spathacea (Hayne) Salisb.	Germany: Saxony-Anhalt 095844 (Hal)	AJ427541	–	AJ416369	–	AJ419166
Gagea sect. *Gagea* (*Holobolbos* K.Koch)						
Gagea aipetriensis Levichev	Levichev 15 (LE)	AM087955	–	AM049259	–	AJ970178
Gagea capusii A.Terracc.	Levichev 24 (LE)	–	–	AM085143	–	AJ969123
Gagea chanae Grossh.	Zarrei 867 (K)(Kew 23270)	EU912082	EU912167	EU939298	EU912245	–
Gagea helenae Grossh.	Levichev 22 (LE)	AM265531	–	AM161461	–	AJ969120
Gagea lutea (L.) Ker-Gawl.	Zarrei 35285 (TUH)(Kew 23148)	EU912052	EU912125	EU939250	EU912202	EU912281

Gagea lutea (L.) Ker-Gawl.	Germany: Saxony-Anhalt, Rothenschirmbach	AJ488569	–	AJ416368	–	AJ488279
Gagea lutea (L.) Ker-Gawl.	Levichev 16 (LE)	AM265530	–	AM161456	–	AM110255
Gagea megapolitana Henker	Henker (HAL)	–	–	AM161455	–	AM084902
Gagea nakaiana Kitag.	Levichev 17 (LE)	AM180454	–	AM161457	–	AM110256
Gagea pratensis (Pers.) Dumort.	Germany: Saxony-Anhalt	AJ437203	–	AJ416372	–	AJ437196
Gagea pratensis (Pers.) Dumort.	Germany: Brandenburg	AJ437202	–	–	–	AJ437195
Gagea pratensis (Pers.) Dumort.	Germany: Saxony-Anhalt	AJ437201	–	–	–	AJ419162
Gagea podolica Schult. & Schult.f.	Levichev 21 (LE)	AM409334	–	AM238525	–	AM084903
Gagea pomeranica R.Ruthe	Germany: Mecklenburg-Western Pomeranica 095846 (HAL)	AJ429193	–	AJ429194	–	–
Gagea pomeranica R.Ruthe	Germany: Saxony-Anhalt 095842 (HAL)	AJ427543	–	AJ416375	–	AJ419167
Gagea pusilla (F.W.Schmidt) Sweet	Levichev 18 (LE)	–	–	AM161458	–	AM180464
Gagea triflora Schult. fl.	Furse & Miyoshi 26159 (K)(Kew 23409)	–	–	–	EU912246	EU912331
Gagea triflora Schult. fl.	Levichev 46 (LE)	AM162674	–	AM049261	–	AJ890377
Gagea transversalis (Pall.) Steven	Levichev 56 (LE)	AM162671	–	AJ973167	–	AJ890370
Gagea subgenus *Hornungia* (Bernh.) Pascher: *Gagea* sect. *Platyspermum* Boiss.						
Gagea afghanica A.Terracc.	Zarrei & Golzarian 35257 (TUH)(Kew 23156)	EU912021	EU912097	EU939221	EU912171	EU912247
Gagea afghanica A.Terracc.	Zarrei & Golzarian 35223 (K, TUH)(Kew 23282)	EU912022	EU912098	EU939222	–	EU912248
Gagea afghanica A.Terracc.	Zarrei & Golzarian 35207 (K, TUH)(Kew 23283)	EU912023	EU912099	EU939223	–	EU912249
Gagea afghanica A.Terracc.	Levichev 52 (LE)	AM087953	–	AJ973160	–	AJ890373
Gagea alexeenkoana Miscz.	TUH-E BOT.EXP. 35305 (TUH)(Kew 23142)	EU912024	EU912100	EU939224	EU912172	EU912250
Gagea alexeenkoana Miscz.	TUH-E BOT.EXP. 35306 (TUH)(Kew 23297)	EU912030	EU912106	EU939229	EU912179	EU912257
Gagea alexeenkoana Miscz.	Levichev 34 (LE)	AM180458	–	AM161460	–	AM110257
Gagea altaica Schischk. & Sumnev.	Levichev 51 (LE)	AM162670	–	AJ973159	–	AJ890374
Gagea bergii Litv.	Zarrei & Golzarian 35222 (TUH)(Kew 23141)	EU912026	EU912102	–	EU912174	EU912252
Gagea bulbifera (Pall.) Salisb.	TUH-E BOT.EXP. 35713 (TUH)(Kew 23140)	EU912027	EU912104	EU939226	EU912176	EU912254
