

# Phylogenetic Relationships and Biogeography within the Eurasian Clade of Amaryllidaceae Based on Plastid *ndhF* and rDNA ITS Sequences: Lineage Sorting in a Reticulate Area?

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**ABSTRACT.** The monophyletic Eurasian clade of Amaryllidaceae was analyzed using plastid *ndhF* and rDNA ITS sequences for 33 and 29 taxa, respectively; all genera were represented by at least one species. Both maximum parsimony and Bayesian analysis were used on each data set and the combined data. Both sequence matrices resolve the Central and East Asian tribe Lycorideae as sister to the Mediterranean-centered genera of the clade, and recognize two large subclades within the greater Mediterranean region: Galantheae, consisting of *Acis*, *Galanthus* and *Leucojum*; and Narcisseae (*Narcissus* and *Sternbergia*)/*Pancratium*. However, there are areas of incongruence between the *ndhF* and ITS trees. When three predominantly monotypic genera, *Hannonia*, *Lapidra*, and *Vagaría*, centered in North Africa, are removed from the alignments, the two sequence matrices produce fully congruent topologies with increased support at many of the nodes, with ILD between partitions rising from  $P = 0.07$  to 0.96. We hypothesize that lineage sorting took place after the divergence of Galantheae and Narcisseae/*Pancratium* from a common genepool with *Hannonia*, *Lapidra*, and *Vagaría* retaining a mosaic of the ancestral haplotypes. We also performed dispersal-vicariance analysis to reconstruct biogeographic scenarios on several of the generic level phylogenies found with and without these three genera included, as well as on a species-level phylogeny of Galantheae. After the vicariant divergence of the Asian Lycorideae, North Africa and the Iberian Peninsula are the most likely areas of origin for the rest of the clade. The results of the dispersal-vicariance analysis are discussed in the context of the complex biogeographic history of the Mediterranean basin.

The Eurasian clade of the Amaryllidaceae contains the members of the family that have adapted to the highest latitudes in the Northern Hemisphere, and also those with the greatest economic value as spring flowering temperate zone garden plants (*Narcissus* L., *Galanthus* L., *Leucojum* L.). The clade has recently been recognized as a monophyletic group, resolved as sister to the endemic American genera by plastid DNA sequences (Ito et al. 1999; Meerow et al. 1999; Lledó et al. 2004) with weak bootstrap support (50–70%). The Eurasian clade encompasses four tribes that were previously recognized (Meerow and Snijman 1998): Galantheae Salisb., Lycorideae Traub, Narcisseae Endl., and Pancratieae Salisb. (Table 1), the overall relationships of which were obscured by their diversity of chromosome number and morphology (Traub 1963). Müller-Doblies and Müller-Doblies (1978a) earlier observed similarities between the internal bulb morphology of *Ungernia* Bunge (Lycorideae) and *Sternbergia* Walst. & Kit. (Narcisseae). With the exception of the Central and East Asian Lycorideae, the clade is centered within the Mediterranean region (Meerow and Snijman 1998; Lledó et al. 2004). For the purposes of our discussion in this paper, Meerow and Snijman (1998) will be followed with slight modification. References to Narcisseae refer to *Narcissus* and *Sternbergia*, Pancratieae to *Pancratium* and *Vagaría*, while references to Galantheae refer collectively to *Acis*, *Galanthus*, and

*Leucojum*. There are 11 genera in the clade, comprising ca. 120 spp., with *Lycoris* (ca. 20 spp.) and *Narcissus* (40 spp.) the largest genera (Meerow and Snijman 1998).

Lledó et al. (2004) recently presented a cladistic analysis of the clade that focused on the relationships of *Leucojum* and *Galanthus* using plastid *matK*, nuclear ribosomal ITS sequences, and morphology. *Leucojum* was revealed as paraphyletic, and the genus *Acis* Salisb. was resurrected to accommodate the linear-leaved Mediterranean *Leucojum* species with solid scapes. While their sampling within these three genera was extensive, only a single species each of the genera *Panocratium*, *Sternbergia*, *Narcissus*, and *Vagaría* Herb., along with the monotypic *Lapidra*, were used as an outgroup. *Hannonia* was not included. Consequently, the phylogenetic relationships of the entire clade were not explicitly examined in their analyses. A similar case holds for Graham and Barrett's (2004) study of floral evolution in *Narcissus* using plastid *ndhF* and *trnL-F* sequences, which included only *Lapidra* and one species each of *Galanthus*, *Leucojum*, and *Sternbergia* as outgroups in their analyses.

ITS was previously used with success to explore the phylogenetic relationships of the American clade of the Amaryllidaceae (Meerow et al. 2000), the tribes Amaryllidaceae (Meerow and Snijman 2001), Haemantheae (Meerow and Clayton 2004), Hymenocallideae (Meerow et al. 2002), and the genus *Crinum* (Meerow et al. 2003).

TABLE 1. Treatment of the Eurasian clade of Amaryllidaceae in the four most recent intrafamilial classification of Amaryllidaceae s.s. *Acis* is included in *Leucojum* in all of these classification. \*As Dahlgren et al. (1985) did not consistently list the component genera in their tribal concepts, their exact generic composition is inferred. Most of their delimitations are presumed to have followed Traub (1963).

Traub (1963)	Dahlgren et al. (1985)*	Müller-Doblies and Müller-Doblies (1996)	Meerow (1995), Meerow and Snijman (1998)
Amarylloideae	Amaryllidaceae	Amaryllidaceae	Amaryllidaceae
Infracolony Amaryllidaceae			
<b>Lycoreae</b>	<b>Lycorideae</b>	<b>Lycorideae</b>	<b>Lycorideae</b>
<i>Lycoris</i> Herb.	<i>Lycoris</i>	<i>Lycoris</i>	<i>Lycoris</i>
<i>Ungernia</i> Bunge	<i>Ungernia</i>	<i>Ungernia</i>	<i>Ungernia</i>
<b>Narcisseae</b>	<b>Narcisseae</b>	<b>Narcisseae</b>	<b>Narcisseae</b>
<i>Narcissus</i> L.	<i>Narcissus</i> (incl. <i>Tapeinanthus</i> Herb.)	<i>Narcissus</i>	<i>Narcissus</i>
<i>Sternbergia</i> Walst. & Kit.	<i>Sternbergia</i>	<i>Sternbergia</i>	<i>Sternbergia</i>
<i>Tapeinanthus</i> Herb.			
<b>Galantheae</b>	<b>Galantheae</b>	subtr. Galanthinae	<b>Galantheae</b>
<i>Galanthus</i> L.	<i>Galanthus</i>	<i>Galanthus</i>	<i>Galanthus</i>
<i>Leucojum</i> L.	<i>Leucojum</i>	<i>Leucojum</i>	<i>Leucojum</i>
<i>Hannonia</i> Braun-Blanq. & Marie			<i>Hannonia</i>
<i>Lapiedra</i> Lag.			<i>Lapiedra</i>
Infracolony Pancratioidinae			
<b>Pancratieae</b>	<b>Pancratieae</b>	<b>Pancratieae</b>	<b>Pancratieae</b>
<i>Pancratium</i> L.	<i>Pancratium</i> (incl. <i>Chapmaniolirion</i> Dint., <i>Klingia</i> Schoen.)	subtr. Pancratiinae <i>Pancratium</i>	<i>Pancratium</i>
<i>Vagaría</i> Herb.	<i>Vagaría</i>	<i>Vagaría</i> <i>Hannonia</i>	<i>Vagaría</i>
<i>Chapmaniolirion</i> Dint.		subtr. Lapiedrinae	
<i>Klingia</i> Schoen.		<i>Lapiedra</i>	

Plastid *ndhF* shows marked difference in nucleotide substitution rate and pattern between the 5' and 3' ends of the gene (Kim and Jansen 1995; Olmstead and Reeves 1995). The 5' region (1,380 bp) is much like *rbcL* while the 3' end (855 bp) has higher base substitutions rates and greater transversion bias (Kim and Jansen 1995). Consequently, the single gene has utility for resolving phylogenetic relationships of both older and more recently evolved taxa (Kim and Jansen 1995). Graham and Barrett (2004) recently utilized *ndhF* to explore the phylogenetic relationships and patterns of floral evolution in *Narcissus*. We were thus optimistic that *ndhF* would provide a well-resolved phylogeny of the entire Eurasian clade of Amaryllidaceae.

In this paper, we present the results of cladistic analyses of the Eurasian clade using plastid *ndhF* and ITS sequences, alone and in combination. We also use dispersal-vicariance analysis (Ronquist 1996, 1997) to reconstruct the biogeographic history of the Eurasian clade, and relate this to the complex paleogeological history of the Mediterranean region.

#### MATERIALS AND METHODS

**Sampling.** *ndhF* sequences were obtained for 32 species, including two outgroups and 29 species for ITS (Appendix 1). All genera from the clade were represented by at least a single species.

**DNA Extraction and Amplification.** EXTRACTION. Genomic DNA was extracted from either 200 mg of fresh or 30 mg of silica gel dried leaf tissue using the FastDNA Kit (BIO 101 Inc., Carlsbad,

CA) according to manufacturer's protocols with a FP 120 FastPrep cell disrupter (Savant Instruments Inc., Holbrook, NY). Samples were quantified with a GeneQuant pro RNA/DNA calculator (Amersham Pharmacia Biotech Inc., Piscataway, CA, USA).

**NDHF.** The plastid *ndhF* gene was amplified and sequenced using the primers of Olmstead and Sweere (1994) and Graham et al. (1998). The gene was amplified and sequenced as described by Pires and Sytsma (2002), but with 4% DMSO added to the 50  $\mu$ l reaction mix.

**ITS.** Amplification of the ribosomal DNA ITS1/5.8S/ITS2 region was accomplished using flanking primers ITS4 and ITS5 and internal ITS2 of White et al. (1990), and one internal primer that we designed (5'-GCA TCG ATG AAG AAC GTA GC-3') to amplify the spacers along with the intervening 5.8S gene, as described in Meerow et al. (2002, 2003) and Meerow and Clayton (2004). Direct sequencing of ITS presented problems within the Eurasian clade due to paralogous variation in some of the genera. In some cases, adding DMSO to the sequencing reaction as per recalcitrant *ndhF* templates allowed direct sequencing. However, for some of the species it was necessary to clone the PCR template. Sequencing of ten clones of each PCR template revealed low to medium levels of paralogy (10–35%), with divergent nucleotides comprising no more than 20% of the clones at any one paralogous position in most cases. If divergent base calls occurred in four or more of the sequenced clones, the base was coded as ambiguous. When we included all of the paralogous, cloned sequences in the analyses, all those from a single species resolved as a single clade. *Lycoris* Herb. and *Ungernia* (Lycorideae) displayed the highest amounts of paralogous variation. We used consensus ITS sequences for *L. radiata* and *U. flava* that contain large numbers of ambiguous nucleotide calls. Congruent resolution of Lycorideae with both ITS and *ndhF* suggested that phylogenetic signal was still present despite the ambiguous base calls.

