Syst. Biol. 52(3):334–351, 2003

ISSN: 1063-5157 print / 1076-836X online
DOI: 10.1080/10635150390196975

The Systematic Utility of Floral and Vegetative Fragrance in Two Genera of Nyctaginaceae

RACHEL A. LEVIN,[1,4] LUCINDA A. MCDADE,[2] AND ROBERT A. RAGUSO[3]

[1]*Department of Ecology & Evolutionary Biology, University of Arizona, Tucson, Arizona 85721, USA*
[2]*Academy of Natural Sciences, 1900 Ben Franklin Parkway, Philadelphia, Pennsylvania 19103, USA*
[3]*Department of Biology, University of South Carolina, Coker Life Sciences Building, 700 Sumter St., Columbia, South Carolina 29208, USA*
[4]*Present address: 2 Mayo Smith House, Amherst, Massachusetts 01002-2316, USA; E-mail: rlevin@lab.si.edu*

Abstract.— We examined relationships between fragrance and phylogeny using a number of approaches to coding fragrance data and comparing the hierarchical information in fragrance data with the phylogenetic signal in a DNA sequence data set. We first used distance analyses to determine which coding method(s) best distinguishes species while grouping conspecifics. Results suggest that interspecific differences in fragrance composition were maximized by coding as presence/absence of fragrance compounds and biosynthetic pathways rather than when quantitative information was also included. Useful systematic information came from both compounds and pathways and from fragrance emitted by both floral and vegetative tissues. The coding methods that emerged from the distance analyses as best distinguishing species were then adapted for use in phylogenetic analysis. Although hierarchical signal among fragrance data sets was congruent, this signal was highly incongruent with the phylogenetic signal in the DNA sequence data. Notably, topologies inferred from fragrance data sets were congruent with the DNA topology only in the most distal portions (e.g., sister group pairs or closely related species that had similar fragrance profiles were often recovered by analyses of fragrance). Examination of consistency and retention indices for individual fragrance compounds and pathways as optimized onto one of the most-parsimonious trees inferred from DNA data revealed that although most compounds were homoplastic, some compounds were perfectly congruent with the DNA phylogeny. In particular, compounds and pathways found in a few taxa were less homoplastic than those found in many taxa. Pathways that synthesize few volatiles also seem to have lower homoplasy than those that produce many. Although fragrance data as a whole may not be useful in phylogeny reconstruction, these data can provide additional support for clades reconstructed with other types of characters. Factors other than phylogeny, including pollinator interactions, also likely influence fragrance composition. [*Acleisanthes*; character coding; four o'clocks; fragrance; *Mirabilis*; phylogenetics; scent; volatiles.]

Volatile chemicals are emitted by many, perhaps all, living organisms. These volatiles give organisms an odor or fragrance that can be detected to differing degrees by many animals, including humans. In plants, these compounds are the products of several biosynthetic pathways (Fig. 1) that are part of secondary metabolism, and they are emitted from vegetative and reproductive structures (Shreier, 1984; Croteau and Karp, 1991; Dudareva and Pichersky, 2000). The fragrances emitted by plants have many functions, including pollinator attraction and associative learning by these animals and the deterrence of herbivores and pathogens (Berenbaum and Seigler, 1992; Dobson, 1994; Raguso, 2001). However, fragrance may not always be functional; it may be simply a by-product of necessary metabolic processes, or the adaptive context within which fragrance evolved may have changed.

Floral fragrances have long been appreciated by humans, and a relationship between fragrance characteristics and pollinators has been noted since at least the late 19th century (Delpino, 1874; Kerner von Marilaum, 1895). These fragrances are typically blends of volatile compounds produced by more than one biosynthetic pathway. The earliest attempts to describe floral odors characterized them in qualitative, metaphorical terminology (e.g., beetle-pollinated flowers tend to have fruity odors and bat-pollinated flowers may be musty; Faegri and van der Pijl, 1979). However, modern analytical chemistry has facilitated the identification of each of the volatile compounds emitted and improved our understanding of the constituents of fragrances that attract certain pollinators. For example, sulfur-bearing compounds have been found in the odors emitted by most bat-pollinated flowers that have been studied (e.g., Knudsen and Tollsten, 1995; Bestmann et al., 1997). In contrast, some pollination "syndromes" seem to permit more variation (e.g., Miyake et al., 1998; Raguso and Willis, 2003). In such cases, variation in fragrance chemistry may reflect factors other than pollinator attraction or plant defense, including current environmental conditions and evolutionary history.

Thus, in addition to the intriguing role that fragrance plays in plant–animal interactions, aspects of fragrance may reflect phylogenetic history. Fragrance data might be used to infer phylogenetic relationships; alternatively, the evolution of fragrance might be studied by optimizing these characters onto phylogenies inferred from other sources of data. To date, few researchers have rigorously examined the evolution of floral fragrance in a phylogenetic context (but see Azuma et al., 1997, 1999; Barkman et al., 1997; Dobson et al., 1997; Williams and Whitten, 1999; Barkman, 2001), in part because such work demands fragrance data from a reasonable sample of the group of interest. A robust phylogeny inferred from independent data is also required if one wishes to study the evolution of fragrance characters.

Any explicit approach to placing fragrance data in a phylogenetic context requires character coding. Unlike DNA sequence data, which are usually straightforward, fragrance data present challenges similar to those faced in coding the information present in frog or bird calls or in a developmental series (Dusenbery, 1992; Mabee and Humphries, 1993; Cannatella et al., 1998; Wiley, 2000). Descriptive terms such as musty or sweet might be mapped onto a phylogeny, but we now know that odors with these qualities can be assembled from a variety

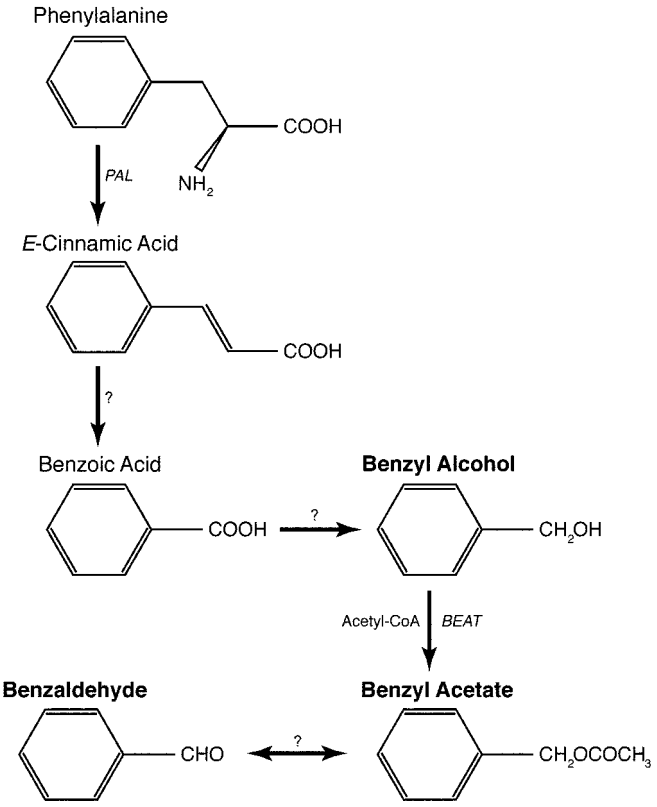

FIGURE 1. Numerous volatiles require a phenylalanine precursor, including those synthesized through the BEAT pathway (BEAT = enzyme acetyl-CoA:benzylalcohol acetyltransferase; Dudareva et al., 1998a). This pathway involves a benzoic acid precursor and forms volatiles that are common in fragrances of Nyctaginaceae and angiosperms in general. Many Nyctaginaceae species emit more than one product from this pathway (e.g., *A. angustifolia* in Fig. 2; also see table 2 in Levin et al., 2001). Volatile products are in bold, and enzymatic steps are in italics: *PAL* = phenylalanine ammonia lyase; ? = enzymes not yet described.

of different chemical compounds. Therefore, it seems best to consider the information content of fragrance in terms of these individual constituents. One approach to such data is to devise a set of binary characters reflecting presence/absence of all observed compounds. However, fragrance also varies quantitatively among plants. Thus, amount of each volatile compound present (i.e., relative or absolute amounts of each compound) might be incorporated in the coding method. Many previous researchers have ignored quantitative differences in volatiles and simply scored presence/absence of compounds or biosynthetic pathways (Barkman et al., 1997; Dobson et al., 1997; Adams, 1999). However, a few have experimented to a limited extent with including quantitative information when coding fragrance data (Azuma et al., 1997; Williams and Whitten, 1999).

Individual compounds are not all independent (Seaman and Funk, 1983; Dobson et al., 1997; Barkman, 2001). Fragrances can contain dozens or even hundreds of compounds produced by many fewer biosynthetic pathways (Steele et al., 1998; Barkman, 2001); for example, three volatiles produced by the BEAT pathway (Fig. 1) were detected in fragrances emitted by the plants in the present study. Information about pathways could be incorporated by coding either quantitatively (relative or absolute amount of volatiles produced by each pathway) or qualitatively (presence/absence of each pathway). Fragrance data also could be coded in a way that recognizes the nonindependence of compounds synthesized by the same pathway while including data on individual compounds. An elegant method for dealing with the nonindependence of compounds involves the use of step matrices (Barkman, 2001). In this approach a biosynthetic pathway is represented as a multistate character, with shifts between compounds defined as states in a step matrix.

The source of emitted volatiles also merits attention. Pollination biologists have focused on fragrance emitted from flowers, but pollinators likely sense odors emitted by the entire plant, especially in long distance attraction. To understand the role that fragrance has played in the evolution of pollinator relationships, it may be important to include both floral and vegetative volatiles.

Clearly, fragrance has a number of attributes that could be defined as characters for phylogenetic analysis. However, to evaluate ideas regarding character coding, it is essential to have a robust phylogeny reconstructed from other sources of data. Because of a lack of previous studies combining phylogenetic analysis with comprehensive collection of fragrance data, there is no consensus as to the best methods of coding fragrance data nor the extent to which these data are useful for phylogenetic analysis.

Here we examine the relationship between fragrance and phylogenetic history in species of two genera of Nyctaginaceae (four o'clocks). We present the results of experiments with a number of coding methods and of analyses using distance and parsimony approaches to address a set of questions. First, does fragrance provide systematically useful characters; that is, do differences in fragrance profiles between species exceed differences among individuals of the same species? If so, which coding methods best capture species-specific differences? Second, do fragrance data contain hierarchical signal, and can these data be used to reconstruct phylogenetic relationships? How do the topologies based on fragrance data compare with phylogenetic hypotheses resulting from DNA sequence data? Do coding methods differ in this regard? Third, given a hypothesis of phylogenetic relationships derived from independent data (e.g., DNA sequence data), do coding methods differ in terms of congruence with this hypothesis? Is congruence related to attributes of fragrance characters (e.g., number of compounds per pathway)?

Our study offers insight into the utility of fragrance data, and chemical data in general, for phylogenetic inference. An understanding of the role of pollinators as agents of selection on fragrance demands first discerning the role that phylogeny plays in constraining or enhancing the responses of plants.

MATERIALS AND METHODS

Study Taxa and Sampling

The plant family Nyctaginaceae is composed of woody or herbaceous perennials (rarely annuals) distributed mainly in subtropical and tropical regions of the New World. We focused on two lineages of Nyctaginaceae that occur primarily in southwestern North America: *Acleisanthes* (16 species; Levin, 2002) and *Mirabilis* section *Quamoclidion* (6 species). Fragrance was collected from multiple individuals of 20 species (Levin et al., 2001), including 11 *Acleisanthes* species and all 6 species in *Mirabilis* sect. *Quamoclidion*. Fragrance profiles of three additional species of *Mirabilis* (*M. bigelovii*, *M. jalapa*, and *M. longiflora*) were also analyzed. Vouchers of all species from which fragrance was collected are at the University of Arizona herbarium (ARIZ; Levin, 2000: table 2).