Gagea bulbifera (Pall.) Salisb.	TUH-E BOT.EXP. 35709 (TUH)(Kew 23166)	EU912028	EU912105	EU939227	EU912177	EU912255
Gagea bulbifera (Pall.) Salisb.	Levichev 2 (LE)	AM162669	–	AM049260	–	AJ969119
Gagea capillifolia Vved.	Levichev 42 (LE)	AM087951	–	AJ973171	–	AJ970177
Gagea caroli-kochii Grossh.	TUH-E BOT.EXP. 35715 (TUH)(Kew 23139)	EU912029	EU912170	EU939228	EU912178	EU912256
Gagea chlorantha (M.Bieb.) Schult. & Schult. f.	Zarrei & Kamrani 35192 (TUH)(Kew 23138)	EU912031	EU912107	EU939230	EU912180	EU912258
Gagea chlorantha (M.Bieb.) Schult. & Schult. f.	Zarrei & Kamrani 35195 (TUH)(Kew 23167)	EU912032	EU912108	EU939231	EU912181	EU912259
Gagea chlorantha (M.Bieb.) Schult. & Schult. f.	Zarrei & Zarre 778 (K, TUH)(Kew 23268)	EU912033	EU912109	EU939232	EU912182	EU912260
Gagea chlorantha (M.Bieb.) Schult. & Schult. f.	Zarrei 872 (K, TUH)(Kew 23269)	EU912034	EU912110	EU939233	EU912183	EU912261
Gagea chlorantha (M.Bieb.) Schult. & Schult. f.	Hikmat Abbas Al-Ani & Danail & Danail Aoraha 9354 (K)(Kew 23275)	EU912035	EU912111	–	–	EU912262
Gagea chomutovae (Pascher) Pascher	Zarrei & Golzarian 35214 (TUH)(Kew 23146)	EU912036	EU912112	EU939234	EU912184	EU912263
Gagea chomutovae (Pascher) Pascher	Zarrei 35814 (TUH)(Kew 23168)	EU912037	EU912113	EU939235	EU912185	EU912264
Gagea chomutovae (Pascher) Pascher	Levichev 37 (LE)	AM087950	–	AM049262	–	AJ970176
Gagea circumplexa Vved.	Carter 721 (K)(Kew 23274)	EU912038	EU912114	EU939236	EU912186	EU912265
Gagea circumplexa Vved.	Levichev 30 (LE)	AM265529	–	AJ973172	–	AJ969122
Gagea commutata K.Koch	Zarrei 876 (K, LE, TUH)(Kew 23336)	EU912039	EU912115	EU939237	EU912187	EU912266
Gagea commutata K.Koch	Dafni s.n. (Kew 23410)	EU912096	–	EU939296	EU912243	EU912329
Gagea dayana Chodat & Beauverd	Davis 8235 (K)(Kew 23273)	EU912042	–	–	EU912190	EU912269
Gagea eleonorae Levichev	Levichev 57 (LE)	AM287274	–	AJ973163	–	AJ970179
Gagea exilis Vved.	Moussavi & Tehrani 29971 (IRAN)(Kew 23182)	–	–	EU939242	EU912193	EU912272
Gagea gageoides (Zucc.) Vved.	Zarrei & Kamrani 35274 (TUH)(Kew 23172)	EU912049	EU912169	EU939247	EU912198	EU912277
Gagea gageoides (Zucc.) Vved.	TUH-E BOT.EXP. 35714 (TUH)(Kew 23145)	EU912048	EU912168	EU939246	EU912197	EU912276
Gagea gageoides (Zucc.) Vved.	Levichev 41 (LE)	AM162673	–	AM161462	–	AM084905
Gagea graeca (L.) Irmisch.	Davis 40591 (K)(Kew 23339)	EU912077	EU912159	EU939285	EU912235	EU912316
Gagea graeca (L.) Irmisch.	Greece: Crete, Lassithi plateau 099962 (HAL)	AJ810089	–	AM049263	–	AJ810090
Gagea graeca (L.) Irmisch.	Greece: Lakonia	AJ810088	–	–	–	–
Gagea helicophylla Levichev” ined.	Levichev 35a (LE)	–	–	AM085145	–	AM084901
Gagea lactea Levichev	Levichev 53 (LE)	AM180452	–	AJ973166	–	AJ969125