PCR products were purified using Qiagen PCR Purification Kit, and used as templates in BD v. 3.1 sequencing reaction on an ABI 9700, using standard dideoxy cycle protocols for sequencing with

dye terminators. Unincorporated dye terminators were removed using Performa DTR Gel Filtration Cartridges, Edge Biosystems, Gaithersburg, MD, USA. DNA was sequenced on either an ABI 3100 or 3730 automated sequencer (according to the manufacturer's protocols; Applied Biosystems, Foster City, CA, USA).

**Sequence Alignment.** The *ndhF* sequences were readily aligned manually and unambiguously using Sequencher 4.1 (Gene Codes, Ann Arbor, MI, USA). The ITS alignment was more problematic as there is substantial sequence divergence among the genera of the Eurasian Amaryllidaceae. We used Clustal X (Higgins and Sharp 1988; Thompson et al. 1997) to align the sequences with varying gap opening and extension penalties, followed by some degree of manual editing in Sequencher. We ran fast heuristic searches to assess tree length, number of trees, and bootstrap values on various iterations of the alignment. We trimmed a continuous 32 bp segment from ITS1 in the original alignment that proved too difficult to align with any confidence.

**Phylogenetic Analyses.** The *ndhF* (33 taxa), and ITS (29 taxa) matrices were analyzed separately and in combination using the parsimony algorithm of PAUP\* for Macintosh (version 4.0b10; Swofford 1998), with the MULPARS option invoked. Tree branches were collapsed if the minimum length = 0. Gaps were coded as missing characters in all of the analyses, as there were only occasional single base indels in *ndhF* and combining the ITS matrix with a strict (no partial homology) gap matrix from the ITS alignment did not alter the tree topology supported by the sequences alone. For all matrices, a heuristic search was conducted under the Fitch (equal) weights (Fitch 1971) criterion with 2000 rounds of random addition sequence, saving no more than 20 minimum length trees per search for swapping using tree branch reconnection (TBR).

*Cyrtanthus herrei* and *Worsleya rayneri* were used as outgroups. *Cyrtanthus* is a primarily South African genus that resolves with *ndhF* sequences as basal in a clade that is sister to the American/Eurasian clade of the family (Meerow and Snijman in press), and *Worsleya* is a basal genus in the American clade (Meerow et al. 2000). Widening the outgroup pool further did not alter ingroup topology. In the combined analysis, species not represented in one of the partitions were coded entirely as missing data. Sequence alignments and parsimony trees are available from TreeBase (study accession S1368).

Before combining the ITS and *ndhF* data sets, we performed a partition homogeneity test (incongruence length difference, ILD) on the matrices (Farris et al. 1994, 1995) to assess the degree of congruence between them. One hundred heuristic searches were conducted, each with 10 random addition replications, saving no more than 20 trees from each for TBR branch swapping.

Internal support was determined by bootstrapping (BS; Felsenstein 1985; 5000 heuristic replicates with simple addition, TBR branch-swapping, saving 20 trees per replicate) and by calculating Bremer (1988) decay indices (DI) using TreeRot v. 2.1 (Sorenson 1996). The cut-off BS value was 50%. A BS value greater than 75% was considered good support, 65–75% was designated moderate support, and less than 65% as weak (Meerow and Snijman 2001; Meerow et al. 2002; Meerow and Clayton 2004). One hundred heuristic searches with random addition sequence were implemented for each constraint statement postulated by TreeRot, saving no more than 10 trees per search. A minimum DI = 2 was considered to represent good support for a clade (Meerow and Snijman 2001; Meerow et al. 2002; Meerow and Clayton 2004).

We also applied Bayesian analysis using MrBayes v. 3.04 (Huelssenbeck and Rohlf 2001; Huelssenbeck et al. 2001) to each sequence matrix, in order to approximate a BS of maximum likelihood estimates of the phylogenetic relationships, and check for congruence with the results of parsimony analysis. We determined the model of nucleotide substitution using ModelTest v. 3.06 (Posada and Crandall 1998) and applied the Akaike information criterion (Akaike 1974). We ran 1,000,000 generations of four simultaneous heated (default value) Markov chains with MrBayes, retaining the tree from every 100<sup>th</sup> generation (10,000 trees, excluding burn-in) from which a 50% majority rule consensus tree was constructed. For ITS, the log likelihood scores stabilized before

10,000 generations of Bayesian analysis; for *ndhF*, before 15,000, and the combined analysis, before 20,000. The results of the Bayesian analyses are reported as the posterior probabilities (PP; Huelssenbeck and Rohlf 2001), which is equal to the percentage of trees sampled where a given clade is resolved. Only PP scores in excess of 50% are shown in our trees. For comparison purposes, PP values were evaluated similarly to BS scores from parsimony. Finally, we also performed individual and combined parsimony and Bayesian analyses of the two individual matrices with several taxa deleted.

**Biogeographic Analyses.** The biogeographic patterns inferred from our gene trees were assessed using the dispersal-variance method of analysis (Ronquist 1997) as modeled by the program DIVA version 1.1 (Ronquist 1996). The program uses vicariance (i.e., allopatric speciation) in its optimization of ancestral distributions but takes into consideration dispersal and extinction events and indicates their direction (Ronquist 1996, 1997). The most parsimonious reconstructions minimize such events. Unlike other biogeographic inference methods based on a strict vicariance model (Nelson and Platnick 1978; Brooks 1990; Page 1994), DIVA does not restrict widespread distributions to terminals or limit ancestral distributions to single areas (Ronquist 1996). By allowing for dispersal and extinction as well as vicariance events within its model, DIVA does not impose adherence of area scenarios to a rigid "area cladogram." It is thus much more amenable for biogeographic analysis within regions that have a complex paleogeological history, which a strict vicariance model can not adequately address. Ancestral area optimizations in DIVA become less certain as the root node of the tree is approached. A weakness of the program is its assignment of nearly every area occupied by the terminal taxa in the tree to the more basal nodes, unless some type of constraint is imposed. Thus, the analyses were performed with a limit on the maximum areas allowed for ancestral nodes set to the minimum (2) to reduce ambiguities at the more basal nodes of the tree (Meerow et al. 2003; Sanmartín 2003). An exact optimization (versus heuristic) was invoked by allowing the maximum number of alternative reconstructions to be held at any node.

Because of our sampling biases, the geographic areas occupied by the species sampled within a number of the genera do not represent the complete distribution of the genus. We therefore reduced our terminal taxa to the level of genus, since all of the genera included are resolved as monophyletic in the parsimony trees. DIVA requires a fully bifurcated tree for analysis. The single generic level phylogeny resolved by a combined sequence analysis with *Hannonia*, *Lapiedra*, and *Vagaría* removed (see Results for why these genera were deleted from the dispersal-variance analysis), as well as three trees from the *ndhF* analysis (the remaining trees differed only in the internal resolution of *Sternbergia*) with *Hannonia*, *Lapiedra*, and *Vagaría* included, were used for optimization. The *ndhF* trees were chosen over the ITS tree because of BS support and high PP values for the resolution of *Lapiedra* and *Vagaría* as sister genera and their inclusion, along with *Hannonia*, within Galantheae (see Results). Fifteen coded geographic areas were used for the optimization, adapted from Sanmartín (2003): A, North Africa (Morocco, Algeria); B, Canary Islands; C, Western Mediterranean (Iberian Peninsula); D, Central Mediterranean (southern France and Italian peninsula); E, Eastern Mediterranean (Greece, Aegan Islands, Crete, western Turkey); F, Middle East (Syria, Israel, Jordan, Iraq, northern Saudi Arabia); G, Caucasus (western Turkey, Armenia, Georgia); H, Central Asia (Turkmenistan, Uzbekistan, Tajikistan, Kirzgistan and Kazakhstan); I, East Asia (China, Japan, Korea); J, South Asia (Indian, Sri Lanka); K, sub-Saharan Africa; L, South America; M, non-Mediterranean Europe; N, Sicily; O, Balkans. Geographic distribution information for terminal taxa was obtained from a variety of sources, including Fernandes (1968a), Mathew (1973), Davis (1999), Lledó et al. (2004), Flora Europaea (Tutin et al. 1972), and the Internet site maintained by Pascal Vigneron (<http://perso.club-internet.fr/v.pascal/amar-ylidaceae/>). East Asia (China and Japan) was not included in our terminal areas for *Narcissus* (*N. tazetta* L.) because this was a human introduction (Zhanhe and Meerow 2002).

To assess biogeographic scenarios within one subclade at the species level, we downloaded the sequences of Lledó et al. (2004) for *Acis*, *Galanthus*, and *Leucojum*, aligned them with CLUSTAL X, and performed a maximum parsimony analysis in PAUP as described above, using *Hannonia hesperidum* as outgroup, followed by dispersal-vicariance analysis on one of the trees found.

## RESULTS

**ndhF.** The *ndhF* matrix consisted of 2085 total characters of which 126 were parsimony informative. The percentage of data cells coded as missing was 2.9%. Eight trees of length = 407 steps were found, with a consistency index (CI) = 0.82, and retention index (RI) = 0.84 (Fig. 1). The Eurasian genera resolved as monophyletic (BS = 99%, DI = 6). Lycorideae (*Lycoris* and *Ungernia*) were monophyletic (BS = 100%, DI = 8) and sister to all remaining genera. The rest of the genera formed two large sister clades (BS = 70%, DI = 1). The first unites a monophyletic Narcisseae (BS = 67%, DI = 1) with *Pancratium* (BS = 100%, DI = 8), but with weak support (BS = 53%, DI = 1). In the second clade (BS = 84%, DI = 3), *Vagaría* and *Lapiedra* formed a sister clade (BS = 88%, DI = 3) to a trichotomy (BS < 50%, DI = 1) comprised of *Galanthus/Leucojum* (BS = 65%, DI = 1), *Acis* (BS = 55%, DI = 1), and an unresolved *Hannonia*. The trees differed from each other only in the terminal resolution within *Sternbergia* and *Pancratium*.

The substitution model that best fit the *ndhF* alignment was the Transversion Model (TvM) with gamma distribution (Rodriguez et al. 1990), which was applied in the Bayesian analysis with the following parameters: base frequencies A = 0.2805, C = 0.1505, G = 0.1815, and T = 0.3875; substitution rates = A → C, 2.0167, A → G, 3.3287, A → T, 0.2972, C → G, 0.3713, C → T, 3.3287, and G → T, 1.0000; gamma distribution shape = 0.3623; and proportion of invariable sites = 0. Bayesian analysis supported many of the clades resolved by parsimony (Fig. 1). Lycorideae and Narcisseae all had PP scores in excess of 90%, as did *Narcissus*, *Pancratium* and *Sternbergia*. *Pancratium zeylanicum* and *P. canariense* were sister species with a PP = 58, a resolution that occurred in only half of the eight parsimony trees. The Galantheae, however, was less well resolved in the Bayesian analysis (Fig. 1).