Phylogenetic Analysis of DNA Sequence Data

Phylogenies were inferred using sequence data from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA and the intergenic region between the *rbcL* and *accD* genes plus a 300 base pair (bp) region from the 5′ end of *accD* on the chloroplast genome (Levin, 2000). Because we collected fragrance from a 20 species subset of the species included in Levin (2000), for the present study we inferred phylogenetic relationships for this reduced sample of taxa. The ITS sequences (including 25 bp of the 18S and 65 bp of the 26S genes in addition to ITS-1, ITS-2, and the 5.8S gene) ranged in length from 656 to 664 bp; the chloroplast sequences ranged in length from 1,007 to 1,046 bp. Parsimony analysis using the branch-and-bound search algorithm was done on a combined data set of the nuclear and chloroplast regions (PAUP*; Swofford, 2002) (see Levin, 2000, for a more complete discussion of these data sets and their combinability). Because *Acleisanthes* and *Mirabilis* are well supported as distinct clades by both molecular and morphological data (Levin, 2000), we rooted all phylogenies between these two groups. Gaps were treated as missing data, although indels >1 bp were coded as a unit as additional characters. To be conservative, indels of 1 bp were not included. Support for individual branches of the tree was estimated using bootstrap values (Felsenstein, 1985) and decay indices (Bremer, 1988; Donoghue et al., 1992; but see DeBry, 2001, for limitations of the decay index). Bootstrap values were from 200 full heuristic replicates with 10 random addition sequence replicates and tree bisection–reconnection (TBR) branch swapping. Decay values for each branch were determined by first drawing the strict consensus of most-parsimonious (MP) trees in MacClade (Maddison and Maddison, 2000) and then creating a batch file containing a set of trees each with a single branch resolved. This file was then executed in PAUP* (Swofford, 2002) using the heuristic search algorithm and 10 random addition sequence replicates to find the shortest tree(s) consistent with each constraint. The decay index (DI) for each branch was the difference in length between the shortest trees consistent with a particular constraint and the globally shortest trees. The 20-taxon data set was also analyzed using maximum likelihood analysis in PAUP* (Swofford, 2002). Likelihood settings corresponding to the general time reversible + G + I model were as follows: empirical nucleotide frequencies, gamma distribution of variable sites, and estimated shape parameter and proportion of invariant sites. Ten random addition heuristic sequence replicates were conducted with TBR branch swapping.

Fragrance Collection and Analysis

Fragrance was collected in the field (rarely in the greenhouse) using the dynamic headspace method (see Raguso and Pellmyr, 1998, and references therein). Flowers of a living plant are enclosed within a polyacetate bag (Reynolds oven bags) where volatile compounds emitted from the plant accumulate and are trapped in adsorbent cartridges through the use of a battery-operated diaphragm pump (KNF Neuberger) (see Levin et al., 2001, for details on methods). Fragrance collections typically commenced at floral anthesis (usually dusk) and continued for 12 hr. Each species has flowers that last only one night; thus, volatiles were collected over the entire period during which a flower was open. In general, fragrance was collected once for each individual plant, and the number of flowers included for each fragrance collection was noted. After fragrance collection, flowers were removed and weighed to obtain a mean fresh weight per flower for each species.

The architecture of these plants makes it impossible to avoid trapping vegetative volatiles while collecting volatiles from a number of flowers. Therefore, to distinguish floral from vegetative volatiles, fragrance collection from only vegetative structures was necessary. Ideally, the mass of the vegetative tissues from which fragrance was collected would be quantified; however, this would have required destructive sampling of plants (in many cases fragrance was collected from an entire plant), which was not feasible. Therefore, we were able to determine which compounds were emitted vegetatively, but we could not quantify emissions on a per leaf area or mass basis.

Fragrance samples were analyzed on a gas chromatograph (GC) equipped with an electron impact quadrupole mass spectrometer (Shimadzu Scientific Instruments). Compounds were tentatively identified using computerized mass spectral libraries (Wiley and NIST libraries, >120,000 mass spectra). The identity of many compounds was verified using retention times of known standards (Levin et al., 2001: table 2) on two different GC columns. Quantification of compound amounts was achieved by integrating individual GC peak areas using Shimadzu Class-5000 software (Shimadzu Co., 1993–1996).

Fragrance Data in Phylogenetics

The result of each analysis of fragrance is a data matrix, the cells of which record the amount of each compound

present in a given individual plant's profile. These matrices may be summarized to yield a single value for each sample (e.g., number of compounds), the cells may be treated separately as individual compounds, or the cells may be combined according to the biosynthetic pathway that produces each compound. We used all three approaches and further experimented with including either quantitative (amount of each volatile or pathway) or qualitative (presence/absence) information and including volatiles emitted by floral structures only (Fl) versus the whole plant (floral + vegetative [Fl + V]). All data sets are available from R.A.L.

Optimizations of summary characters.—Fragrance profiles were summarized as single values for each species representing (1) total amount of floral and vegetative volatiles emitted (mean of all samples per species), (2) total number of volatile compounds produced (Fl + V), (3) total amount of floral volatiles released per unit floral mass, and (4) percentage of total volatiles released from vegetative tissues (mean of all samples per species). We used linear and squared-change parsimony in MacClade to optimize these continuous summary characters onto an unrooted MP tree inferred from DNA sequence data and, thus, to evaluate congruence between these summary features of the fragrance profiles and phylogenetic history.

Distance analyses: Impact of coding on intra- versus interspecific variation.—If fragrance data have systematic signal, there should be less variation among conspecific individuals than between species, and intraspecific distances should be shorter than interspecific distances. To examine the presence of signal, we prepared matrices that treated compounds (data sets 1 and 2; Table 1) or biosynthetic pathways (data sets 3 and 4; Table 1) as separate characters for each individual plant (Fig. 2 provides examples of the structure of the data sets and of relationships among them). Researchers have characterized major biosynthetic enzymes and metabolites of plant volatiles in several metabolic pathways (reviewed by Mahmoud and Croteau, 2002; Pichersky and Gershenzon, 2002). We used this literature to infer the biosynthetic precursors for each of the compounds present in the fragrance profiles. Each set of precursors and associated enzymes acting in a cascade between metabolic intermediates was coded as separate pathway branches (hereafter referred to simply as pathways).

Quantitative data were first standardized to z scores, with mean = 0 and SD = 1. Pairwise dissimilarity matrices were calculated using Euclidean distance as the distance metric (SPSS, 1999). Wilcoxon rank sum tests were used to determine whether dissimilarity in fragrance composition between pairs of conspecific individuals differed from dissimilarity between interspecific pairs (JMPIN 3.2.1, SAS Institute, 1997). We employed the results of these analyses to address four issues regarding how best to code fragrance data for use in systematics.

First, do quantification methods differ in their ability to differentiate species by their fragrance profiles? Quantities of compounds and pathways were expressed relative to total volatiles produced (data sets 1a, 1b, 3a, and 3b; Table 1) and to amount of floral tissue from which fragrance was collected (data sets 1c and 3c; Table 1). To avoid confounding the comparison with differences in tissues sampled, we compared only 1b and 3b (i.e., solely floral fragrance) to 1c and 3c, respectively. We predicted that expressing quantities of floral volatiles relative to amount of tissue sampled (i.e., 1c and 3c) would best differentiate species.

Second, does quantification of the data more clearly group conspecifics and distinguish species compared with coding as presence/absence? We compared quantitative data sets 1a and 3a, which included fragrance from both floral and vegetative tissues, with data sets 2a and 4a (i.e., presence/absence of compounds or pathways in samples from Fl + V tissues; Table 1). We also compared the quantitative data sets that emerged from the first analysis (i.e., the best of 1b or 1c and 3b or 3c) with data sets 2b and 4b (presence/absence, Fl only). Because including quantity of volatiles incorporates more information than simple presence/absence, we predicted that quantification would enhance interspecific differences in fragrance profiles.

Third, does including fragrance from vegetative tissues enhance the systematic utility of these data? For these comparisons, we held other aspects of coding constant; for example, data sets 1a and 1b differ only in whether compounds emitted from vegetative tissues were (1a) or were not (1b) included. Similarly, we compared data sets 2a and 2b, 3a and 3b, and 4a and 4b (Table 1). The inclusion of vegetatively emitted volatiles adds information. Therefore, we predicted that the data sets that included vegetative tissues would

TABLE 1. Coding methods for fragrance data. Data were coded as characters according to fragrance compounds (data sets 1 and 2) or biosynthetic pathways (data sets 3 and 4), quantities of compounds or pathways (data sets 1 and 3), and presence/absence (P/A) of compounds or pathways (data sets 2 and 4). Fragrance data were quantified in two ways: relative to total volatiles emitted per sample (1a, 1b, and 3a, 3b) and relative to amount of floral tissue sampled (1c and 3c). Amounts relative to total volatiles are from floral (Fl) and vegetative (V) (1a and 3a) or Fl tissue only (1b and 3b).

Compounds as characters	Biosynthetic pathways as characters
Data set 1. Amount of each compound	Data set 3. Amount of compounds per pathway
1a. Relative amount of each compound (Fl + V)	3a. Relative amount of each pathway (Fl + V)
1b. Relative amount of each compound (Fl only)	3b. Relative amount of each pathway (Fl only)
1c. Amount per unit floral tissue (Fl only)	3c. Amount per unit floral tissue (Fl only)
Data set 2. P/A of each compound	Data set 4. P/A of each pathway
2a. P/A of each compound (Fl + V)	4a. P/A each pathway (Fl + V)
2b. P/A of each compound (Fl only)	4b. P/A each pathway (Fl only)

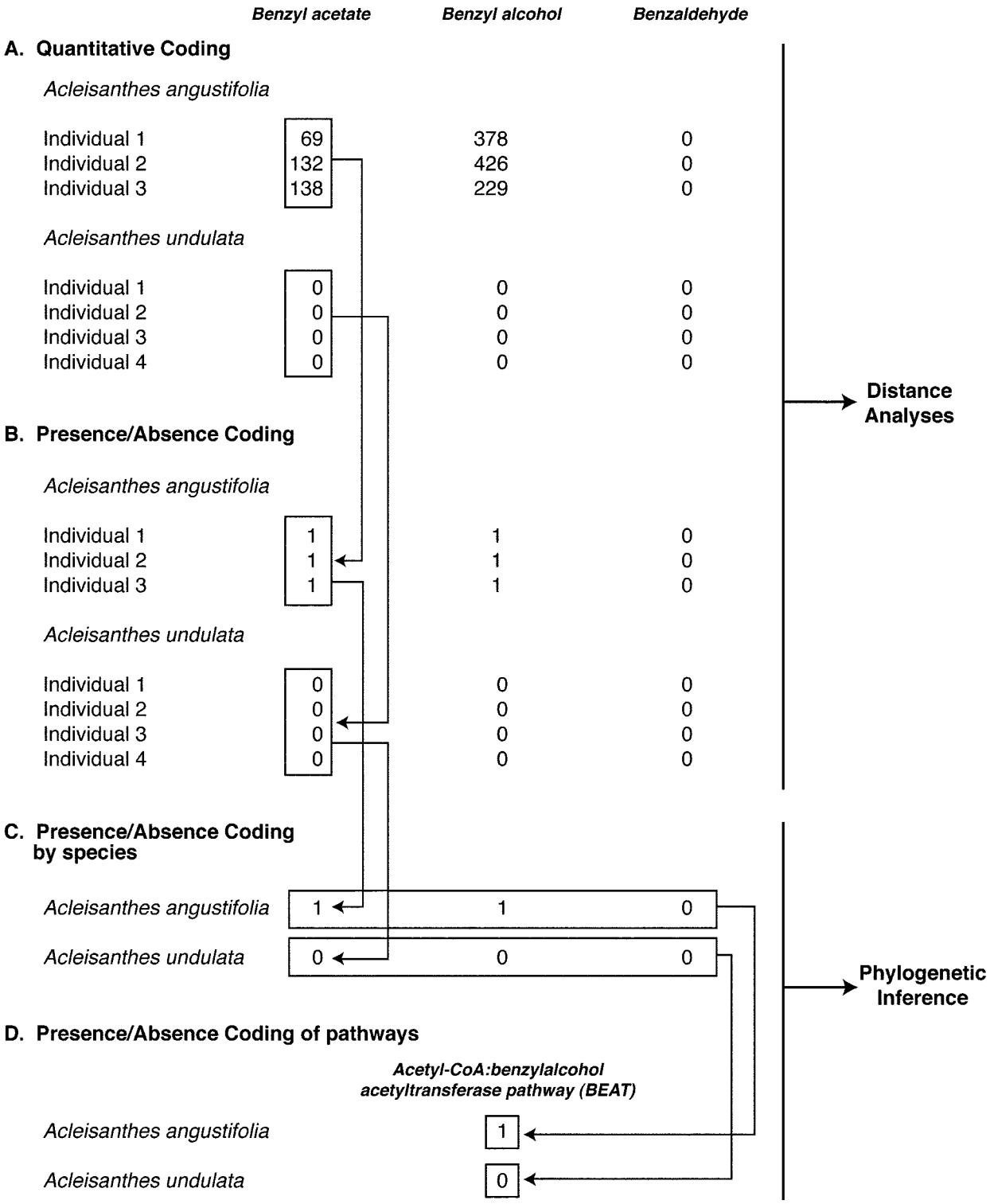

FIGURE 2. Overview of the composition of the data sets used for distance and phylogenetic analyses and the relationships among them. Data for the three compounds from the BEAT pathway (Fig. 1) are presented for 2 of the 20 species included here. For distance analyses (A, B), fragrance data were coded for all individuals per species. (A) Compound quantities emitted per unit floral tissue, corresponding to values in data set 1c (Table 1). (B) For presence/absence coding, values in (A) reduce to scores of 0 (compound absent) or 1 (any detectable amount) for each individual; values correspond to those in data set 2b. For phylogenetic analysis (C, D), fragrance data were scored by species. (C) Presence of any amount of a compound in at least one individual of a species was scored as presence of that compound for that species. These numbers correspond to those in data set 2b. (D) A pathway character was coded as present if at least one compound synthesized by that pathway is present in a species' fragrance profile. These data correspond to those in data set 4b.

better differentiate species than those that included only floral fragrance compounds.