Gagea libanotica (Hochst.) Greuter	Townsend 74/38 (K)(Kew 23338)	–	EU912160	EU939286	EU912236	EU912317
Gagea olgae Regel	Levichev 3 (LE)	–	–	AM085144	–	AM161465
Gagea ova Stapf	Levichev 39b (LE)	AM287277	–	AM265588	–	AM180466

Continued

Appendix *Continued*

Taxa	Voucher information	ITS	*matK*	*psbA-trnH*	*rpl16*	*trnL-trnF* region
Gagea ova Stapf	Levichev 39a (LE)	AM287276	–	AM238526	–	AM180465
Gagea reticulata (Pall.) Schult. & Schult. f.	Zarrei & Golzarian 35260 (TUH)(Kew 23150)	EU912056	EU912129	EU939254	EU912206	EU912285
Gagea reticulata (Pall.) Schult. & Schult. f.	Zarrei & Kamrani 35196 (TUH)(Kew 23174)	EU912058	EU912131	EU939256	EU912208	EU912287
Gagea reticulata (Pall.) Schult. & Schult. f.	Zarrei & Kamrani 35186 (K, TUH)(Kew 23284)	EU912060	EU912133	EU939258	EU912210	EU912289
Gagea reticulata (Pall.) Schult. & Schult. f.	Zarrei & Ajani 832 (IRAN, K, M, TUH)(Kew 23287)	EU912061	EU912134	EU939259	EU912211	EU912290
Gagea reticulata (Pall.) Schult. & Schult. f.	Levichev 56 (LE)	–	–	AJ973162	–	–
Gagea cf. *reticulata* (Pall.) Schult. & Schult. f.	Zarrei & Zarre 1032 (K)(Kew 23271)	EU912059	EU912132	EU939257	EU912209	EU912288
Gagea setifolia Baker	Zarrei 35289 (TUH)(Kew 23149)	EU912055	EU912128	EU939253	EU912205	EU912284
Gagea setifolia Baker	Zarrei & Golzarian 35246 (TUH)(Kew 23151)	EU912063	EU912136	EU939261	EU912213	EU912292
Gagea setifolia Baker	Zarrei & Golzarian 35254 (TUH)(Kew 23175)	EU912064	EU912137	EU939262	EU912214	EU912293
Gagea setifolia Baker	Heydari 30547 (K)(Kew 23267)	EU912065	EU912138	EU939263	EU912215	EU912294
Gagea setifolia Baker	Zarrei 1017 (K)(Kew 23272)	EU912066	EU912139	EU939264	EU912216	EU912295
Gagea setifolia Baker	Mohammadi 35198 (K, TUH)(Kew 23285)	EU912067	EU912140	EU939265	EU912217	EU912296
Gagea setifolia Baker	Zarrei & Zarrei 35268 (K, TUH)(Kew 23290)	EU912068	EU912141	EU939266	EU912218	EU912297
Gagea setifolia Baker	Zarrei & Golzarian 35213 (K, TUH)(Kew 23291)	EU912069	EU912142	EU939267	EU912219	EU912298
Gagea cf. *setifolia* Baker	Zarre 1009 (K, TUH)(Kew 23286)	EU912025	EU912101	EU939225	EU912173	EU912251
Gagea cf. *setifolia* Baker	Zarrei & Golzarian 35252 (K, TUH)(Kew 23292)	EU912062	EU912135	EU939260	EU912212	EU912291
Gagea stipitata Merckl. ex Bunge	Zarre & Zarrei 35297 (TUH)(Kew 23154)	EU912070	EU912143	EU939268	EU912220	EU912299
Gagea stipitata Merckl. ex Bunge	Zarrei & Kamrani 35275 (TUH)(Kew 23176)	EU912071	EU912144	EU939269	EU912221	EU912300
Gagea stipitata Merckl. ex Bunge	Zarrei & Kamrani 35197 (TUH)(Kew 23177)	EU912072	EU912145	EU939270	EU912222	EU912301
Gagea stipitata Merckl. ex Bunge	Zarrei & Golzarian 35215 (K, TUH)(Kew 23293)	EU912073	EU912146	EU939271	EU912223	EU912302
Gagea tenera Pascher	Zarrei & Golzarian 35256 (TUH)(Kew 23152)	EU912074	EU912147	EU939272	EU912224	EU912303