**ITS.** The ITS matrix consisted of 659 characters, of which 318 were parsimony informative. The percentage of data cells coded as missing was 1.7%. A single most parsimonious tree was found (Fig. 2). The tree was 1025 steps long with CI = 0.64 and RI = 0.76. The Eurasian clade was monophyletic (BS < 50%, DI = 1). A monophyletic Lycorideae (BS = 100%, DI = 39) was the sister to the rest of the clade (BS < 50% DI = 1), which formed two subclades. The first was the tribe Galantheae sensu Meerow and Snijman (1998) less *Hannonia* and *Lapiedra* (< 50% BS, DI = 1). *Galanthus* was monophyletic (BS = 100%, DI = 56), and sister to

*Leucojum* (BS = 98%, DI = 11). *Acis* was monophyletic (BS = 99%, DI = 8). The second subclade (BS < 50%, DI = 1) included a monophyletic *Sternbergia* (BS = 99%, DI = 10), sister to a diverse and poorly supported clade consisting of *Vagaría* and *Hannonia* as poorly supported sister genera, resolved in turn as sister (BS < 50%, DI = 1) to a monophyletic *Narcissus* (BS = 100%, DI = 16). However, branch lengths for *Hannonia* and *Vagaría* pointed to the possibility of a long branch attraction effect (Felsenstein 1978). *Lapiedra* was resolved as sister (BS < 50%, DI = 1) to a monophyletic *Pancratium* (BS = 87%, DI = 3).

The substitution model that best fit the ITS alignment was the Tamura-Nei model (TrN) with gamma distribution (Tamura and Nei 1993), which was applied in the Bayesian analysis with the following parameters: state frequencies fixed at A = 0.2126, C = 0.2626, G = 0.3114, and T = 0.2134; substitution rates = A → C, 1.000, A → G, 3.0010, A → T, 1.0000, C → G, 1.0000, C → T, 5.8168, and G → T, 1.0000; gamma distribution shape = 1.0867; and proportion of invariable sites = 0. Overall, the Eurasian clade received a PP = 81%. The sister relationship of *Lapiedra* to *Pancratium* had a PP = 55. *Vagaría* and *Hannonia* were unresolved. While *Acis*, Lycorideae, *Galanthus*, *Narcissus*, *Pancratium* and *Sternbergia* had PP = 100% (Fig. 2), tribal clades received less than 50 % PP scores (Fig. 2).

**Combined.** The ILD suggested that the two sequence matrices were largely incongruent (P = 0.07), an unsurprising conclusion given the lack of support for the internal branches of the ITS phylogeny (Fig. 2). The ILD may not always accurately assess the relative congruence of independent data sets (Yoder et al. 2001), and combining data sets can be a springboard for focused discussion of apparent discontinuities in the tree topologies generated by each data set independently (Reeves et al. 2001; Hipp et al. 2004). Nonetheless, aside from differences in the resolution of some terminal taxa in the phylogenies generated independently by each sequence matrix, the two data sets resolved trees with many of the same major clades (Figs. 1, 2). The combined sequence matrix yielded 444 potentially parsimony informative characters out of 2744 total. The percentage of data cells coded as missing was 8.2%. The heuristic search found six trees of length = 1445 steps, CI = 0.69 and RI = 0.77, one of which is shown (Fig. 3). Two of the six were identical in topology except for the terminal resolution within *Pancratium*. The trees were incongruent in terms of their placement of *Hannonia*, *Lapiedra*, and *Vagaría*, and, in one of the six, the position of *Pancratium*. *Acis*, *Galanthus*, *Narcissus*, *Pancratium*, and *Sternbergia* each resolved as monophyletic with BS > 90%. The monophyletic Lycorideae (BS = 83%, DI = 2) were sister to all remaining Eurasian Amaryllidaceae (BS = 98%, DI = 7). BS support for the two main clades resolved by



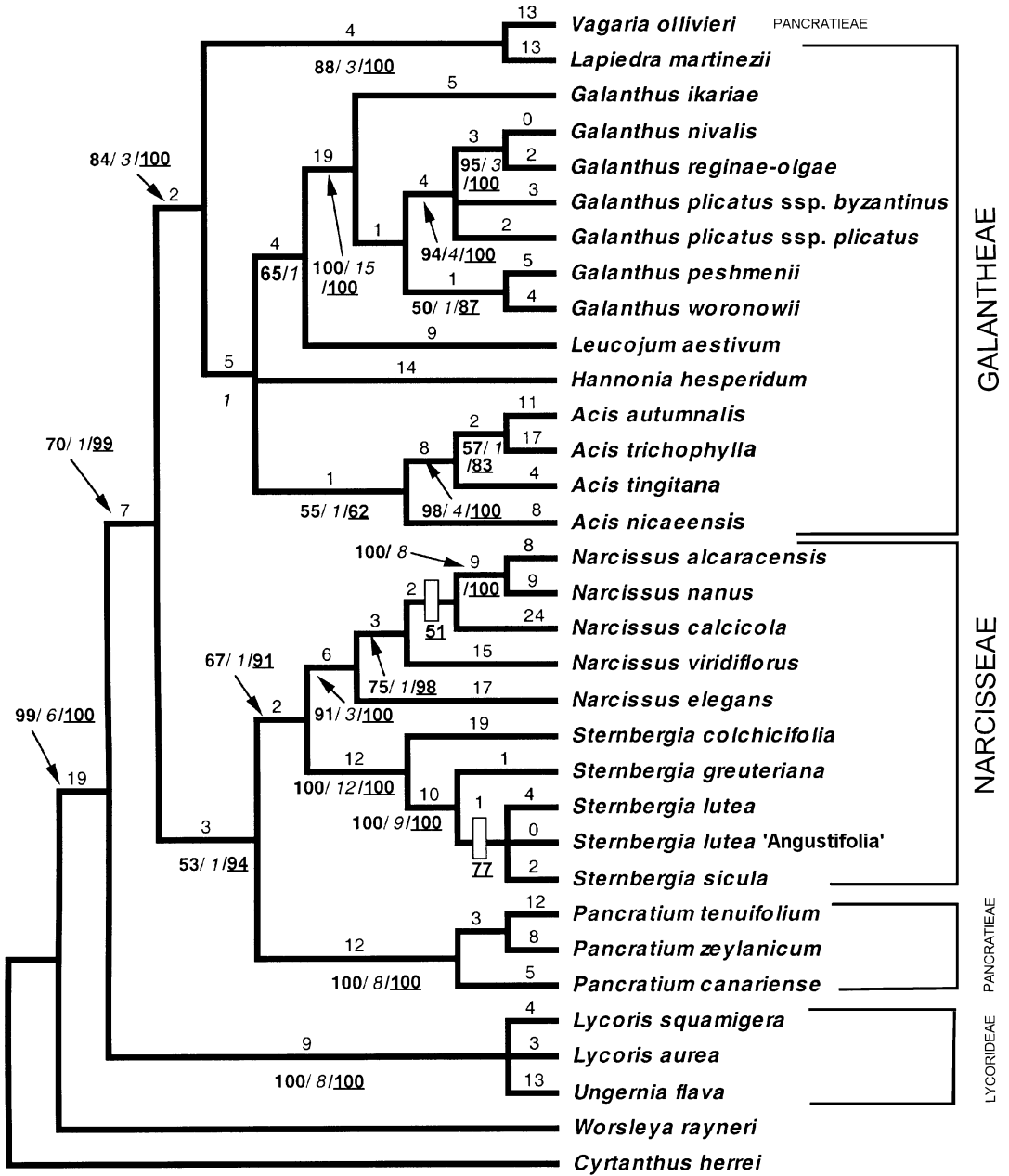


FIG. 1. One of eight equally most parsimonious trees based on plastid *ndhF* data. Numbers above branches are branch lengths. Numbers below branches or indicated by arrow are BS support percentages/decay indices (italic)/PP score (if  $\geq 50\%$ ) based on 9900 trees from Bayesian analysis (underlined). A vertical white bar indicates branches that collapse in the strict consensus of all four trees.

*ndhF* alone (Fig. 1) was lost. *Pancratium* was sister to a *Narcissus*/*Sternbergia* clade in five of the six trees. While *Hannonia*, *Lapiedra*, and *Vagaria* were placed within the Galanthaceae clade in four of the six trees, their terminal resolution varied among the four trees. These three genera were grouped within a Narcisseae/Pantratieae clade in the other two trees. In one tree (not shown), *Pancratium* was sister to both *Narcisseae*

and *Galanthaceae*, a topology found only by the combined data set. In the tree shown (Fig. 3), long branch attraction between *Vagaria* and *Lapiedra* was again a distinct possibility.

Bayesian analysis of the combined data set, retaining the respective models of nucleotide substitution previously applied to each partition, produced a fully resolved 50% majority rule consensus tree that con-