Fourth, does coding the data as individual compounds (data sets 1 and 2; Table 1) or as biosynthetic pathways (data sets 3 and 4) best differentiate the fragrance profiles of species? The comparisons made were based upon the results from the second and third analyses.

Fragrance characters in phylogenetics.—Four fragrance data sets emerged from the distance analyses as best distinguishing species and, thus, most appropriate for analyses of relationships among species. For parsimony analyses, when a compound or pathway was present in at least one individual, then that species was coded as having that compound/pathway (see Fig. 2). Experience indicates that if one individual synthesizes a particular compound/pathway, other conspecifics probably do so as well, albeit perhaps in quantities below the threshold level for detection. All phylogenetic analyses were conducted with PAUP* using default settings, except as noted.

Presence of hierarchical signal in the fragrance data sets was assessed using the g_1 statistic (Hillis and Huelsenbeck, 1992) as calculated by PAUP*. As structure in the data increases, the distribution of tree length frequencies becomes more left skewed, resulting in a more negative value for g_1 (Hillis and Huelsenbeck, 1992).

Trees were inferred from the fragrance data using the heuristic search option and 100 random addition sequence replicates. Because *Acleisanthes* and *Mirabilis* are well supported as distinct clades by both molecular and morphological data (Levin, 2000), we rooted all phylogenies between these two groups. Strict consensus trees were then constructed.

Congruence among data sets was assessed using partition-homogeneity tests (Farris et al., 1994) and tree-to-tree distances. Partition-homogeneity tests were done for each fragrance data set in combination with the DNA sequence data set and for all fragrance data sets together. For these tests, 100 replicates were conducted using the heuristic search option with 10 random addition sequence replicates. We also calculated tree-to-tree distances from the strict consensus tree topology inferred from DNA sequence data to each of the strict consensus trees inferred from the fragrance data sets. For comparison, we calculated tree-to-tree distances from each of the 36 MP trees inferred from the DNA sequence data to the strict consensus of these 36 trees. The tree-to-tree distance metric was the symmetric difference, which counts the number of groups present on only one of the two tree topologies. Therefore, tree topologies that are more similar will have a lower value of symmetric difference.

To further compare the tree topologies inferred from the fragrance and DNA sequence data sets, we used Templeton's test (Templeton, 1983) as implemented in PAUP*. This test calculates differences in the number of steps required by each character when a data set is optimized on one tree versus another. Pairwise tests were used to compare the results of optimizing each fragrance data set onto both the strict consensus tree inferred from that data set and the strict consensus tree inferred from DNA sequence data. The magnitude of the difference between the lengths of characters from a given data set optimized on the two trees is indicative of the degree to which the topologies supported by the fragrance versus DNA data sets differ. Differences in lengths of characters were assessed statistically with PAUP* using one-tailed Wilcoxon signed ranks tests. Goldman et al. (2000) argued that this test is inappropriate for a posteriori comparisons. We concur and follow their advice in using a one-tailed rather than two-tailed test; however, caution is warranted in interpreting these results.

If the DNA and fragrance data sets are congruent, then partition-homogeneity tests will not be significant, all tree-to-tree distances will be low, and Templeton's tests will be nonsignificant. Differences among the fragrance data sets in degree of congruence with the DNA sequence data provide a further basis for evaluating coding methods in terms of systematic utility.

The final approach to evaluating the phylogenetic information content of the fragrance data involved optimizing fragrance characters onto the phylogeny inferred from DNA sequence data. Using MacClade 4.0 (Maddison and Maddison, 2000), characters from the four data sets that emerged from the distance analyses as best distinguishing species were mapped onto one of the MP topologies inferred from DNA sequence data. MacClade was used to calculate the consistency index (CI) and retention index (RI) for each parsimony-informative character from these fragrance data sets as optimized onto this MP tree. As discussed by Archie (1989a, 1989b) and Farris (1989), CIs and RIs emphasize different aspects of character congruence with topological patterns of relationship. In particular, RIs are higher when unique and unreversed character transitions occur deep in the phylogeny, whereas CIs are not sensitive to the location of such character transitions. CIs and RIs were also calculated for each parsimony-informative character from the DNA sequence data set as mapped onto the DNA topology.

The nonparametric Wilcoxon rank sum test (JMPIN 3.2.1) was used to compare homoplasy (i.e., CI and RI values) among data sets. We hypothesized that coding as biosynthetic pathways is less subject to homoplasy than coding as individual compounds; a single evolutionarily conservative pathway may comprise many highly labile compounds. We predicted that the biosynthetic pathway characters would evolve deeper in the topology than would the compound characters and, thus, predicted higher RI values for pathway than for compound data. We also predicted that there would be no pronounced differences in levels of homoplasy in compounds/pathways produced by vegetative versus floral tissue. Both are likely subject to some degree of selection (by herbivores versus pollinators, respectively) in addition to phylogenetic constraint.

We used the nonparametric Spearman's rank correlation test (JMPIN 3.2.1) to examine the relationship between CI and RI values of fragrance characters (as

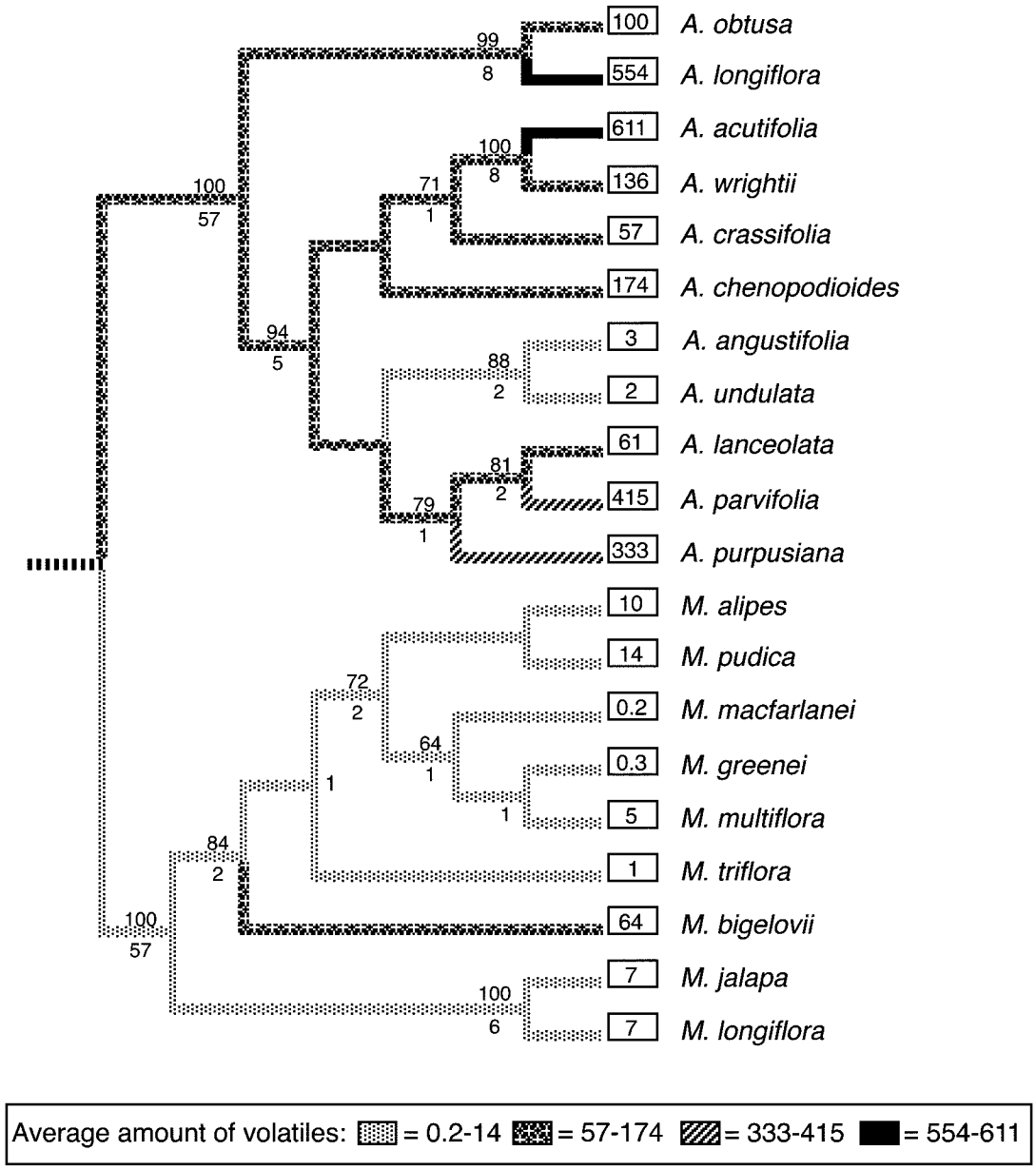

FIGURE 3. One of the most-parsimonious (MP) trees inferred using nuclear and chloroplast DNA sequence data for species of *Acleisanthes* and *Mirabilis*; this tree was used for all other analyses in this study (tree length = 287). Bootstrap values >50 are indicated above the branches, and decay indices are below (those clades not resolved in the strict consensus lack both bootstrap and decay index values). Mean total amount of floral plus vegetative volatiles emitted per 12 hr per sample per species was optimized onto this tree using minimum linear parsimony. These values represent total unit area under the chromatogram peaks (divided by 10^6 for ease of presentation). Boxed numbers are the observed character value for each terminal taxon. Purely for ease of visualizing character evolution, branches are patterned to reflect four "states" that were delineated based upon gaps in the data. This character was mapped onto an unrooted tree; thus, the character state at the root is equivocal (vertical hatching).

optimized onto the MP topology inferred from DNA data) and some attributes of these characters. We predicted that pathways represented by many compounds in these fragrance profiles would be more homoplastic than those with fewer compounds. Similarly, we predicted that compounds and pathways present in many taxa would be more homoplastic than those present in only a few taxa.

RESULTS

Figure 3 shows one of 36 MP trees from the phylogenetic analysis of DNA sequence data. All but four nodes were resolved in the strict consensus of MP trees (of these, two were present in 50% and two were present in 33% of MP trees). Where support for branches is weak, there are often morphological characters that support the branch (e.g., sister taxa *M. pudica* and *M. alipes* have

morphological synapomorphies). Exclusion of taxa from this analysis because of lack of fragrance data had no effect on the overall tree topology (see also Levin, 2000).

ML analysis yielded one optimal tree (−ln likelihood = 4072.94076; tree not shown). This tree is identical to the MP tree shown in Figure 3, except that *A. chenopodioides* is part of a polytomy with the clade of *A. purpusiana* + (*A. lanceolata* + *A. parvifolia*) and the clade of *A. undulata* + *A. angustifolia*.