Gagea tenera Pascher	Zarrei & Golzarian 35219 (K, TUH)(Kew 23281)	EU912075	EU912148	EU939273	EU912225	EU912304
Gagea uliginosa Siehe & Pascher	TUH-E BOT.EXP. 35304 (TUH)(Kew 23153)	EU912089	EU912155	EU939281	EU912233	EU912312
Gagea uliginosa Siehe & Pascher	Moussavi *et al.* 30018 (IRAN)(Kew 23288)	EU912090	EU912157	EU939283	–	EU912314
Gagea uliginosa Siehe & Pascher	Rawi & Serhang 18286 (K)(Kew 23277)	–	EU912156	EU939282	EU912234	EU912313
Gagea vegeta Vved.	Shafii 475 (Shahed University Herbarium)(Kew 23181)	EU912076	EU912149	EU939274	EU912226	EU912305
Gagea vegeta Vved.	Zarrei and Zarre, 1033 (K, TUH)(Kew 23335)	–	EU912150	EU939275	EU912227	EU912306
Gagea vegeta Vved.	Levichev 32 (LE)	AM287275	–	AM238520	–	AM180468
Gagea wendelboi Rech.f.	Matin 35605 (IRAN)(Kew 23183)	EU912091	EU912158	EU939284	–	EU912315
Gagea sp.	Zarrei & Kamrani 35194 (TUH)(Kew 23155)	EU912057	EU912130	EU939255	EU912207	EU912286
Gagea sect. *Plaecostigma* (Turcz.) Pascher						
Gagea iranica Zarrei & Zarre	Zarrei & Golzarian 35210 (TUH)(Kew 23147)	EU912050	EU912123	EU939248	EU912200	EU912279
Gagea iranica Zarrei & Zarre	Zarrei & Golzarian 35251 (TUH)(Kew 23173)	EU912051	EU912124	EU939249	EU912201	EU912280
Gagea pauciflora (Turcz. ex Trautv.) Turcz. ex Ledeb.	Mongolia: Ulan Bator 070423 (HAL)	AM409330	–	AJ973168	–	AJ890372
Lloydia delicatula Noltie	AGSES 212 (K)(Kew 23340)	EU912079	–	–	–	EU912320
Lloydia flavonutans H.Hara	AGSES 77 (K)(Kew 23341)	EU912080	–	–	EU912238	EU912321
Lloydia oxycarpa Franch.	ACE 137 (K)(Kew 23342)	EU912081	–	EU939289	–	EU912322
Lloydia serotina (L.) Rchb.	Jones s.n. (K)(Kew 1004)	EU912092	AY624471	EU939288	–	EU912319
Lloydia serotina (L.) Rchb.	Levichev 45a (LE)	AM087956	–	AM238530	–	AJ890376
Lloydia serotina (L.) Rchb.	Bulgaria: Ovtscharez 074806 (HAL)	–	–	AJ585048	–	AJ585049
Lloydia yunnanensis Franch.	Luo, Yi-bo 64 (K)(Kew 23337)	EU912078	EU912161	EU939287	EU912237	EU912318
Outgroup members						
Tulipa clusiana DC.	Zarrei 35183 (TUH)(Kew 23348)	EU912093	EU912162	EU939290	EU912239	EU912323
Tulipa lehmanniana Merckl.	Zarrei & Golzarian 35228A (TUH)(Kew 23349)	EU912094	EU912163	EU939291	EU912240	EU912324
Tulipa uniflora (L.) Besser ex Baker	Chase 751 (K)	–	EU912164	EU939292	EU912241	EU912325
Amana erythronioides (Baker) D. Y. Tan & D. Y. Hong	Chase 742 (K)	EU912095	AY624472	EU939293	EU912020	EU912326
Erythronium japonicum Decne.	Chase 780 (K)	EU912083	AF485323	EU939295	AF485323*	EU912332
Fritillaria persica L.	Chase 3496 (K)	AY616736	AY624451	AY624399	AY624399	EU912327
Lilium ledebourii (Baker) Boiss.	Zarrei s.n. (TUH)(Kew 23346)	–	EU912165	EU939299	EU912242	EU912328

* Voucher differs from those on the voucher list; it has been taken from NCBI.