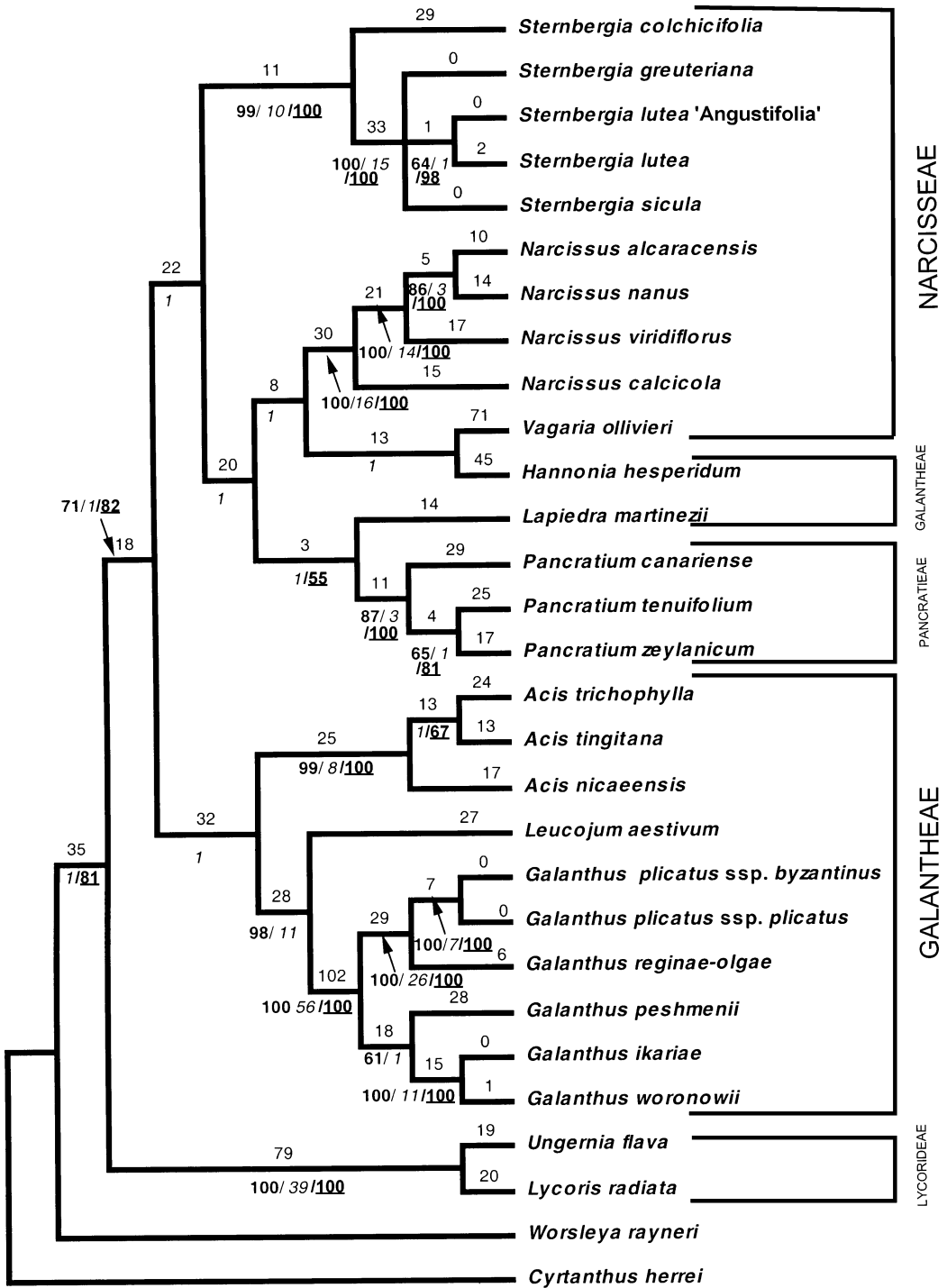


FIG. 2. Single most parsimonious tree based on rDNA ITS data. Numbers above branches are branch lengths. Numbers below branches are BS support percentages/decay indices (italic)/ PP score (if  $\geq 50\%$ ) based on 9800 trees from Bayesian analysis (underlined).

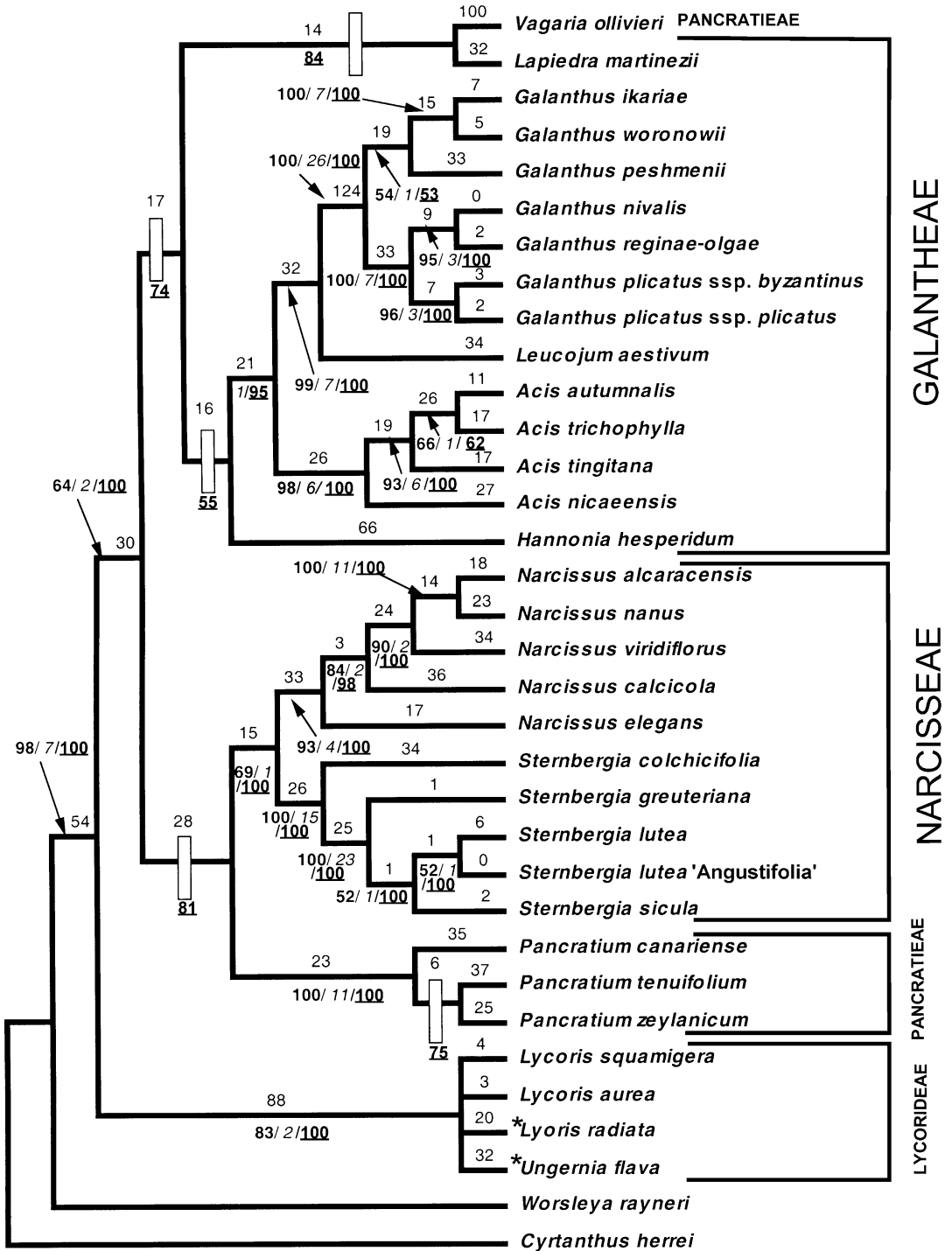


FIG. 3. One of six equally parsimonious trees based on combined plastid *ndhF* and rDNA ITS sequences. Numbers above branches are branch lengths. Numbers below branches are BS support percentages/decay indices (italic)/PP score (if  $\geq 50\%$ ) based on 9700 trees from Bayesian analysis (underlined). A vertical white bar indicates branches that collapse in the strict consensus of all six trees.

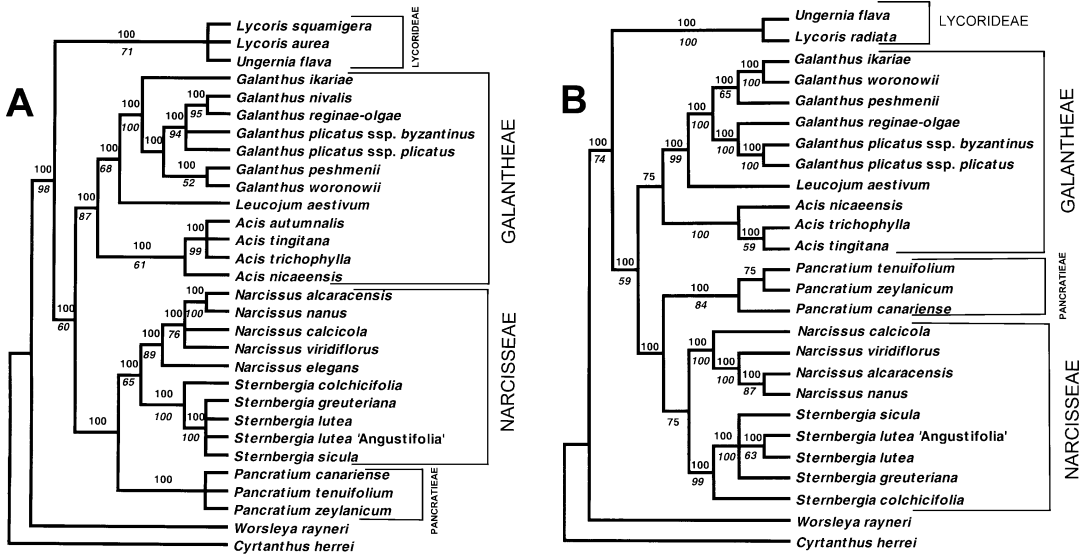


FIG. 4. 50% majority rule consensus trees from parsimony analyses of plastid *ndhF* and rDNA ITS, with *Hannonia*, *Lapiedra* and *Vagaría* deleted from the matrices. A. Consensus of sixteen *ndhF* trees. B. Consensus of four ITS trees. Numbers above branches are consensus intervals; number below (italic) are BS percentages.

formed in topology to one of the six parsimony trees (Fig. 3), except for the resolution of a sister relationship between *Lycoris radiata* and *Ungernia flava* (thereby rendering *Lycoris* paraphyletic). Despite a PP = 100, this should be considered spurious as *L. radiata* was the only *Lycoris* species for which an ITS sequence was obtained. The sister relationship of *Pancratium* and Narcisseae had a PP = 81. A Galantheae clade containing *Hannonia*, *Lapiedra*, and *Vagaría* had a PP = 74. *Vagaría* and *Lapiedra* were sister genera (PP = 84), and *Hannonia* as sister to *Acis*/*Galanthus*/*Leucojum* received the lowest PP in the tree = 55. The genera with BS > 90% in the parsimony analysis had PP = 100 (*Acis*, *Galanthus*, *Lycorideae*, *Narcissus*, *Pancratium* and *Sternbergia*).

**Reanalyses with *Hannonia*, *Lapiedra*, and *Vagaría* Removed.** Because the incongruence between the *ndhF* and ITS phylogenies appeared attributable to the inclusion of *Lapiedra*, *Hannonia*, and *Vagaría*, we reanalyzed both matrices separately and together without these three genera, using both maximum parsimony and Bayesian analysis (for the combined analysis only) as previously described. Four equally parsimonious trees were found with ITS (875 steps long, CI = 0.68, RI = 0.80) and 16 with *ndhF* (370 steps long, CI = 0.83, RI = 0.86). The 50% majority rule consensus trees from each of the two data sets were almost completely congruent (Fig. 4). The results of the ILD on the combined data set with *Hannonia*, *Lapiedra*, and *Vagaría* removed was P = 0.96, indicating quite clearly that virtually all of the incongruence between the two sequence matrices was due to these three genera. The combined data set less these three taxa found four trees (1247 steps, CI = 0.73, RI = 0.81) that were extremely well-resolved

(Fig. 5), differing only in a few terminal positions of species within a genus.

We reanalyzed the combined matrix with parsimony three more times, each time including only one of the three genera. With only *Hannonia* added, one tree was found (1322 steps, CI = 0.71, RI = 0.80). *Hannonia* was resolved as sister to Galantheae, but with BS < 50% (not shown). When only *Lapiedra* is added, a single tree was found (1278 steps, CI = 0.72, RI = 0.81). *Lapiedra* was resolved as sister to *Pancratium* (not shown). In the BS consensus, *Lapiedra* was unresolved within a polytomy inclusive of *Pancratium* and *Narcisseae*. When only *Vagaría* is added to the alignment, two trees were found (1343 steps, CI = 0.71, RI = 0.79) that differ only in the resolution among the three *Pancratium* species. *Vagaría* was resolved as sister to *Galanthus*/*Leucojum*, with < 50% BS support (not shown). If the individual resolutions of these three taxa were imposed as constraints on the combined data set, the cost was an increase of six steps from the trees produced without constraints imposed (Fig. 3).