Plants of all 20 taxa emitted fragrance from flowers, vegetative tissues, or both, although the amount and number of volatiles released varied greatly. Amount of volatiles emitted was measured using the integrated peak area $\times 10^{-6}$ of all compounds (Fl + V) released over the 12 hr collection period for each sample. The average volatile emission across all samples per species spanned four orders of magnitude, from 0.2 in *M. macfarlanei* to 611 in *A. acutifolia* (Fig. 3), with a mean volatile emission of 128 units. An average of 45 compounds (range, 5–108 compounds) were present in each species' floral and vegetative fragrance (Fig. 4). Across all species' fragrance profiles, 71 of the total 199 compounds were

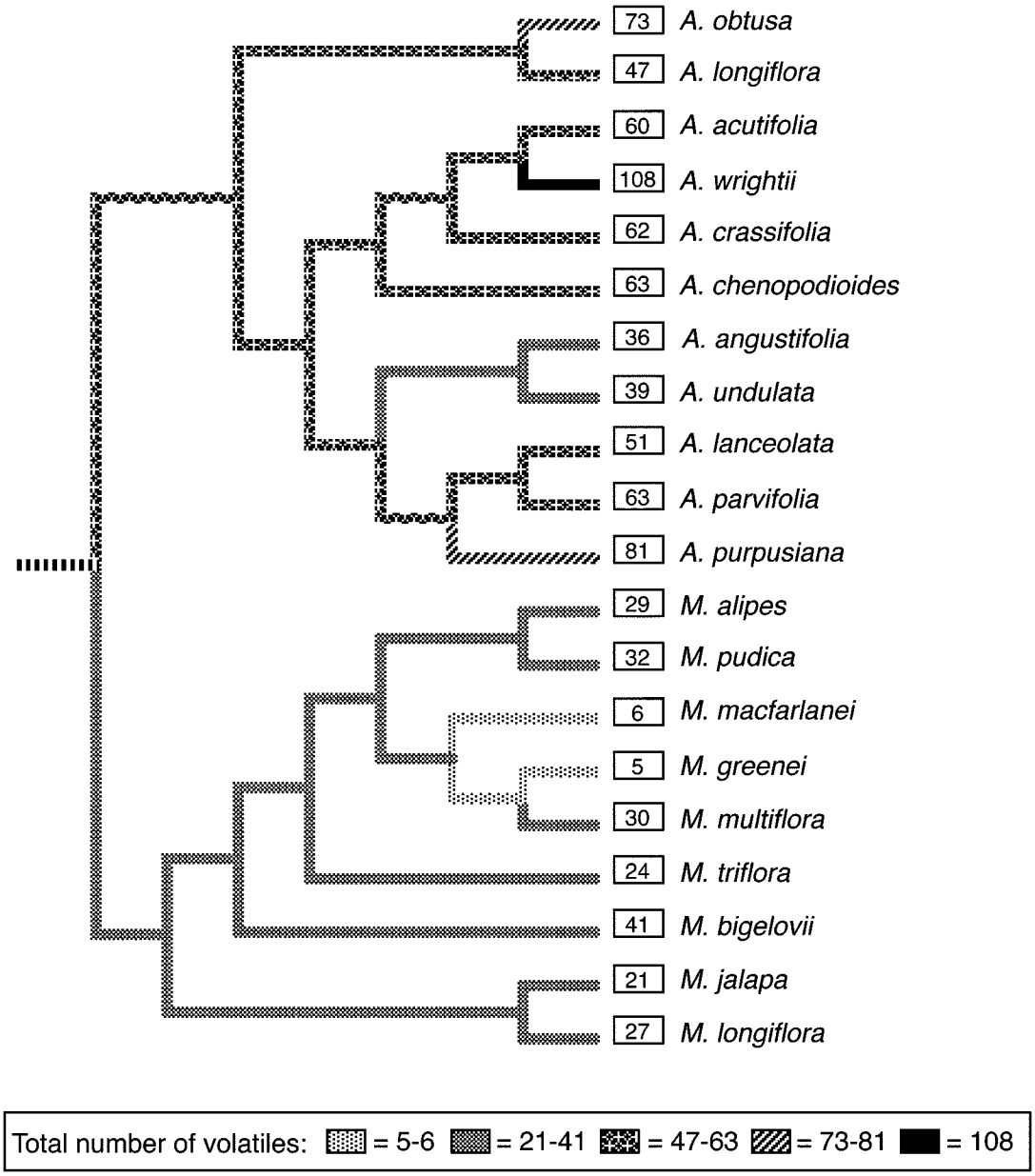

FIGURE 4. Total number of floral plus vegetative fragrance compounds emitted by plants of each species of *Acleisanthes* and *Mirabilis* optimized on the MP tree in Figure 3 using minimal linear parsimony. Boxed numbers are the observed character value for each terminal taxon. For ease of visualizing character evolution, branches are patterned to reflect five "states" that were delineated based upon gaps in the data. This character was mapped onto an unrooted tree; thus, the character state at the root is equivocal (vertical hatching).

autapomorphies, and ca. 25% of them could be identified only as from a particular biosynthetic pathway or, rarely, could not be identified. Two of the 17 biosynthetic pathways were autapomorphic.

Fragrance Data as Summary Characters

Regardless of optimization method, summary measures of fragrance profiles show phylogenetic pattern when mapped onto the tree inferred from DNA data, although there is also considerable homoplasy (Figs. 3–5). We present minimum values assigned by linear parsimony; this optimization concentrates changes in distal portions of the tree. In comparison, using maximum values (under linear parsimony) optimizes transitions deeper in the phylogeny with reversals toward the tips. Squared-change parsimony posits many gradual changes and often requires evolution in different directions for sister taxa.

The average volatile emission across all samples per species is much lower in the *Mirabilis* clade than in the *Acleisanthes* clade (Fig. 3); a similar pattern was observed for the amount of volatiles produced per unit floral tissue (not shown). Plants of *Acleisanthes* species also emit more floral and vegetative compounds (Fig. 4). The *Acleisanthes* lineage apparently evolved from a common ancestor that emitted a lot of fragrance composed of many compounds. Only in the sister taxa *A. angustifolia* + *A.*

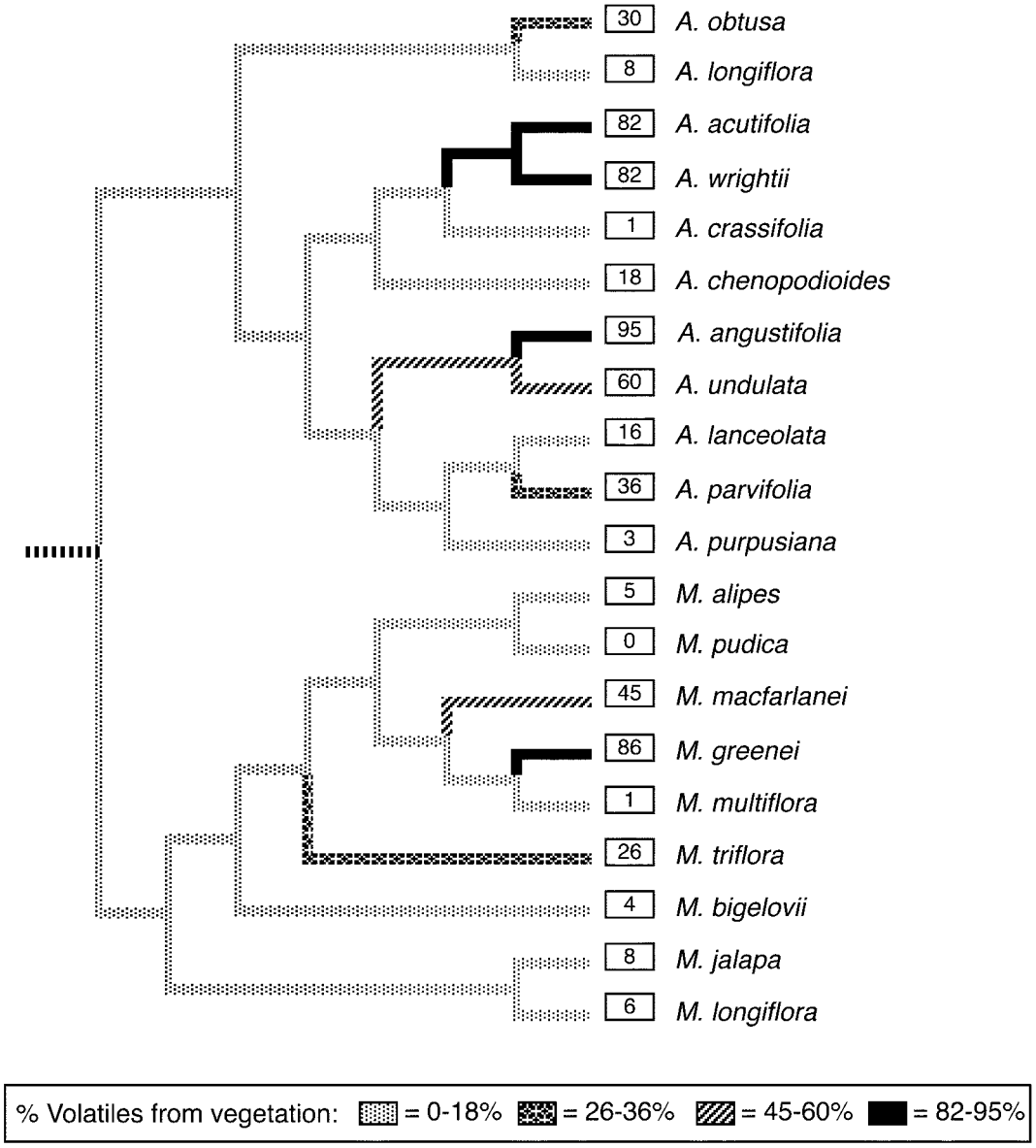

FIGURE 5. Percentage of total volatiles produced that were emitted by vegetative tissues of *Acleisanthes* and *Mirabilis* species optimized on the MP tree in Figure 3 using minimal linear parsimony. Boxed numbers are the observed character value for each terminal taxon. For ease of visualizing character evolution, branches are patterned to reflect four "states" that were delineated based upon gaps in the data. This character was mapped onto an unrooted tree; thus, the character state at the tree root is equivocal (vertical hatching).

undulata do both amount and number of volatiles decrease.

Percentage of the total amount of volatiles released vegetatively appears to show phylogenetic pattern at a lower level than total amount or number of volatiles (Fig. 5). The sister species *A. wrightii* + *A. acutifolia* and *A. angustifolia* + *A. undulata* share high percentages of volatile production from vegetative tissues. *Mirabilis greenei* and *M. macfarlanei* also share high percentages of vegetative volatile production because of an overall low volatile production, with almost all of what little fragrance is emitted being released from vegetative tissues. These data can be inverted to give percentage of volatiles released from floral structures, such that the sister species produce little of their total fragrance from flowers.

Compounds and Pathways as Characters

Data sets 1a and 2a include 199 characters reflecting relative amount and presence/absence of each compound, respectively, in floral and vegetative fragrance. About two-thirds of the 199 compounds were emitted entirely from floral tissue; hence the floral only data sets 1b, 1c, and 2b have 133 characters, representing amount (1b and 1c) and presence/absence (2b) of each floral compound. Across all 20 species, very few compounds were produced only by vegetative tissues, although many were emitted by both flowers and vegetative tissues. The compounds detected were produced by 17 biosynthetic pathways. Thus, data set 3a, the relative amount contributed by each pathway, and data set 4a, presence/absence of compounds produced by a given biosynthetic pathway, both have 17 characters (see Fig. 2 for examples of the relationship between the compound and pathway data sets). Across all species sampled, no pathway was unique to vegetative tissues, such that data sets 3b, 3c, and 4b (Fl only) also have 17 characters. However, these data sets are not identical to data sets 3a and 4a because at the level of individual species some pathways did produce compounds emitted only by vegetative tissues.

Distance Analyses: Impact of Coding on Intra- Versus Interspecific Variation

For all methods of coding, intraspecific variation in fragrance profiles was lower than interspecific variation (Table 2), indicating that fragrance data have potential as systematically useful characters. However, the magnitude of the differences between mean values and the degree of overlap between inter- and intraspecific distances varied among coding methods. These differences permit evaluation of coding methods in the context of the issues that we sought to explore.

First, do quantification methods differ (data sets 1b vs. 1c, 3b vs. 3c; Table 2)? Contrary to our prediction that expressing amounts of compounds or pathways relative to amount of tissue sampled would best differentiate species' fragrance profiles, the two quantification methods did not differ markedly. Therefore, we retained both quantitative coding methods for subsequent comparisons.

Second, does quantification group conspecifics and distinguish species better than qualitative coding (data sets 1 vs. 2, 3 vs. 4; Table 2)? In all comparisons, coding the data as presence/absence more clearly distinguished species; there were no overlapping rank values in the 10–90% quantile and lower mean ranks for intraspecific differences in data sets 2 and 4 compared with data sets 1 and 3, respectively (Table 2). Thus, we omitted quantitative coding schemes from subsequent comparisons.