**Biogeographic Analyses.** Optimal reconstruction of biogeographic scenarios on three *ndhF* trees collapsed to the generic level required 30 dispersals. The three trees differed in their positioning of *Hannonia* relative to *Acis*, *Galanthus*, and *Leucojum*, and only one is shown (Fig. 6A). DIVA found 32 equally optimal area reconstructions, with most of the ambiguity at the three innermost nodes (Fig. 6A). The first event was a vicariance that separated the ancestor of Lycorideae (Central and East Asian distribution) from the rest of the Eurasian clade, the ancestral distribution of which was restricted to North Africa and either the Central



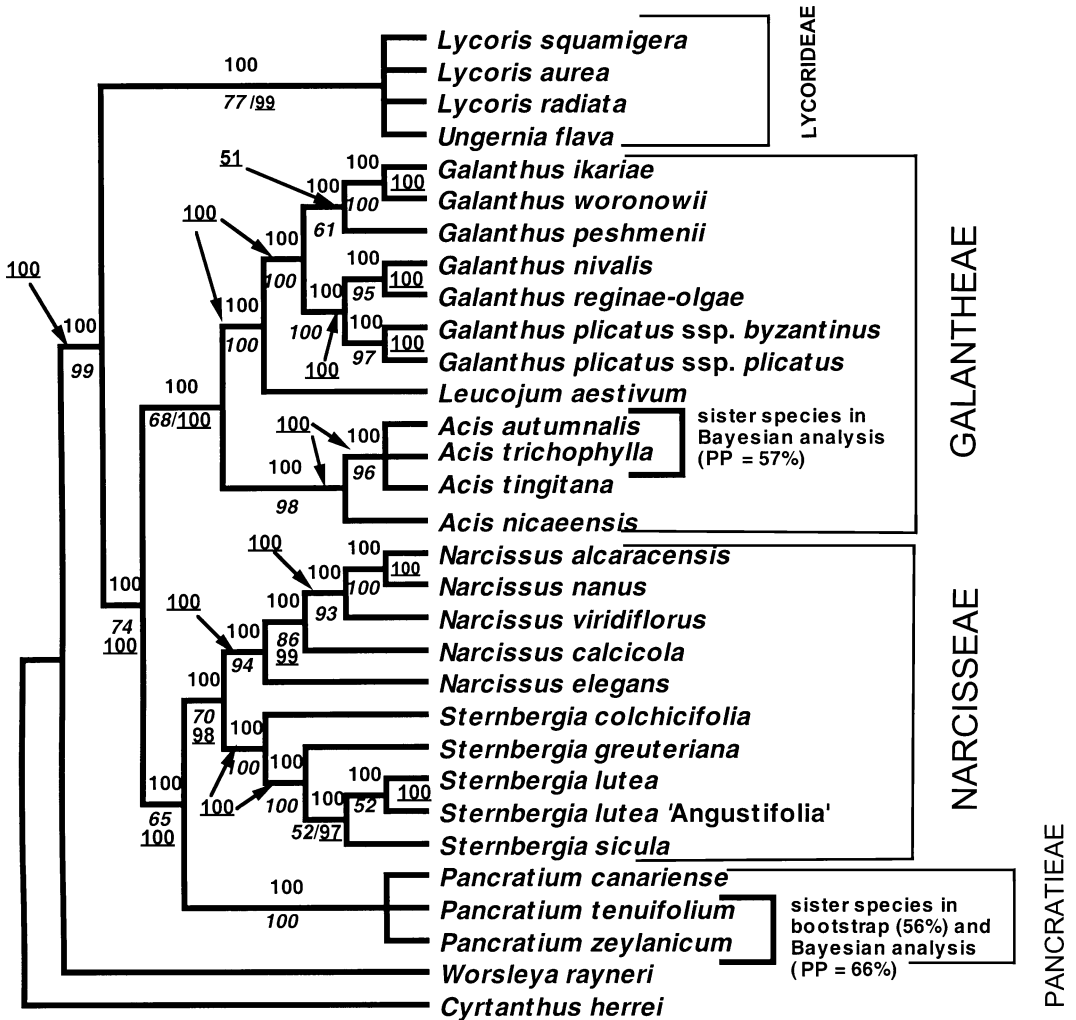


FIG. 5. 50% majority rule consensus tree of four equally parsimonious trees from parsimony analysis of combined plastid *ndhF* and rDNA ITS with *Hannonia*, *Lapiedra* and *Vagaría* deleted from the matrices. Numbers above branches are consensus intervals; number below branches (italic) are BS percentages; underlined numbers are PP scores from 1,000,000 generations of Bayesian analysis.

or Western Mediterranean. The next vicariance separated the North African ancestor of the Galantheae (including *Vagaría*), with subsequent dispersal to the Caucasus and non-Mediterranean Europe, from the ancestor of Narcisseae/*Pancratium* in either the Western or Central Mediterranean. These two areas remained the two alternative optimal areas for the ancestor of the Narcisseae.

One of three trees found with the combined sequence data without *Hannonia*, *Lapiedra*, and *Vagaría* included (Fig. 5) collapsed to the generic level required 29 dispersal events (Fig. 6B). DIVA found a total of 24 alternative scenarios, with most of the ambiguity concentrated in the Galantheae (Fig. 6B). Without *Hannonia*, *Lapiedra*, and *Vagaría* included, DIVA hypothesized a Central or Western Mediterranean/Central or East

Asian ancestral area for the entire Eurasian clade. The first event was a vicariance event that separated the ancestor of Lycorideae (Central and East Asian distribution) from the rest of the Eurasian clade, the ancestral distribution of which was restricted to the central or western Mediterranean. This ancestral distribution was then retained for the ancestors of Pancratieae and Narcisseae, with subsequent dispersal to the broad range now encompassed by both tribes. The area optimization for the ancestral node of Galantheae was highly ambiguous, and included all possible combinations of either the Central and Western Mediterranean with the Caucasus or non-Mediterranean Europe, as well as isolation in the Central Mediterranean. Subsequent dispersal into North Africa and Sicily took place (*Acis*). Although considerable ambiguity was re-

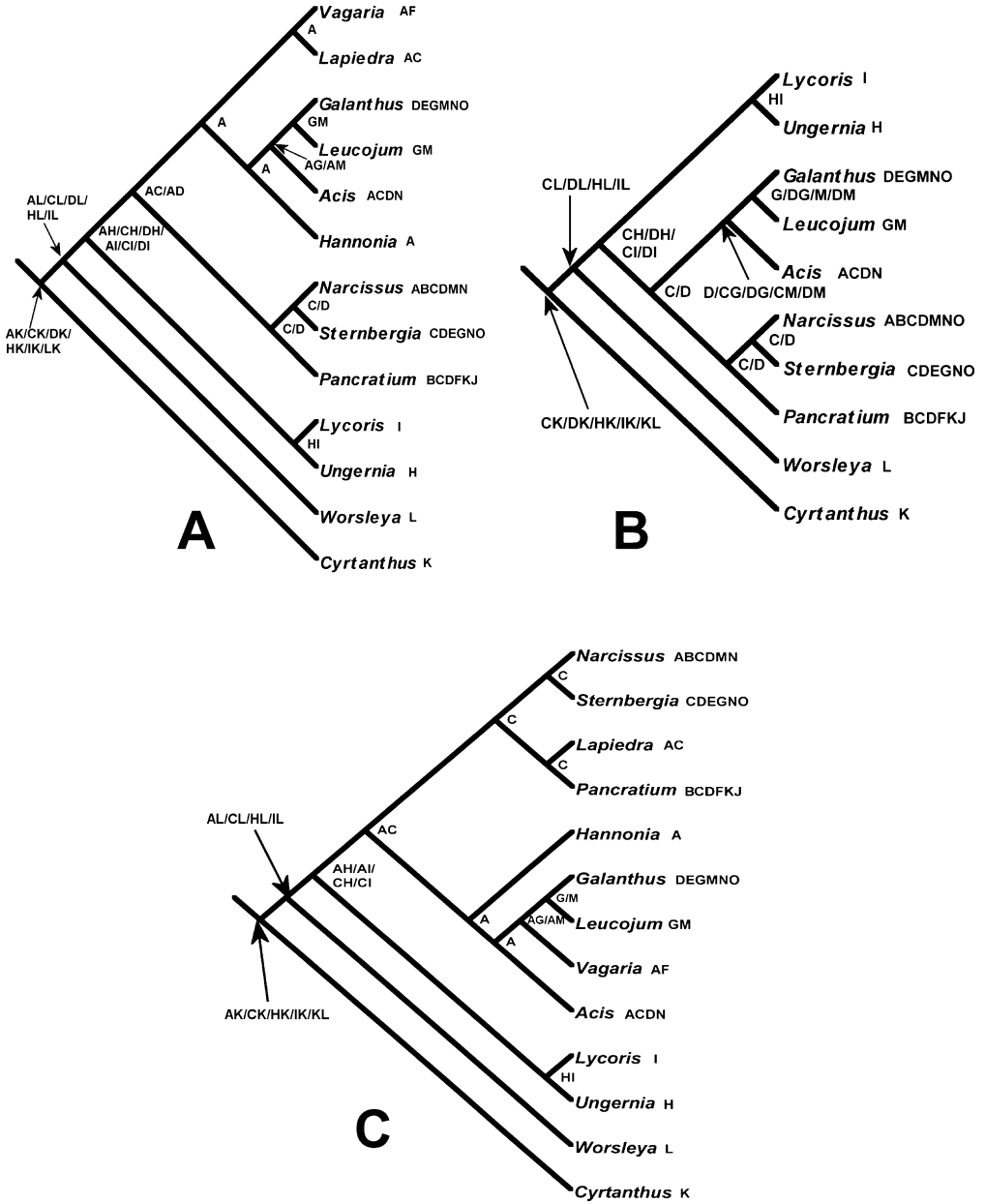


FIG. 6. Selected trees from phylogenetic analyses of combined *ndhF* and ITS sequence matrices, collapsed to the generic level, and analyzed by dispersal vicariance analysis. Area optimizations assigned are indicated by letter codes. A. One of four *ndhF* trees. B. Tree from Fig. 5. C. Constrained combined *ndhF* and ITS topology incorporating the individual resolutions of *Hannonia*, *Lapiedra* and *Vagaria*. A, North Africa (Morocco, Algeria); B, Canary Islands; C, Western Mediterranean (Iberian peninsula, i.e., Spain and Portugal); D, Central Mediterranean (southern France and Italian peninsula); E, Eastern Mediterranean (Greece, Aegan Islands, Crete, western Turkey); F, Middle East (Syria, Israel, Jordan, Iraq, northern Saudi Arabia); G, Caucasus (western Turkey, Armenia, Georgia); H, Central Asia (Turkmenistan, Uzbekistan, Tajikistan, Kirzigitan and Kazakhstan); I, East Asia (China, Japan, Korea); J, South Asian (Indian, Sri Lanka); K, sub-Saharan Africa; L, America; M, non-Mediterranean Europe; N, Sicily; O, Balkans. Letter codes separated by a forward slash represent alternative optimizations of area along that branch.

tained at the ancestral node of *Galanthus* and *Leucojum*, the latter ultimately was restricted to the Caucasus and non-Mediterranean Europe, and *Galanthus* expanded into the Balkans and Eastern Mediterranean.

We also analyzed a constrained topology wherein *Hannonia*, *Lapiedra*, and *Vagararia* were placed in the combined tree (Fig. 6B) according to positions resolved when each was added in turn to the combined matrix (Fig. 6C). The optimization required 32 dispersals, and 16 alternative scenarios were found by DIVA. This scenario indicated a major vicariance at the root node of Narcisseae/*Pantratum* + *Lapiedra* (Western Mediterranean) and (Galantheae + *Vagararia*)/*Hannonia* (North Africa). It should be noted, however, that the supporting tree for this scenario was six steps longer than the most parsimonious trees found by the combined analysis (Fig. 3).

Our alignment of Lledó et al.'s (2004) ITS sequences of *Acis*, *Galanthus*, and *Leucojum*, using our *Hannonia hesperidum* sequence as outgroup [based on its position in both the *ndhF* (Fig. 6A) and "forced" combined tree (Fig. 6C)] was 707 characters long, of which 336 were parsimony informative). Five trees were found of 934 steps, CI = 0.68 and RI = 0.84. They differed only in the resolution within *Acis*, and one of them matched the ingroup resolution in the ITS tree shown by Lledó et al. (2004). All were tested with DIVA, and one of the five (Fig. 7) generated half the number of alternative scenarios (24) as the other four trees (48), and required 23 dispersals. That tree will be the focus of our discussion.

## DISCUSSION

Lledó et al. (2004) combined the plastid matrices of Meerow et al. (1999; *rbcL*, *trnL-F*) and Ito et al. (1999, *matK*) for the entire Amaryllidaceae, using a single species each of every genus in the Eurasian clade except *Ungernia* and *Hannonia*, and recovered a somewhat different phylogeny for the clade than Meerow et al. (1999). As in Meerow et al. (1999), Lycorideae was sister to the rest of the Eurasian clade, and *Galanthus* and *Leucojum* were sister genera. A sister relationship between *Narcissus* and *Sternbergia* was found in at least one tree, but without strict consensus support and < 50% BS (versus *Pantratum* and *Sternbergia* with 74% BS in Meerow et al. 1999). *Lapiedra* was grouped as sister to *Vagararia* with a 70% BS (versus *Lapiedra* and *Narcissus* with < 50% support in Meerow et al. 1999).

Our *ndhF* phylogeny (Fig. 1) is congruent with Lledó et al.'s (2004) more skeletal phylogeny of the Eurasian clade based on *matK* (Ito et al. 1999) and *rbcL* and *trnL-F* (Meerow et al. 1999), but with increased support for some of the internal nodes. The Eurasian clade (BS = 99%) consists of three monophyletic groups: Lycorideae (*Lycoris* and *Ungernia*), Galantheae sensu Meerow and Snijman (1998) with the addition

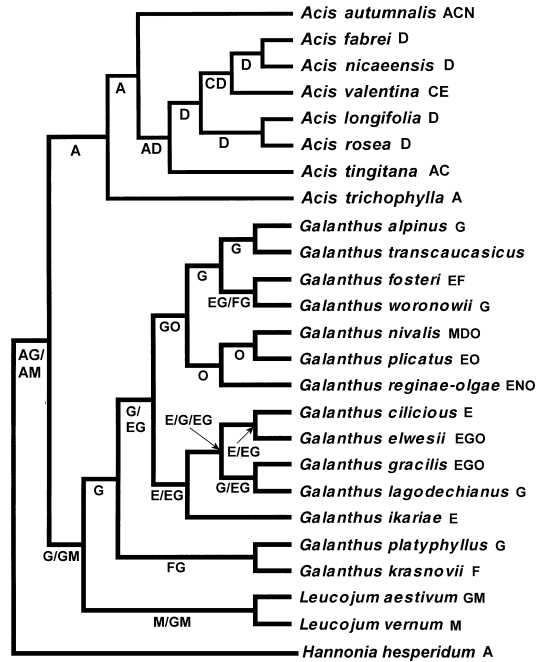


FIG. 7. One of four most parsimonious trees found by cladistic analysis of sequences of tribe Galantheae from Lledó et al. (2004) with area optimizations (letter codes) assigned by dispersal-vicariance analysis. A, North Africa (Morocco, Algeria); C, Western Mediterranean (Iberian peninsula, i.e., Spain and Portugal); D, Central Mediterranean (southern France and Italian peninsula); E, Eastern Mediterranean (Greece, Aegan Islands, Crete, western Turkey); F, Middle East (Syria, Israel, Jordan, Iraq, northern Saudi Arabia); G, Caucasus (western Turkey, Armenia, Georgia); M, non-Mediterranean Europe; N, Sicily; O, Balkans. Letter codes separated by a forward slash represent alternative optimizations of area along that branch.

of *Vagararia* (BS = 84%), and Narcisseae (*Narcissus* and *Sternbergia*) + *Pantratum*. BS support for the latter clade is the weakest in the tree (53%, the same as Lledó et al. 2004), and even the sister relationship of *Sternbergia* and *Narcissus* is not strongly supported (67%; vs. < than 50% in Lledó et al. 2004). In Lledó et al.'s (2004) larger analysis of *Galanthus* and *Leucojum* using plastid *matK* and ITS, it would appear that the resolution of *Lapiedra* and *Vagararia* was constrained by including them in a large outgroup with one species each of *Narcissus*, *Sternbergia*, and *Pantratum*, despite the fact that their overall analysis of Amaryllidaceae in the same paper, using previously published plastid sequences from Ito et al. (1999) and Meerow et al. (1999), indicated that the two former taxa were more closely allied to Galantheae.

Both matrices group Lycorideae as sister to the rest of the Eurasian clade. Both are also congruent with Lledó et al.'s (2004) segregation of *Acis* from *Leucojum*, with very strong support in ITS (Fig. 2). The sister status of *Narcissus* and *Sternbergia* is also supported by

*ndhF* alone and in combination with ITS, although not by ITS alone unless *Hannonia*, *Lapiedra*, and *Vagaría* are removed from the matrix (Fig. 4B). Both plastid *ndhF* and ITS ally *Pancreatium* with *Narcissus* and *Sternbergia* (Figs. 1, 2).

The most significant incongruence among the trees generated by *ndhF* and ITS respectively is the phylogenetic position of three monotypic (*Lapiedra*, *Hannonia*) or small (*Vagaría*) genera. Plastid sequences (Fig. 1, this paper; Lledo et al. 2004) clearly ally these genera with the Galantheae (BS = 84%, PP = 100%), albeit based on only a few base substitutions, while ITS indicated a relationship with Pancreatiae sensu Müller-Doblies and Müller-Doblies (1996) and Narcisseae sensu Traub (1963) but without support (Fig. 2). Bayesian PP scores are also much lower at the deeper nodes of the ITS tree (Fig. 2) relative to the *ndhF* topology (Fig. 1). Which is a more accurate estimate of phylogeny, and what are the reasons for the incongruence?

Incongruence between or among independent data partitions in molecular systematics is more the rule than the exception (Rodrigo et al. 1993; Hipp et al. 2004). Topological differences between gene phylogenies of the same organisms can arise due to sampling or analytical error (Hipp et al. 2004). Genealogical discordance (Baum et al. 1998) between independent character sets can be caused by lineage sorting (Maddison 1997; Wendel and Doyle 1998; Avise 2000) and hybridization (Dumolin-Lapégue et al. 1997; Rieseberg 1997; McKinnon et al. 1999; Avise 2000). Rokas et al. (2003) go so far as to suggest that a minimum of 20 gene sequences are necessary to overcome incongruent phylogenetic signals.

ITS sequences in the Eurasian clade are marked not only by great divergence among the genera, but the most significant amount of paralogy that we have yet encountered in the Amaryllidaceae. The reasons for paralogous variation in the ITS region have been ascribed to factors as diverse as hybridization, polyploidy, agamospermy, and slow concerted evolution (Buckler and Holsford 1996a, b; Campbell et al. 1997; Fierres Aguilar et al. 1999; Booy et al. 2000; Kita and Ito 2000; Vasquez et al. 2000; Gernandt et al. 2001). Álvarez and Wendel (2003) reviewed the entire suite of issues with ITS as a reliable phylogenetic marker and concluded that these collectively reduce the robustness of phylogenies based on ITS alone. Bailey et al. (2003) were more optimistic, and suggested several methods by which polymorphisms in ITS sequences could be tested for phylogenetic signal.

Interspecific hybridization has been documented in *Lycoris* (Bose and Flory 1963), and *Narcissus* (Fernandes 1951), and is at least suspected in *Galanthus* (Davis 1999; Lledo et al. 2004). Polyploidy is less of an issue; chromosome number has decreased rather than increased in more cases in *Narcissus* (Fernandes 1967,

1968b) and *Lycoris* (Kurita 1986, 1987a, b, c, 1989). Constraints on the homogenization of rDNA sequences via concerted evolution include large population size—evident in at least some species of these genera (pers. obs.), and rapid or recent speciation (Gernandt et al. 2001). How significant was paralogous variation among the taxa included in our analysis? It characterized one of three *Acis* spp., *Lapiedra martinezii*, one of four *Narcissus* spp., three of four *Sternbergia* spp., and both *Lycoris radiata* and *Ungernia flava*. All *Galanthus*, *Leucojum* and *Pancreatium* spp. were directly sequenced from the initial PCR without any problem. There is no indication that paralogy had a significant effect on the results of our analyses. In our initial tests, all ITS paralogs found for any one species clustered together as a single clade.

Maternally inherited plastid DNA could fail to accurately position taxa that arose via reticulation (Small et al. 2004). Though there is no evidence that any genus of the Eurasian clade has a hybrid origin, it is a possibility that cannot be positively ruled out, given the “reticulate” history of the Mediterranean region (Sanmartín et al. 2001; Sanmartín 2003). The possibility of reticulation was raised by Meerow et al. (2000) to account for the polyphyletic resolution of two genera of endemic American Amaryllidaceae with ITS. Ribosomal DNA on the other hand may result in inaccurate phylogenetic reconstructions due to the presence of pseudogenes that become preferentially amplified over functional loci (Buckler and Holsford 1996; Buckler et al. 1997; Hartman et al. 2001; Mayol and Rossello 2001; Chase et al. 2003; Bailey et al. 2003; Small et al. 2004). However, the high level of congruence between our two gene phylogenies when *Hannonia*, *Lapiedra*, and *Vagaría* are dropped from the analyses (Figs. 4, 5), and the high bootstrap support for all genera represented by more than one species, suggest that the ITS alignment has accurate phylogenetic signal for the rest of the Eurasian clade.