Third, does including information on fragrance emitted from vegetative tissues enhance the systematic utility of these data (2a vs. 2b; 4a vs. 4b; Table 2)? Including vegetatively emitted compounds had little impact on the distinction between intra- and interspecific distances. There

TABLE 2. Intra- versus interspecific variation in fragrance profiles based upon dissimilarity between pairs of individuals; data sets are as described in Table 1. For ease of interpretation, mean percent dissimilarity (Euclidean distance) for intra- and interspecific comparisons are presented by data set. Because of nonnormality, all analyses were conducted on ranks rather than on raw data (Wilcoxon rank sum test; in all analyses, interspecific distances exceeded intraspecific distances, $P < 0.0001$). Values reported are the mean (range in the 10–90% quantile) of ranks for intra- and interspecific distances for each data set. Sample sizes are the number of intraspecific (167) and interspecific (3,319) pairwise comparisons; the total number of ranks is the sum of these (3,486). Fl = floral; V = vegetative; P/A = presence/absence; Amts = amounts relative to total volatiles (1a and 1b; 3a and 3b) or to amount of floral tissue sampled (1c and 3c).

	Raw mean % dissimilarity (range)		Mean ranks (10–90%)	
Data set	Intraspecific	Interspecific	Intraspecific	Interspecific
Compounds as characters				
1a (Amts, Fl + V)	12.0 (1.9–36.2)	19.1 (4.8–47.7)	818.1 (16.8–2566.2)	1790.1 (420–3152)
1b (Amts, Fl only)	10.3 (0.1–25.9)	15.7 (0.1–34.8)	833.5 (20.8–2299.8)	1789.3 (402–3150)
1c (Amts, Fl only/amount floral tissue)	9.8 (0–38.7)	14.0 (0–50.0)	1242.6 (23.6–2622.4)	1768.7 (371–3148)
2a (P/A, Fl + V)	3.1 (1.0–6.6)	6.5 (1.4–10.6)	194.4 (16–288)	1821.4 (451–3138)
2b (P/A, Fl only)	2.5 (0–5.6)	5.6 (1–7.8)	183.3 (11–339)	1822 (452–3149)
Pathways as characters				
3a (Amts, Fl + V)	2.4 (0.1–8.9)	5.6 (0.4–12.6)	431 (20.8–1537.4)	1809.5 (463–3154)
3b (Amts, Fl only)	2.3 (0.1–8.0)	5.6 (0.1–12.3)	378.6 (21.8–1040.8)	1812.2 (461–3155)
3c (Amts, Fl only/amount floral tissue)	2.3 (0–13.6)	4.4 (0–19.3)	1113.6 (34–2810.2)	1775.2 (390–3149)
4a (P/A, Fl + V)	0.8 (0–2.0)	2.2 (0–3.6)	215.8 (1–241)	1820.4 (241–2991)
4b (P/A, Fl only)	1.0 (0–2.2)	2.5 (0–3.9)	165.1 (1–238)	1822.9 (467–3036)

were similar mean ranks for intra- and interspecific distances and no overlap of values in the 10–90% quantile for both data sets 2a and 2b. For the data sets coded by biosynthetic pathway, data set 4b (i.e., Fl only) distinguished species slightly better than did data set 4a (Fl + V) (mean rank intraspecific differences were lower and ranges of rank values in the 10–90% quantile were distinct for data set 4b compared with data set 4a).

Fourth, does coding the data as individual compounds or as biosynthetic pathways best differentiate the fragrance profiles of species (2a vs. 4a; 2b vs. 4b; Table 2)? Differences were not marked; mean rank intraspecific differences were somewhat higher in data set 4a compared with data set 2a, but the opposite was true in comparing data sets 2b and 4b.

Thus, the main distinction among the 10 data sets was that the presence/absence data performed better than both approaches to quantification (Table 2). Compound and pathway data were equally successful in grouping conspecifics and separating heterospecifics as were data from floral + vegetative and floral only emissions. Therefore, based upon these distance results, we used all four presence/absence data sets in phylogenetic inference.

Fragrance Characters in Phylogenetics

All data sets had significant hierarchical structure as indicated by g_1 statistics (Table 3). The DNA sequence data had the most negative value; among fragrance data sets, 2a (presence/absence of Fl + V compounds) and 4b (presence/absence of floral pathways) had the most hierarchical structure. Data set 4b resolved more nodes than did any other fragrance data set and even resolved more nodes than did the DNA sequence data. This is because the analysis of data set 4b yielded a single MP tree, such that no resolution was lost from construction of a consensus tree. Compared with data set 4a, data set 4b also had more parsimony-informative characters (Table 3).

Partition-homogeneity tests indicated congruence among fragrance data sets ($P = 0.86$) but rejected the null hypothesis that the fragrance and DNA sequence data sets were congruent (Table 3). Barker and Lutzoni (2002) and Darlu and Lecointre (2002) questioned the utility of this test. However, in this case, all resampled data sets yielded longer trees than the summed tree lengths from the original data sets (Table 3); except for data set 4a, all resampled trees were also substantially longer than the summed length of trees from the original data sets. The tree-to-tree distances suggested that regardless of how fragrance data were coded the tree based on DNA sequence data was different from all trees inferred from fragrance data (Table 3). Results of Templeton's tests concur in pointing to significant incongruence between the DNA and fragrance data sets (Table 4) (but see Goldman et al., 2000, for problems with these types of analyses). Thus, all these approaches point to significantly different signals between the fragrance and DNA sequence data sets.

In terms of topology, the phylogenies inferred from fragrance data (e.g., Fig. 6) corresponded to the topology inferred from DNA sequence data (Fig. 3) only in the most distal portions. When sister species or closely related species were similar in terms of fragrance profile, these groupings were often recovered by the analyses of fragrance (Fig. 6). For example, *M. greenei* and *M. macfarlanei* emit small amounts of the same few compounds (Figs. 3, 4), and these close relatives were always placed near each other by fragrance characters. However, larger scale phylogenetic patterns were never reconstructed correctly by fragrance data. Instead, these analyses mixed members of the two main clades.

TABLE 3. Comparison of DNA and fragrance data for phylogenetic inference in the plant family Nyctaginaceae. Shown are the number of parsimony informative (PI) characters (total number of characters) in each data set (data sets are as described in Table 1). g_1 values measure hierarchical structure in the data sets (more negative indicates greater structure). Pairwise partition-homogeneity tests (ILD) were between the DNA sequence data set and each fragrance data set and are expressed as the range of tree lengths from the 100 resampled data sets, with the sum of the tree lengths of the original data sets in parentheses. Tree-to-tree distances are symmetric differences between the strict consensus topology based on DNA sequence data and each of the four strict consensus topologies inferred from fragrance data.

Data set	No. PI (total) characters	g_1[a]	No. nodes resolved in strict consensus tree	ILD with DNA data[b]	Tree–tree distance
DNA	132 (1,811)	−0.49**	11	–	4[c]
Fragrance					
Compounds as characters					
2a (P/A, Fl + V)	128 (199)	−0.42**	5	770–783 (752)	14
2b (P/A, Fl)	98 (133)	−0.32**	14	670–685 (644)	28
Pathways as characters					
4a (P/A, Fl + V)	11(17)	−0.30*	3	322–329 (319)	17
4b (P/A, Fl)	14 (17)	−0.37**	16	339–347 (330)	29

[a]Conservative significance values estimated from Hillis and Huelsenbeck (1992): $^*P < 0.05$; $^{**}P < 0.01$.

[b]$P \leq 0.01$ for all comparisons.

[c]For comparison, the mean tree-to-tree distance between the 36 MP trees based on the DNA sequence data and the strict consensus of these trees is also presented.

TABLE 4. Congruence between DNA and fragrance data: Templeton's tests. Lengths of fragrance characters when optimized onto the tree inferred from DNA sequence data are compared with lengths on trees inferred from each particular fragrance data set. When $n < 25$ (n = the number of characters that differ in length when reconstructed onto the two-tree topologies) is, $P < 25$ values were determined from a table of critical values of the Wilcoxon T distribution (Zar, 1996: Appendix B, Table B.12). Data set labels are as in Table 1.

Data set	Pairwise tree comparison	z value (n)	P value (one-tailed)
Compounds as characters			
2a (P/A, Fl + V)	DNA vs. data set 2a	−4.28 (46)	<0.0001
2b (P/A, Fl only)	DNA vs. data set 2b	−5.16 (52)	<0.0001
Pathways as characters			
4a (P/A, Fl + V)	DNA vs. data set 4a	−2.16 (11)	<0.05
4b (P/A, Fl only)	DNA vs. data set 4b	−2.51 (11)	<0.01

Congruence of Fragrance and DNA Sequence Topologies

The highest median CI and RI values were for the DNA characters optimized onto the topology inferred from these same data (Table 5). All fragrance data sets had much lower median CI and RI values (Table 5). However, some characters from data sets 2a and 2b were perfectly congruent with the DNA topology, whether measured by CI or RI. By contrast, no biosynthetic pathway was perfectly congruent with the DNA phylogeny. However, CI and RI values did not differ between data sets 2a (compounds) and 4a (pathways) (Wilcoxon rank sum test; $P = 0.62$ for CIs, $P = 0.35$ for RIs), perhaps because the small number of pathways limited our power to detect such a pattern.

The relationship between number of compounds per pathway and RI is negative for data set 4b, as predicted, and almost significant (Table 6). However, data set 4a (CI and RI) and the CI values for 4b provide no support for this prediction (Table 6). The hypothesized relationship between homoplasy (CI) and number of taxa in which compounds are present was confirmed (data set 2a), but the pathway data set (4a) showed no such relationship (Table 6). RIs were not significantly related to the number of taxa in which compounds or pathways were present (Table 6).

No pathway and only six parsimony-informative compounds were emitted solely from vegetative tissues. As a result, we were unable to test our hypothesis that there would be similar levels of homoplasy in compounds/pathways produced by vegetative versus floral tissue. However, contrary to our prediction, comparison of CI and RI values (Table 5) suggests that inclusion of data from vegetative emissions improved the fit of the fragrance data to the DNA sequence topology. Data sets 2a and 4a have slightly higher median CI and RI values than their counterparts (2b and 4b) that lack vegetative compounds (Table 5). Four of the six vegetatively emitted compounds are sesquiterpenoids that were perfectly congruent with the DNA sequence phylogeny: α,β-eudesmol, α-muurolene, calamenene, and α-cubebene (VS; Fig. 7).

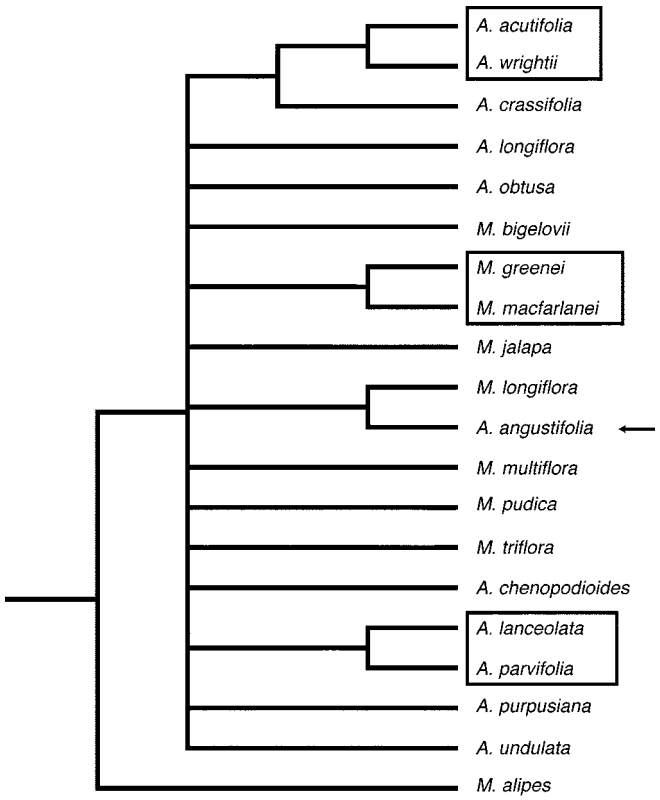

FIGURE 6. Strict consensus tree from parsimony analysis of data set 2a, presence/absence of all compounds (Fl + V) in species of *Acleisanthes* and *Mirabilis*. Boxed taxa illustrate that phylogenetic analysis of fragrance data places sister or closely related taxa together when they share similar fragrance profiles (compare with Fig. 3, phylogeny inferred from DNA sequence data). *Acleisanthes angustifolia* (arrow) differs from other members of the genus in fragrance profile and, thus, is placed by phylogenetic analysis of fragrance data with a species of *Mirabilis*.

TABLE 5. Congruence between fragrance data and topology inferred from DNA sequence data. Median CI and RI values are for parsimony-informative characters from the DNA sequence data and fragrance data sets 2a and 2b and 4a and 4b when optimized onto one of the MP trees inferred from DNA data (Fig. 3). Data set labels are as in Table 1.

Data set	CI (range)	RI (range)
DNA	1 (0.25–1)	1 (0–1)
Fragrance		
Compounds as characters		
2a (P/A, Fl + V)	0.33 (0.11–1)	0.33 (0–1)
2b (P/A, Fl only)	0.25 (0.13–1)	0.17 (0–1)
Pathways as characters		
4a (P/A, Fl + V)	0.33 (0.13–0.5)	0.5 (0–0.78)
4b (P/A, Fl only)	0.23 (0.13–0.5)	0.24 (0–0.78)

TABLE 6. Tests of hypotheses regarding the relationship between homoplasy (CI and RI) of fragrance characters and traits of those characters (i.e., number of compounds per biosynthetic pathway and number of taxa emitting each compound or pathway). CI and RI values were determined by optimizing fragrance characters onto one of the MP trees (Fig. 3) inferred from DNA sequence data. Data set labels are as in Table 1; sample sizes are in parentheses.

		CI		RI	
Hypothesis	Data set	ρ^a	P	ρ^a	P
Pathways represented by more compounds are more homoplastic than those with fewer compounds	4a ($n = 11$)	−0.215	0.95	−0.0449	0.90
	4b ($n = 14$)	0.4172	0.14	−0.5063	0.06
Compounds and pathways present in many taxa are more homoplastic than those emitted by few taxa	2a ($n = 99$)	−0.6465	<0.0001	−0.0451	0.66
	4a ($n = 11$)	0.2513	0.46	−0.5189	0.10

[a]From Spearman's rank correlation.

DISCUSSION

Fragrance is a complex feature with numerous attributes (e.g., number, identity, and quantities of compounds and biosynthetic pathways). In Nyctaginaceae, we have documented remarkable fragrance diversity: plants of 20 taxa emitted ca. 200 compounds synthesized by 17 pathways (Levin et al., 2001). Although caution is warranted when comparing our results to others because of differences in tissues (i.e., flowers, vegetation), methods, and number of taxa sampled, these values are high. Recent studies of angiosperms that included multiple species per genus documented a range of ca. 130 compounds belonging to ca. 10 biosynthetic pathways for 21 taxa of *Geonoma* (Arecaceae) (Knudsen, 1999) to only 27 compounds from ca. 4 pathways in 10 species in four genera of Winteraceae (Pellmyr et al., 1990). Azuma et al.'s (1997) study of Magnoliaceae was similar in scope to ours, in that fragrance data were collected for 16 species in three genera. Azuma et al. found about half as many compounds in the magnolias as we did in flowers of the Nyctaginaceae (76 vs. 133 floral compounds), and these compounds were produced by ca. 11 versus 17 biosynthetic pathways, respectively.

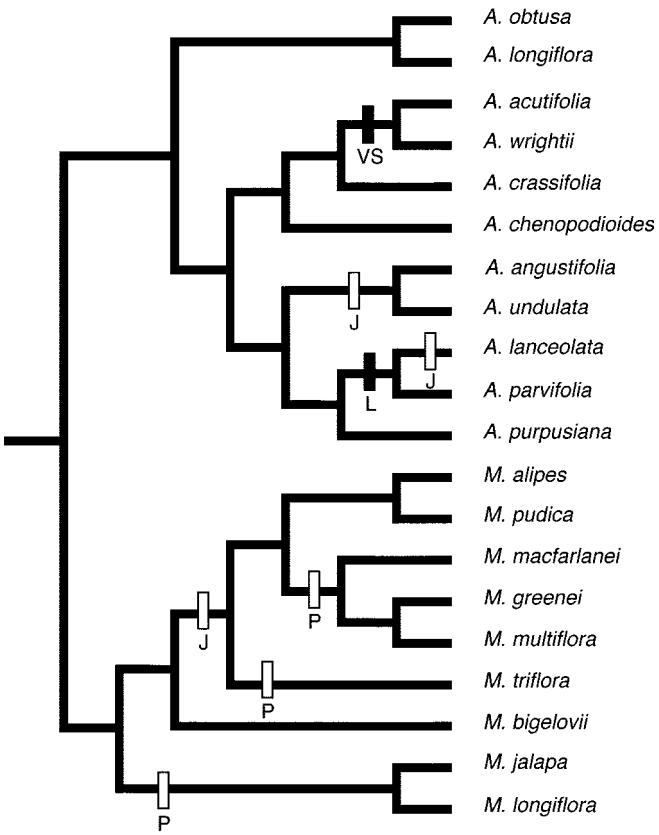

FIGURE 7. The MP tree in Figure 3 with evolutionary losses (open bars) and gains (solid bars) of selected characters. It is most parsimonious to infer that cis-jasmone (J) and phenyl acetic acid derivatives (P) were present in the common ancestor of both these lineages. L = δ-octalactone and δ-nonalactone; VS = the vegetatively produced sesquiterpenoids α,β-eudesmol, α-muurolene, calamenene, and α-cubebene.

Fragrance Data as Summary Characters

When optimized onto a phylogeny reconstructed from DNA sequence data (Figs. 3–5), summary characters showed some degree of congruence with phylogenetic relationships. For example, the two main lineages were generally rich (*Acleisanthes*) versus poor (*Mirabilis*) in both number of compounds and amount of fragrance emitted. Percentage of volatiles emitted from flowers versus vegetative tissues varied at lower phylogenetic levels, with close relatives sometimes differing markedly (Fig. 5). The finding that these single value summaries of fragrance are phylogenetically congruent at various levels (Figs. 3–5) suggests that, regardless of individual compounds and amounts thereof selection or phylogenetic constraint is operating at the level of overall fragrance attributes. Various factors may be responsible for these patterns.

Compounds and Pathways as Characters

Distance analyses: Impact of coding on intra- versus interspecific variation.—Regardless of how fragrance data were coded, interspecific variation exceeded intraspecific variation (Table 2). Barkman et al. (1997) documented a similar pattern for species of *Cypripedium* (Orchidaceae), showing that the considerable intraspecific variation in fragrance profiles is lower than that observed among species. These results indicate that each species has its own unique fragrance profile, even though the fragrances of different species may share many compounds. These findings also suggest that fragrance is

under genetic control and is not completely swamped by plastic responses to environmental conditions (Raguso and Pichersky, 1999). Because we sampled only one population of most species and the majority of species did not occur sympatrically with individuals of other species, we cannot rule out environmental variation as a factor contributing to the interspecific differences that we observed. However, we did collect fragrance from sympatric individuals of *A. crassifolia* and *A. wrightii* and of *A. longiflora* and *A. parvifolia*, and interspecific fragrance differences were notable (Levin et al., 2001). Data for *Mirabilis multiflora* (unpubl.) indicate that greenhouse-grown individuals have fragrance profiles more similar to those of field-collected conspecifics than to those of other species.

Although mean interspecific differences exceeded intraspecific differences for all coding methods, variation among these results permitted us to address our questions about coding fragrance data. Contrary to expectations, quantifying per unit floral mass distinguished species no better than quantifying relative to total fragrance produced. Standardizing by amount of tissue is analogous to standardizing morphometric data by size, which is key to comparing individuals and species. We may have used a less than optimal method to standardize floral tissues (e.g., dry weight may be better than fresh weight). Alternatively, emitting surface area might be a more appropriate standard. This approach would first require identification of the source(s) of emissions within the flower. Different floral structures are known to emit distinct compounds in *Clarkia* (Onagraceae; Pichersky et al., 1994; Wang et al., 1997; Dudareva et al., 1998b), *Boronia* (Rutaceae; Mactavish and Menary, 1997), and *Ranunculus* (Ranunculaceae; Bergström et al., 1995). Thus, it would also be necessary to link particular emissions to specific structures.

Our comparison of methods of quantitative coding was also affected by the fact that we could not quantify vegetative tissues from which fragrance was sampled; plant architecture and rarity precluded removal of vegetation for quantification. As with flowers, we need to learn more about emitting vegetative structures before we can determine the best way to quantify vegetative tissues. We are addressing quantification of vegetative tissues in subsequent research using plants that can be cultivated.

More generally, coding fragrance data as presence/absence of compounds or pathways distinguished species better than did either quantitative method. This result was unexpected; quantities clearly add information, and it is apparent simply from being around the plants that there is interspecific variation in fragrance quantity (see also Fig. 3). However, intraspecific quantitative variation appears to blur differences among species. Thus, high levels of intraspecific variation, rather than the quantitative nature of the data *per se*, may be at issue.

Thus, we tentatively concur with Barkman (2001), who suggested that amounts of compounds are too homoplastic for use in phylogenetic studies. However, fragrance collection methods require evaluation before quantification is totally rejected. For example, our methods may not have been sufficiently sensitive to yield strictly comparable quantitative results. Certain compounds will not even be detected unless fragrance is collected from a threshold amount of tissue (Raguso and Pellmyr, 1998). Solvent desorption also introduces methodological complexity that might be eliminated by other methods (e.g., thermal desorption with internal standards; Agelopoulos and Pickett, 1998). We are currently testing such approaches with *Nicotiana* (Solanaceae).

More research is also needed to understand the sources of intraspecific fragrance variation, and environmentally induced phenotypic plasticity (e.g., due to changes in temperature, soil, nutrient/water status; Jakobsen and Olsen, 1994) and genetic factors are likely involved. One approach would be to grow plants and sample fragrance in a controlled environment (most of our data were collected in the field). Ideally, quantitative genetic studies should be conducted to evaluate the effects of genetic and environmental variation on fragrance composition.

Results were equivocal as to the systematic utility of fragrance emitted from vegetative tissues and of coding individual compounds versus biosynthetic pathways. This finding suggests that useful systematic information comes from compounds and pathways and from fragrance emitted by both floral and vegetative tissues. Contra Azuma et al. (1999), valuable information would be ignored if fragrance data were coded only by pathway.

We have certainly not exhausted options for coding fragrance data for systematics. For some groups, Barkman's (2001) step matrix coding method may be useful. However, in the Nyctaginaceae species studied here, single biosynthetic pathways often contributed several individual compounds (e.g., many species included in this study emitted multiple compounds from the BEAT pathway; Fig. 1). The step matrix approach would have required overlooking much of this information or scoring most taxa as polymorphic for each character. A step matrix even more complex than that of Barkman (2001) might successfully encompass the diversity of fragrance compounds that we observed.

Fragrance characters in phylogenetics.—The four presence/absence fragrance data sets all had significant hierarchical signal (g_1 tests; Table 3), but this signal was not congruent with signal from DNA sequence data (partition homogeneity tests and tree-to-tree distances; Table 3). Thus, although the fragrance data sets could be used to infer trees, these trees have little in common with the hypothesis of phylogenetic relationships inferred from DNA sequence data. A partition-homogeneity test indicates that the fragrance data sets are mutually congruent; regardless of coding method, fragrance information is hierarchically structured in the same way. Below we discuss alternative explanations for the hierarchical but nonphylogenetic structure that we detected in the fragrance data.

Congruence of fragrance with DNA topology.—Although trees inferred from fragrance characters were not congruent with the phylogenetic hypothesis inferred from DNA sequence data, some aspects of fragrance are clearly consistent with phylogenetic patterns. In terms of congruence with the DNA phylogeny, as indicated by CI and RI values, fragrance characters were as variable as the DNA data themselves (Table 5). Although the median CI and RI values for the DNA sequence data were 1 (i.e., perfect congruence), individual DNA characters had CI and RI values as low as 0.25 and 0, respectively. Median CI and RI values for the fragrance data sets were substantially lower than those for the DNA sequence data, but some compounds were perfectly congruent with the DNA phylogeny (Table 5).

In contrast, no pathway characters had CI or RI values of 1; contrary to our prediction, these characters were no less homoplastic than compounds. However, data set 4a (presence/absence of Fl + V pathways) does provide limited support for our hypothesis that pathways would evolve deeper in the phylogeny than compounds and, thus, have higher RI values. Most biosynthetic pathways are present in almost all species that we studied; therefore, they are optimized as evolving deep in the phylogeny with subsequent loss(es) in the few species that lack them. The phenyl acetic acid pathway (Fig. 7; RI = 0.60) exemplifies this pattern. Five compounds from this pathway were detected in our fragrance samples, and at least one of these appears in 14 taxa. This pathway was likely present in the common ancestor of all of these plants (its precursor, phenylalanine, is essential for primary metabolic processes), and variation among species in the presence of compounds from this pathway probably is due to its parallel loss within the *Mirabilis* lineage (Fig. 7). These results suggest that pathways are more evolutionarily labile than we expected. However, these apparent losses of pathways may reflect silencing, perhaps related to a lack of the compounds that serve as substrates (Kolosova et al., 2001). Thus, these transitions should probably be viewed as losses from the fragrance profiles but not necessarily from plant biochemistry.