There is little doubt that the incongruence between our two partitions is due to *Hannonia*, *Lapiedra*, and *Vagaría*. Removal of these three taxa not only increases support in general for the other clades in the trees, but eliminates virtually all of the incongruence between the partitions ( $p = 0.96$  vs.  $0.07$  when the three are included). What is extraordinary is how the addition of merely one of these three genera back into the combined data set causes the well-resolved and well-supported topology to lose BS support.

Long-branch attraction (Felsenstein 1978) is a possibility in the ITS topology (Fig. 2). While in the *ndhF* trees, *Vagaría* and *Lapiedra* form a sister clade to the Galantheae, in the ITS tree it is *Vagaría* and *Hannonia* that are sister genera, forming a sister clade to Narcisseae. If long branch attraction were at work, it would be expected that a likelihood approach, in our case

Bayesian analysis, would not be congruent with parsimony for the position of these three genera (Siddall 1998; Sanderson and Shaffer 2002). In the case of ITS, neither parsimony nor Bayesian analysis is able to resolve the position of *Hannonia* and *Vagaría* with even 50% confidence (Fig. 2), while the sister relationship of *Vagaría* and *Lapiedra*, resolved with 88% BS support by parsimony analysis of the *ndhF* matrix, receives a PP = 100% (Fig. 1). Long branch attraction thus seems a credible explanation for the position of *Vagaría* and *Hannonia* in the ITS phylogeny.

Another explanation for the incongruence attributable to these three, primarily North African genera, in the absence of any evidence of hybrid origin or horizontal transfer (Amáñile-Cuevas and Chicurel 1993; Kidwell 1993; Syvanen 1994), is lineage sorting (Wendel and Doyle 1998). If lineage sorting occurred, it must have taken place in the ancestral lineages that ultimately led to *Narcisseae/Pancreátium* and *Galantheae*. Analyzing our combined matrix with each of the three problematic genera added in turn (Fig. 6C), sorts them into both main clades resolved with the three absent (Fig. 5). Both *Hannonia* and *Vagaría* (Fig. 6C) are positioned within the *Galantheae*, while *Lapiedra* is sister to *Pancreátium* (Fig. 6C). The usual expectations of lineage sorting are that 1) it is much more likely for nuclear genes than organellar (Moore 1995) and 2) that it should be expected at lower taxonomic ranks. In the light of the first expectation, one might argue for acceptance of the plastid *ndhF* resolution of these three genera over the topology supported by ITS. However, exceptions to both expectations have been noted (Mason-Gamer et al. 1995; Hoelzer 1997; Wendel and Doyle 1998). Lineage sorting could also explain the marked divergence we see in ITS sequences among the major clades of the Mediterranean-centered genera of the Eurasian group, as evidenced by the long branch lengths and low BS support (Fig. 2).

*Hannonia*, *Lapiedra*, and *Vagaría* have certain shared morphological characteristics. The first two genera are monotypic; *Vagaría* arguably contains two species (Meerow and Snijman 1998). All three have very generalized floral morphology (white tepals with a green abaxial keel, actinomorphic), and solid scapes. *Vagaría* and *Lapiedra* have lorate leaves with a conspicuous adaxial midrib stripe (the latter observed as well in some *Galanthus*, *Lycoris*, and *Pancreátium*); *Hannonia* has linear, almost filiform leaves similar to those of *Acis*. *Vagaría* has broad, appendaged staminal filaments and a long floral tube; the filaments of both *Lapiedra* and *Hannonia* are filiform, and a floral tube is obsolete or very short. Moreover, both are entirely (*Hannonia*) or partially (*Vagaría* and *Lapiedra*) distributed in North Africa. *Lapiedra* is diploid, with the ancestral chromosome number for the *Amaryllidaceae* ( $2n = 22$ ; Fernandes 1952); no reports are available for *Hannonia* or *Vagaría*. Fernandes

(1952) remarked on the similarities of the karyotype of *Lapiedra* to that of both *Acis* (as *Leucojum*) and *Galanthus*. Müller-Doblies and Müller-Doblies (1978b) moved *Lapiedra* and *Hannonia* from *Galantheae* to an alliance with *Pancreátium* on the basis of seed and internal bulb morphology. Such a relationship for *Lapiedra* is credible, though not well supported, based on our analyses (Figs. 2, 6C), but is never resolved for *Hannonia*. Their shared character states, presence in North African, and the difficulty in resolving their phylogenetic position suggest that the three genera may represent relict taxa from the early differentiation of the Mediterranean clades, particularly the *Galantheae*. Certainly, their position as resolved by maternally inherited *ndhF* sequences points in that direction (Fig. 1).

Area optimization of the clade without *Hannonia*, *Lapiedra*, and *Vagaría* included (Fig. 6B) results in much higher ambiguity at the ancestral node of *Galantheae*, clearly indicating that information is lost by excluding these taxa. In the absence of any further sequence data at this time, two of the trees analyzed with DIVA, the *ndhF* tree (Fig. 6A) and the "forced" topology (Fig. 6C), will be used as the background for discussion of the biogeographic history of the Eurasian clade. Congruent areas optimizations between the two trees are thus perceived as better supported hypotheses than optimizations occurring in only one of the trees. We believe that the incongruence between our plastid and nuclear two data sets, clearly attributable to three, possibly relict taxa, as well as the breakdown of concerted evolution for rDNA encountered in some of Eurasian *Amaryllidaceae*, is inextricably linked to the complex paleohistory of the Mediterranean.

**Biogeographic History of the Eurasian Clade.** The Mediterranean basin formed during the Tertiary due to the confluence of the African and Eurasian tectonic plates and several lesser plates (Dewey et al. 1973; Dercourt et al. 1986; Krijgsman 2002). The modern western Mediterranean land mass (Iberia, France, Italy in part) is thought to date to the Eocene, ca. 35 MYR, while the eastern area (Aegean) formed during the mid-Miocene, ca. 16 MYBP (Krijgsman 2002). It has been suggested that land bridges existed on and off across the entire Mediterranean basin during the Oligocene and Miocene, alternately separating and re-joining the Tethys and Paratethys seas (Rogl and Steininger 1983; Osterbroek and Arntzen 1992). Such a scenario would have allowed dispersal across the entire region, followed by east-west vicariances when the connections were disrupted by rising sea levels. The cyclic nature of these events resulted in complex patterns of fragmentation and coalescence of Mediterranean biotas (Oosterbroek and Arntzen 1992; Martin-Piera and Sanmartin 1999). Thus, the biogeography of the Mediterranean region can be considered "reticulate" (Ronquist 1997), characterized by successive periods of vicariance and dis-



persal (Sanmartín et al. 2001). As a result, Mediterranean taxa often exhibit disjunct distributions between the eastern and western Mediterranean, or even between Central Asia and the western Mediterranean. Such "Kiermack" disjunctions (Ribera and Blasco-Zumeta 1998) are characterized by a sizable percentage of endemic taxa in northwest Africa, the islands of the Mediterranean, the Middle East, the Balkans, and the Caucasus, which is precisely the case for most of the genera of the Eurasian clade (Fernandes 1968a; Davis 1999; Lledó et al. 2004).

Both dispersal-vicariance scenarios suggest an ancestral distribution for the Eurasian clade encompassing Central Asia (current distribution of *Ungernia*) or East Asia (current distribution of *Lycoris*) and either North Africa or Western Mediterranean (Fig. 6A, C). In the *ndhF* tree alone (Fig. 6A), the Central Mediterranean is an additional alternative. Central Asia seems the more likely of the two Asian optimizations, as a Central Asian/Mediterranean pattern is well known in European biogeography (Ribera and Blasco-Zumeta 1998). Similar distributions have been reported for a number of plant taxa (Braun-Blanquet et al. 1957; Davis and Hedge 1971; Thorne 1972; Willis 1996), and their origins are considered pre-Pleistocene. Further support for Central Asia is the fact that *Ungernia* species are elements of temperate steppe vegetation (Artyushenko 1970), which has had a strong continuity with similar vegetation in the Mediterranean throughout the late Tertiary and Quaternary (Ribero and Blasco-Zumeta 1998; Sanmartín 2003), while *Lycoris* species occur in mesic warm temperate and subtropical forests (Kurita 1986; Zhanhe and Meerow 2001). In the absence of a fossil record, it is impossible to date this primary vicariance event within the clade, but it must have occurred early enough to allow the ancestor of *Lycoris* to reach East Asia. Sanmartín (2003) presented a somewhat similar scenario for the genera of the beetle subfamily Pachydemiae, in which the Central Asian endemic genus *Hemictenius* represents a vicariance from North Africa via Sicily. Voelker (1999) suggested that a land bridge existed before the Pleistocene between Sicily and Tunisia. Much of the subsequent diversification within the Eurasian clade after the origin of the Lycorideae took place sympatrically in the western and/or central Mediterranean, as well as in North Africa. Both *ndhF* (Fig. 6A) and our "forced" topology (Fig. 6C) suggest a subsequent vicariance between North Africa and the Mediterranean at the root node of the rest of the clade after the vicariance of Lycorideae. One might speculate that an African lineage was lost by extinction in the early history of the entire clade, but there is no evidence to indicate such occurred. However, the Iberian microplate (western Mediterranean) was part of Africa from the late Cretaceous to the Eocene (110–54 MYBP; Dewey et al.

1973; Dercourt et al. 1986) and didn't connect with Eurasia until the late Eocene (35 MYBP). Thus, the vicariance event that separated Lycorideae from the rest of the clade may date to the early Tertiary. Without any confirming fossil record, which is absent for Amaryllidaceae in this region, any dating hypothesis is ultimately speculative.

There were two opportunities for biotic interchange between the Mediterranean and North Africa during the later Tertiary. The first, during the mid-Miocene (14–13 MYBP), was via a landmass that separated the Tethys and Paratethys seas (Rogl and Steininger 1983), corresponding roughly to the eastern Mediterranean/Balkan region (Sanmartín 2003). This was eliminated by the late Miocene (10 MYBP) by interposing water bodies. This may have engendered the early vicariance event that separated the North African ancestor of *Acis* from the ancestor of *Galanthus* and *Leucojum* located in either the Caucasus or the Caucasus and extra-Mediterranean Europe (Fig. 7), with subsequent isolation of the ancestor of *Galanthus/Leucojum*.

In the mid-Pliocene, ca. 6 MYBP, connections between North Africa and the western Mediterranean (Iberian peninsula) were re-established during a period of hyper-aridity (Krijgsman 2002), and sundered 1 MY later by the Strait of Gibraltar. The vicariance event between the African and Mediterranean species of *Acis* (Fig. 7) may reflect the loss of this migration corridor. This later event may also correlate with patterns of intraspecific variation in a decidedly western Mediterranean genus like *Narcissus* wherein a number of species co-occur in both Spain or Portugal and North Africa, but for which subspecific taxa in each region have been described (Fernandes 1968a). Sympatric speciation within *Acis* (Fig. 7) took place twice in the eastern Mediterranean (*A. longifolia*-*A. rosea* and *A. fabrei/A. nicaeensis*). Terminal taxa eventually dispersed to the Eastern Mediterranean (E) and Sicily (N).