We found limited support for our hypothesis of a negative relationship between number of compounds per pathway and homoplasy (Table 6). The relationship between RI and number of compounds per pathway is negative and almost significant ($P = 0.06$). The jasmonate pathway (Fig. 7), with its single compound and high RI value (0.78), exemplifies the negative relationship between number of compounds per biosynthetic pathway and homoplasy. Small sample size (only 11 [data set 4a] or 14 [data set 4b] pathways were parsimony informative) restricts our power to test hypotheses involving pathways.

There was stronger support for the hypothesis of a negative relationship between number of taxa in which a compound occurs and CI ($P < 0.0001$). For example, compounds produced by the lactone pathway (individual compound CIs, 0.5–1) and phenylpropanoid pathway (individual compound CIs = 0.5) occur in few taxa. Conversely, more common compounds such as the benzoic acid esters show higher levels of homoplasy (individual compound CIs, 0.13–1).

Some of the biosynthetic pathways in our data set that are congruent with phylogenetic relationships have to date been detected in relatively few angiosperm floral fragrances (Knudsen et al., 1993). For example, lactones are infrequent in floral fragrances (Knudsen et al., 1993) but are more common in fruit odors (Tressl and Albrecht, 1986). In Nyctaginaceae, many *Acleisanthes* species emit lactones in floral fragrance, but these are absent from *Mirabilis* (Levin et al., 2001). In addition, two lactones (δ-octalactone and δ-nonalactone) are synapomorphies for the sister species *A. lanceolata* and *A. parvifolia* (Fig. 7).

At the level of individual compounds and pathways, the patterns we detected may be limited to Nyctaginaceae. However, we predict that the more general relationships (e.g., the negative relationship between CI and ubiquity of compounds) that we detected will be more widespread. For example, in groups where the presence of vegetative sesquiterpenoids is the rule rather than the exception (e.g., Asteraceae; Johnson and Lincoln, 1987), we would expect greater homoplasy in the phylogenetic distribution of specific sesquiterpenoids. Clearly, similar studies with other groups of plants are needed for comparison with the results reported here for Nyctaginaceae.

In general, fragrance characters may be too evolutionarily labile to be useful in phylogenetic inference (Williams and Whitten, 1999; Barkman, 2001). Cannatella et al. (1998) suggested that the same is true of frog vocalizations. However, our results clearly indicate that some fragrance characters are patterned phylogenetically. Further, whereas some fragrance compounds are conserved at the level of the largest lineages studied here (e.g., lactones and many sesquiterpenoids are limited to the *Acleisanthes* lineage), there is more congruence between fragrance and phylogeny at the level of sister-group relationships. The congruence of fragrance and phylogenetic relationships at low taxonomic levels is consistent with results of a number of previous studies of volatiles (Azuma et al., 1997, 1999; Adams, 1999; Williams and Whitten, 1999). As with other types of characters, the general lack of congruence of the fragrance data sets with the DNA sequence data does not mean that we should exclude all fragrance characters from phylogenetic studies. Rather, we need to learn to distinguish phylogenetically informative characters from those that reflect factors other than phylogenetic history.

Factors Influencing Fragrance Composition

An understanding of why some fragrance characters are congruent with phylogenetic patterns whereas others are not demands an understanding of the factors beyond phylogeny that influence fragrance. Selection related to pollinator relationships is likely important in determining fragrance composition. In general, multiple independent plant lineages that have experienced selection for

production of a suite of compounds associated with a particular pollinator would yield the pattern that we observed: fragrance data sets with congruent hierarchical signal that conflicts with phylogeny. Many of the taxa that we studied have hawkmoth-pollinated flowers, and Levin (2001) and Levin et al. (2001) showed that nitrogenous compounds are correlated with moth pollination in these plants. The sister taxa *A. angustifolia* + *A. undulata* are the only species of *Acleisanthes* in this study with pollinators other than hawkmoths (Levin et al., 2001). Perhaps as a result, these species differ from other *Acleisanthes* in emitting smaller amounts of fragrance composed of fewer compounds.

Behavioral studies of bats (Winter and von Helversen, 2001), noctuid moths (Plepys et al., 2002), and hawkmoths (Raguso and Willis, 2003) have indicated that these animals' sensory requirements for foraging are more flexible than previously appreciated. No one compound or ingredient is absolutely necessary for attraction and feeding by these animals. Compounds of similar polarity and functionality may play interchangeable roles in pollinator attraction or learning (Raguso et al., 1996). For example, methyl benzoate and methyl salicylate may be equally attractive to hawkmoths. To further examine the association between chemistry and pollination, we are currently using naïve hawkmoths in behavioral bioassays and are conducting parallel studies involving other plant groups in which hawkmoth pollination has evolved.

Further complicating our ability to understand the role of individual compounds in pollinator relationships is the idea that there may be synergistic interactions among compounds. Fragrance profiles, like animal courtship songs (e.g., of frogs and birds), have many compounds or notes, respectively, creating the potential for "harmonics" among blended components (Ryan and Rand, 1990, 1999). Harmonic properties of sound are reasonably well understood, but such emergent properties are only beginning to be explored in olfaction (see review by Smith and Getz, 1994). We are exploring the use of multivariate techniques to identify "clusters" of fragrance compounds that should provide hints about olfactory harmonics.

Many of the pathways involved in the biosynthesis of floral fragrance compounds also synthesize compounds that play roles in herbivore defense. This close association may be related to the dual roles played by odor as both attractant for pollinators and repellant for herbivores/florivores (Galen, 1985, 1999; Mullin et al., 1991; Berenbaum and Seigler, 1992; Dobson, 1994; Euler and Baldwin, 1996). Fragrance blends are often composed of both attractive and repellent compounds (Euler and Baldwin, 1996; Omura et al., 2000), with certain compounds being at once attractive to some insects and repellent to others (Berenbaum and Seigler, 1992; Birkett et al., 2000; Dobson and Bergström, 2000). Floral odor has been shown to act as a feeding and/or oviposition cue for herbivores (Metcalf and Metcalf, 1992; Dobson, 1994), thus suggesting a cost to the olfactory apparency provided by fragrance (Baldwin et al., 1997). Under these circumstances, floral fragrance should be lost in evolutionary shifts from pollinators that use odor to locate flowers to pollination systems that do not require fragrance.

One of the most notable groups of compounds involved in both insect attraction and deterrence are the jasmonates. Jasmonic acid and its metabolites are important signal transductants in herbivore-induced plant defenses (see reviews by Birkett et al., 2000; Dicke and van Loon, 2000; Ryan, 2000). Jasmonates are also found in moth-pollinated flowers in many angiosperm lineages (Kaiser, 1993). One of these compounds, cis-jasmone, is released from the flowers of a number of Nyctaginaceae species (Fig. 7). This compound appears to have been present ancestrally and was then lost multiple times; these parallel losses may reflect selection for increased plant defense. Enzymes that synthesize compounds involved in herbivore deterrence or pathogen defense likely compete for substrates with enzymes that synthesize floral fragrance. Thus, the presence of cis-jasmone in floral fragrance may be limited by demands for its precursors in synthesis of defense compounds. A similar argument can be made for the many aromatic compounds derived from salicylic acid, which also mediates the biosynthesis of plant defense compounds (Yalpani et al., 1993; Hardie et al., 1994; Ross et al., 1999).

The metabolic costs of compound biosynthesis also are likely to affect the composition of fragrance. Selection may favor replacement of very costly compounds (e.g., in nitrogen or ATPs) with functional equivalents that are less costly to produce. However, plant secondary metabolism is notably complex and plastic, and determination of the limiting biochemical steps or nutrients is difficult at best. Euler and Baldwin (1996) argued that the metabolic costs of fragrance, based on nitrogen expenditure, are negligible in comparison to the costs of nonvolatile defenses and of herbivory due to volatile-mediated increases in apparency to enemies. This idea is supported by the low cost of volatiles in terms of carbon investment reported for *Ficus carica* (Grison-Pigé et al., 2001).

Conclusions

Although fragrance characters may not be especially useful for phylogeny reconstruction, many of these characters can provide support for relationships inferred using other data. As with other types of characters, we need to learn to distinguish fragrance characters that are likely to reflect phylogenetic history from those that are free to respond to selection. Improved knowledge of the development (i.e., biosynthesis), including metabolic costs, of fragrance compounds will help. Enhanced understanding of the function of fragrance compounds will also be useful in this regard. Certain compounds may be more homoplastic than others because they are under selection by pollinators or herbivores or are simply by-products of the synthesis of other compounds. More studies such as this in combination with investigations of the function, cost, and genetic background of these compounds are necessary to understand fully the evolution of fragrance as a complex phenotype.

ACKNOWLEDGMENTS

We are grateful to T. Tully, A. Boyd, M. Manktelow, D. Hearn, and A. Chetochine for assistance with fragrance collections. We thank Big Bend National Park, the Jornada LTER, and J. Duft for access to plants and A. Le Duc and M. Fishbein for providing seeds. For use of a GC-MS and injection of many of the samples, we thank E. Pichersky and J. D'Auria (University of Michigan). We are grateful for the helpful comments provided by J. G. Lundberg on an earlier version of the manuscript and for the assistance of J. S. Miller with figure preparation. This manuscript was greatly improved by constructive criticisms from C. Simon, R. Mason-Gamer, M. Whitten, and an anonymous reviewer. The National Science Foundation provided major support for the project (DEB9806840 to L. A. McDade and R. A. Raguso); further support came from grants to R. A. Levin from the American Society of Plant Taxonomists, Sigma Xi, the Department of Ecology & Evolutionary Biology at the University of Arizona, and the University of Arizona Research Training Group in the Analysis of Biological Diversification (NSF DIR-9113362, BIR-9602246).

REFERENCES

ADAMS, R. P. 1999. Systematics of multi-seeded eastern hemisphere *Juniperus* based on leaf essential oils and RAPD DNA fingerprinting. Biochem. Syst. Ecol. 27:709–725.

AGELOPOULOS, N. G., AND J. A. PICKETT. 1998. Headspace analysis in chemical ecology: Effects of different sampling methods on ratios of volatile compounds present in headspace samples. J. Chem. Ecol. 24:1161–1172.

ARCHIE, J. 1989a. A randomization test for phylogenetic information in systematic data. Syst. Zool. 38:239–252.

ARCHIE, J. 1989b. Homoplasy excess ratios: New indices for measuring levels of homoplasy in phylogenetic systematics and a critique of the consistency index. Syst. Zool. 38:253–269.

AZUMA, H., L. B. THIEN, AND S. KAWANO. 1999. Molecular phylogeny of *Magnolia* (Magnoliaceae) inferred from cpDNA sequences and evolutionary divergence of the floral scents. J. Plant Res. 112:291–306.

AZUMA, H., M. TOYOTA, Y. ASAKAWA, R. YAMAOKA, J. G. GARCIA-FRANCO, G. DIERINGER, L. B. THIEN, AND S. KAWANO. 1997. Chemical divergence in floral scents of *Magnolia* and allied genera (Magnoliaceae). Plant Species Biol. 12:69–83.

BALDWIN, I. T., C. PRESTON, M. EULER, AND D. GORHAM. 1997. Patterns and consequences of benzyl acetone floral emissions from *Nicotiana attenuata* plants. J. Chem. Ecol. 23:2327–2343.

BARKER, F. K., AND F. M. LUTZONI. 2002. The utility of the incongruence length difference test. Syst. Biol. 51:625–637.

BARKMAN, T. J. 2001. Character coding of secondary chemical variation for use in phylogenetic analyses. Biochem. Syst. Ecol. 29:1–20.

BARKMAN, T. J., J. H. BEAMAN, AND D. A. GAGE. 1997. Floral fragrance variation in *Cypripedium*: Implications for evolutionary and ecological studies. Phytochemistry 44:875–882.

BERENBAUM, M., AND D. SEIGLER. 1992. Biochemicals: Engineering problems for natural selection. Pages 89–121 *in* Insect chemical ecology: An evolutionary approach (B. D. Roitberg and M. B. Isman, eds.). Chapman and Hall, New York.