The ancestor of the *Leucojum/Galanthus* clade represents a vicariance between North Africa and the Caucasus (or the Caucasus and non-Mediterranean Europe). The ancestor of *Galanthus*, however, was isolated in the Caucasus, while that of *Leucojum* either evolved in non-Mediterranean Europe or retained the ancestral broad distribution. When and how did the ancestor of *Galanthus/Leucojum* reach the Caucasus? In Sanmartín's (2003) biogeographic scenario for the beetle subfamily Pachydemiae, dispersal to the Caucasus via the Iran-Afghanistan region was inferred (an area within which no Amaryllidaceae is known), but only in the more terminal nodes of the molecular-based tree. In the Miocene, the Great Caucasus was an island situated centrally within the Paratethys Sea (Ruggieri 1967; Kholodov and Nedumov 1996) that in the late Miocene was connected to Asia Minor (Vereshchagin 1959; Tuniyev 1990). This would correspond well with

our scenario (Fig. 7) of an isolated Caucasian lineage that later dispersed to the eastern Mediterranean.

Entries into northern Europe, which on the whole are rare within the clade, are likely very recent, as the last full glacial (10,000–100,000 years ago) would have maintained their distribution in the Iberian, Italian, and Balkan peninsulas, as well as a linkage belt encompassing southern France, northern Italy and Slovenia (Willis 1996). This would favor the Caucasus as the likely area of origin for the Galantheae, versus non-Mediterranean Europe (Fig. 7), which would have been largely covered by ice. The Near East and south-west Asia may have been warmer than these peninsulas as well as drier (Willis 1996), which would have favored geophytic plants. Allopatric derivation of subspecific taxa in *Acis*, *Galanthus*, *Narcissus*, and *Sternbergia* may have occurred during that time.

*Galanthus* (Fig. 7) overall dispersed twice into the Middle East (F) and the eastern Mediterranean (E), and three times into the Balkans (O). The ancestor of *G. platyphyllus* and *G. krasnovii* dispersed to the Middle East from the Caucasus, followed by vicariance between the terminal taxa. The Balkan subclade (*G. nivalis*, *G. plicatus*, and *G. reginae-olgae*) speciated sympatrically then dispersed into non-Mediterranean Europe and both the central and eastern Mediterranean, as well as Sicily. Its sister Caucasian subclade (G) also speciated sympatrically then dispersed either to the eastern Mediterranean or the Middle East. The most ambiguous biogeographic scenario within *Galanthus* occurs in the ancestrally eastern Mediterranean or eastern Mediterranean/Caucasian subclade (*G. cilicius*, *G. elwesii*, *G. gracilis*, *G. ikariae*, and *G. lagodechianus*). It is unclear if dispersal or vicariance separated the ancestor of this clade from its Caucasian/Balkan sister group. Subsequent divergences in the subclade could represent sympatric speciation, dispersal, or vicariance within and between the Caucasus, eastern Mediterranean, or both. Additionally, two terminal taxa dispersed to the Balkan region independently.

*Pancretium* is the most widespread of all of the genera in the clade, throughout sub-Saharan Africa to the Mediterranean and the Middle East and into tropical Asia, including one species endemic to the Canary Islands. *Narcissus* is the only other genus of the Amaryllidaceae that occurs in the Canaries (Fernandes 1968a). The Canary Islands are a volcanic archipelago that has never been connected to the mainland (Juan et al. 2000), thus long-distance dispersal must be involved in both cases. The remarkable similarity of the flowers of *Pancretium* to those of *Hymenocallis* Salisb., *Ismene* Salisb., *Pamianthe* Stapf, and *Paramongaia* Velarde, all endemic American genera, led Traub (1963) to ally *Pancretium* with those genera in an informal “infracolony” Pancretioidinae. Meerow and Dehgan (1985) suggested a similar relationship on the basis of

pollen morphology, but it is now clear that these shared character states are homoplastic adaptations (Meerow et al. 1999), ostensibly for sphingid moth pollination (Morton 1965; Bauml 1979; Grant 1983). A single tree from the combined analysis that resolves *Pancretium* as sister to the rest of the Mediterranean-centered genera of the clade (not shown) is noteworthy insofar as it implies a long enough time frame for such a broad distribution of *Pancretium* species to be engendered. The relationship of *Pancretium* to Narcisseae is consistently resolved (Figs. 2–5) but only with weak BS support at best.

The sister relationship between *Narcissus* and *Sternbergia* received highest support in the Bayesian analyses of *ndhF* (Fig. 1) and the combined matrix (Figs. 3, 5), but BS percentages for this resolution in the parsimony analyses never rises above 75%, even when *Hannonia*, *Lapiedra* and *Vagararia* are dropped from the matrices (Figs. 4, 5). They are the only genera within the Mediterranean-centered subclade that express carotenoid pigments in the floral organs. The great diversity of *Narcissus* species occurs on the Iberian peninsula (Fernandes 1968b) and it is the only genus of Amaryllidaceae that has evolved heterostyly (Graham and Barrett 2004). The inclusion of only a few *Narcissus* species in our analyses does not allow for any significant discussion of speciation patterns in that genus (but see Graham and Barrett 2004). Our trees support treating *Sternbergia sicula* as a synonym of *S. lutea*. The taxon we referred to as *S. lutea* ‘*Angustifolia*’ has been variously treated as a variety of *S. lutea* or *S. sicula*.

The most strongly supported subclade in the Mediterranean-centered genera is the sister relationship between *Galanthus* and *Leucojum*. The segregate genus *Acis* is sister to them, but only with weak to moderate BS support. As a group, the three genera are linked by the synapomorphy of pseudo-porandrous anthers (Traub 1963; Meerow and Snijman 1998).

The complex phylogenetic relationships of the Eurasian clade of the Amaryllidaceae reflect the complex paleohistory of the Mediterranean region. After the initial and vicariant separation of the Asian Lycorideae, the early diversification of the rest of the clade was most likely centered in North Africa and the Iberian peninsula (Figs. 6A, C). We hypothesize that lineage sorting took place early in the diversification of the group, after the early vicariance of the ancestor of Lycorideae, but this hypothesis remains to be tested more rigorously. Three genera, *Hannonia*, *Lapiedra*, and *Vagararia*, have likely retained a mosaic of ancestral ITS haplotypes that introduce incongruence into our gene trees of the clade, incongruence that disappears if these taxa are removed from the analyses (Figs. 4, 5).

Ultimately, resolution of the incongruent positions of *Hannonia*, *Vagararia*, and *Lapiedra* would benefit from phylogenetic analysis of multiple single or low-copy

nuclear genes with sufficient phylogenetic information to resolve a well-supported tree topology. Until this is accomplished, we are reluctant to make any tribal assignments for these genera, though plastid sequences support inclusion of all three within Galanthaeae.

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## APPENDIX 1

Species, vouchers, and GenBank accession numbers (or literature citations for previously published sequences) of DNA sequences used in this paper. All vouchers deposited at FTG unless otherwise stated.

*Acis autumnalis* (L.) Herb.—Meerow 2604: *ndhF* AY434489. *A. nicaeensis* (Ardoino) Lledó, A.P.Davis & M. B. Crespo—Meerow 2613: *ndhF* AY747088, ITS AY751419. *A. tingitana* (Baker) Lledó, A.P.Davis & M. B. Crespo—Meerow 2614: *ndhF* AY747089, ITS AY751418. *A. trichophylla* (Schoub.) Sweet—Meerow 2601: *ndhF* AY747090, ITS AY751417.

*Cyranthus herrei* (F. M. Leight.) R. A. Dyer—van Zyl 104 (NBG): *ndhF* AY434484, ITS AY751428.

*Galanthus ikariae* Baker—Meerow 2627: *ndhF* AY747080, ITS AY751432. *G. nivalis* L., Meerow 2608, *ndhF* AY747081. *G. peshmenii* A. P. Davis & C. D. Brickell—Meerow 2609: *ndhF* AY434490, ITS AY751424. *G. plicatus* subsp. *byzantinus* (Bak.) D. A. Webb—Meerow 2600: *ndhF* AY747082, ITS AY751421. *G. plicatus* M. Bieb. subsp. *plicatus*—Meerow 2610: *ndhF* AY747083, ITS AY751422. *G. reginae-olgae* Orph.—Meerow 2611: *ndhF* AY747084, ITS AY751423. *G. woronowii* Losink.—Meerow 2612: *ndhF* AY747085, ITS AY751433.

*Hannonia hesperidum* Braun-Blanquet & Maire—Meerow 2626: *ndhF* AY747086, ITS AY751427.

*Lapidra martinezii* Lag.—Meerow 2607: *ndhF* AY434488, ITS AY751425.



*Leucojum aestivum* L.—Meerow 2599: *ndhF* AY747087, ITS AY751420.

*Lycoris aurea* Herb.—Meerow 2625: *ndhF* AY747091. *L. radiata* Herb.—Meerow 2606: ITS AY751430. *L. squamigera* Maxim—M. W. Chase 2014 (K): *ndhF* AY747079.

*Narcissus alcaracensis* S.Ríos Ruiz, D. Rivera Nuñez, F.Alcaraz Ariza & C. Obón de Castro—Meerow 2616: *ndhF* AY747092, ITS AY751413. *N. calcicola* Mendonca—Meerow 2617: *ndhF* AY747093, ITS AY751414. *N. elegans* Spach—M. W. Chase 617 (K): *ndhF* AY747094. *N. nanus* Steud.—Meerow 2618: *ndhF* AY747095, ITS AY751415. *N. virdiflorus* Schousb.—Meerow 2619: *ndhF* AY747086, ITS AY751416.

*Pancratium canariense* Ker Gawl.—Meerow 1142: *ndhF* AY747097, ITS Meerow et al. 2000. *P. tenuifolium* Hochst. Ex A. Rich—Meerow

2427: *ndhF* AY747098, ITS Meerow et al. 2000. *P. zeylanicum* L.—Preuss s. n. (FLAS): *ndhF* AY747099, ITS AY751431.

*Sternbergia colchicifolia* Walst. & Kit—Meerow 2628: *ndhF* AY747100, ITS AY751408. *S. greuteriana* G. Kamari & R. Artelari—Meerow 2605: *ndhF* AY747101, ITS AY751409. *S. lutea* Ker-Gawl. ex Schult.f.—Meerow 2621: *ndhF* AY747102, ITS AY751411. *S. lutea* 'Angustifolia'—Meerow 2622: *ndhF* AY747103, ITS AY751410. *S. sicula* Tineo ex Guss.—Meerow 2602: *ndhF* AY747104, ITS AY751412.

*Ungernia flava* Boiss. & Haussk.ex Boiss.—M. W. Chase 3640 (K), *ndhF* AY434483, ITS AY751429.

*Vagaría ollivieri* Maire—Archibald et al. 4484 (RSA): *ndhF* AY747078, ITS AY751426.

*Worsleya rayneri* (Hook.f.) Traub & Moldenke—Meerow 2302: *ndhF* AY434477, AY747105, ITS Meerow et al. (2000).