BERGSTRÖM, G., H. E. M. DOBSON, AND I. GROTH. 1995. Spatial fragrance patterns within the flowers of *Ranunculus acris* (Ranunculaceae). Plant Syst. Evol. 195:221–242.

BESTMANN, H. J., L. WINKLER, AND O. VON HELVERSEN. 1997. Headspace analysis of volatile flower scent constituents of bat-pollinated plants. Phytochemistry 46:1169–1172.

BIRKETT, M. A., C. A. M. CAMPBELL, K. CHAMBERLAIN, E. GUERRIERI, A. J. HICK, J. L. MARTIN, M. MATTHES, J. A. NAPIER, J. PETTERSSON, J. A. PICKETT, G. M. POPPY, E. M. POW, B. J. PYE, L. E. SMART, G. H. WADHAMS, L. J. WADHAMS, AND C. M. WOODCOCK. 2000. New roles for *cis*-jasmone as an insect semiochemical and in plant defense. Proc. Natl. Acad. Sci. USA 97:9329–9334.

BREMER, K. 1988. The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. Evolution 42:795–803.

CANNATELLA, D. C., D. M. HILLIS, P. T. CHIPPINDALE, L. WEIGT, A. S. RAND, AND M. J. RYAN. 1998. Phylogeny of frogs of the *Physalaemus pustulosus* species group, with an examination of data incongruence. Syst. Biol. 47:311–335.

CROTEAU, R., AND F. KARP. 1991. Origin of natural odorants. Pages 101–126 *in* Perfumes: Art, science and technology (P. M. Müller and D. Lamparsky, eds.). Elsevier, London.

DARLU, P., AND G. LECOINTRE. 2002. When does the incongruence length difference test fail? Mol. Biol. Evol. 19:432–437.

DEBRY, R. W. 2001. Improving interpretation of the decay index for DNA sequence data. Syst. Biol. 50:742–752.

DELPINO, F. 1874. Ulteriori osservazioni e considerazioni sulla dicogamia nel regno vegetale. 2 (IV). Delle piante zoidifile. Atti Soc. Ital. Sci. Nat. 16:151–349.

DICKE, M., AND J. J. A. VAN LOON. 2000. Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. Entomol. Exp. Appl. 97:237–249.

DOBSON, H. E. M. 1994. Floral volatiles in insect biology. Pages 47–81 *in* Insect–plant interactions, Volume V (E. A. Bernays, ed.). CRC Press, Boca Raton, Florida.

DOBSON, H. E. M., J. ARROYO, G. BERGSTRÖM, AND I. GROTH. 1997. Interspecific variation in floral fragrances within the genus *Narcissus* (Amaryllidaceae). Biochem. Syst. Ecol. 25:685–706.

DOBSON, H. E. M., AND G. BERGSTRÖM. 2000. The ecology and evolution of pollen odors. Plant Syst. Evol. 222:63–87.

DONOGHUE, M. J., R. G. OLMSTEAD, J. F. SMITH, AND J. D. PALMER. 1992. Phylogenetic relationships of Dipsacales based on *rbcL* sequences. Ann. Mo. Bot. Gard. 79:333–345.

DUDAREVA, N., J. C. D'AURIA, K. H. NAM, R. A. RAGUSO, AND E. PICHERSKY. 1998a. Acetyl-CoA:benzylalcohol acetyltransferase—An enzyme involved in floral scent production in *Clarkia breweri*. Plant J. 14:297–304.

DUDAREVA, N., AND E. PICHERSKY. 2000. Biochemical and molecular genetic aspects of floral scents. Plant Physiol. 122:627–633.

DUDAREVA, N., R. A. RAGUSO, J. WANG, J. R. ROSS, AND E. PICHERSKY. 1998b. Floral scent production in *Clarkia breweri*: III. Enzymatic synthesis and emission of benzenoid esters. Plant Physiol. 116:599–604.

DUSENBERY, D. B. 1992. Sensory ecology: How organisms acquire and respond to information. W. H. Freeman and Co., New York.

EULER, M., AND I. T. BALDWIN. 1996. The chemistry of defense and apparency in the corollas of *Nicotiana attenuata*. Oecologia 107:102–112.

FAEGRI, K., AND L. VAN DER PIJL. 1979. The principles of pollination ecology, 3rd edition. Pergamon Press, Oxford.

FARRIS, J. S. 1989. The retention index and rescaled consistency index. Cladistics 5:417–419.

FARRIS, J. S., M. KÄLLERSJÖ, A. G. KLUGE, AND C. BULT. 1994. Testing significance of incongruence. Cladistics 10:315–319.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791.

GALEN, C. 1985. Regulation of seed-set in *Polemonium viscosum*: Floral scents, pollination, and resources. Ecology 66:792–797.

GALEN, C. 1999. Flowers and enemies: Predation by nectar-thieving ants in relation to variation in floral form of an alpine wildflower, *Polemonium viscosum*. Oikos 85:426–434.

GOLDMAN, N., J. P. ANDERSON, AND A. G. RODRIGO. 2000. Likelihood-based tests of topologies in phylogenies. Syst. Biol. 49:652–670.

GRISON-PIGÉ, L., J.-L. SALAGER, M. HOSSAERT-MCKEY, AND J. ROY. 2001. Carbon allocation to volatiles and other reproductive components in male *Ficus carica* (Moraceae). Am. J. Bot. 88:2214–2220.

HARDIE, J., R. ISAACS, J. A. PICKETT, L. J. WADHAMS, AND C. M. WOODCOCK. 1994. Methyl salicylate and (-)-(1R,5S)-myrtenal are plant-derived repellents for black bean aphid, *Aphis fabae* Scop. (Homoptera: Aphididae). J. Chem. Ecol. 20:2847–2855.

HILLIS, D. M., AND J. P. HUELSENBECK. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. J. Hered. 83:189–195.

JAKOBSEN, H. B., AND C. E. OLSEN. 1994. Influence of climatic factors on emission of flower volatiles in situ. Planta 192:365–371.

JOHNSON, R. H., AND D. E. LINCOLN. 1987. A phytochemical and physiological comparison of *Artemisia tridentata* mountain and basin populations. Am. J. Bot. 74:702–703.

KAISER, R. 1993. The scent of orchids: Olfactory and chemical investigations. Elsevier, Amsterdam.

KERNER VON MARILAUM, A. 1895. The natural history of plants: Their forms, growth, reproduction and distribution. Blackie and Son, London.

KNUDSEN, J. T. 1999. Floral scent chemistry in geonomoid palms (Palmae: Geonomeae) and its importance in maintaining reproductive isolation. Mem. NY Bot. Gard. 83:141–157.

KNUDSEN, J. T., AND L. TOLLSTEN. 1995. Floral scent in bat-pollinated plants: A case of convergent evolution. Bot. J. Linn. Soc. 119:45–57.

KNUDSEN, J. T., L. TOLLSTEN, AND L. G. BERGSTRÖM. 1993. Floral scents—A checklist of volatile compounds isolated by head-space techniques. Phytochemistry 33:253–280.

KOLOSOVA, N., N. GORENSTEIN, C. M. KISH, AND N. DUDAREVA. 2001. Regulation of circadian methyl benzoate emission in diurnally and nocturnally emitting plants. Plant Cell 13:2333–2347.

LEVIN, R. A. 2000. Phylogenetic relationships within Nyctaginaceae tribe Nyctagineae: Evidence from nuclear and chloroplast genomes. Syst. Bot. 25:738–750.

LEVIN, R. A. 2001. Relationships among fragrance, phylogeny and pollination in southwestern Nyctaginaceae. Ph.D. Dissertation, Univ. Arizona, Tucson.

LEVIN, R. A. 2002. Taxonomic status of *Acleisanthes*, *Selinocarpus*, and *Ammocodon* (Nyctaginaceae). Novon 12:58–63.

LEVIN, R. A., R. A. RAGUSO, AND L. A. MCDADE. 2001. Fragrance chemistry and pollinator affinities in Nyctaginaceae. Phytochemistry 58:429–440.

MABEE, P. M., AND J. HUMPHRIES. 1993. Coding polymorphic data: Examples from allozymes and ontogeny. Syst. Biol. 42:166–181.

MACTAVISH, H. S., AND R. C. MENARY. 1997. Volatiles in different floral organs, and effect of floral characteristics on yield of extract from *Boronia megastigma* (Nees). Ann. Bot. 80:305–311.

MADDISON, D. R., AND W. P. MADDISON. 2000. MacClade 4: Analysis of phylogeny and character evolution. Sinauer, Sunderland, Massachusetts.

MAHMOUD, S. S., AND R. B. CROTEAU. 2002. Strategies for transgenic manipulation of monoterpene biosynthesis in plants. Trends Plant Sci. 7:366–373.

METCALF, R. L., AND E. R. METCALF. 1992. Plant kairomones in insect ecology and control. Chapman and Hall, New York.

MIYAKE, T., R. YAMAOKA, AND T. YAHARA. 1998. Floral scents of hawkmoth-pollinated flowers in Japan. J. Plant Res. 111:199–205.

MULLIN, C. A., A. A. ALFATAFTA, J. L. HARMAN, A. A. SERINO, AND S. L. EVERETT. 1991. Corn rootworm feeding on sunflower and other Compositae: Influence of floral terpenoid and phenolic factors. Pages 278–292 *in* Naturally occurring pest bioregulators (P. A. Hedin, ed.). American Chemical Society, Washington, D.C.

OMURA, H., K. HONDA, AND N. HAYASHI. 2000. Floral scent of *Osmanthus fragrans* discourages foraging behavior of cabbage butterfly, *Pieris rapae*. J. Chem. Ecol. 26:655–666.

PELLMYR, O., L. B. THIEN, G. BERGSTRÖM, AND I. GROTH. 1990. Pollination of New Caledonian *Winteraceae*: Opportunistic shifts or parallel radiation with their pollinators? Plant Syst. Evol. 173:143–157.

PICHERSKY, E., AND J. GERSHENZON. 2002. The formation and function of plant volatiles: Perfumes for pollinator attraction and defense. Curr. Opin. Plant Biol. 5:237–243.

PICHERSKY, E., R. A. RAGUSO, E. LEWINSOHN, AND R. CROTEAU. 1994. Floral scent production in *Clarkia* (Onagraceae): I. Localization and developmental modulation of monoterpene emission and linalool synthase activity. Plant Physiol. 106:1533–1540.

PLEPYS, D., F. IBARRA, W. FRANCKE, AND C. LÖFSTEDT. 2002. Odour-mediated nectar foraging in the silver Y moth, *Autographa gamma* L. (Lepidoptera: Noctuidae): Behavioural and electrophysiological responses to floral volatiles. Oikos 99:75–82.

RAGUSO, R. A. 2001. Floral scent, olfaction and scent-driven foraging behavior. Pages 83–105 *in* Cognitive ecology of pollination: Animal behavior and floral evolution (L. Chittka and J. D. Thomson, eds.). Cambridge Univ. Press, Cambridge, U.K.

RAGUSO, R. A., D. M. LIGHT, AND E. PICHERSKY. 1996. Electroantennogram responses of *Hyles lineata* (Sphingidae: Lepidoptera) to volatile compounds from *Clarkia breweri* (Onagraceae) and other moth-pollinated flowers. J. Chem. Ecol. 22:1735–1766.

RAGUSO, R. A., AND O. PELLMYR. 1998. Dynamic headspace analysis of floral volatiles: A comparison of methods. Oikos 81:238–254.

RAGUSO, R. A., AND E. PICHERSKY. 1999. A day in the life of a linalool molecule: Chemical communication in a plant–pollinator system. Part 1. Linalool biosynthesis in flowering plants. Plant Species Biol. 14:95–120.

RAGUSO, R. A., AND M. A. WILLIS. 2003. Hawkmoth pollination in Arizona's Sonoran Desert: Behavioral responses to floral traits. *In* Butterflies: Ecology and evolution taking flight (C. L. Boggs, W. B. Watt, and P. R. Erhlich, eds.). Rocky Mountain Biological Lab Symposium Series, Univ. Chicago Press, Chicago.

ROSS, J. R., K. H. NAM, J. C. D'AURIA, AND E. PICHERSKY. 1999. S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. Arch. Biochem. Biophys. 367:9–16.

RYAN, C. A. 2000. The systemin signaling pathway: Differential activation of plant defensive genes. Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 1477:112–121.

RYAN, M. J., AND A. S. RAND. 1990. The sensory basis of sexual selection for complex calls in the Túngara frog, *Physalaemus pustulosus* (sexual selection for sensory exploitation). Evolution 44:305–314.

RYAN, M. J., AND A. S. RAND. 1999. Phylogenetic influence on mating call preferences in female Túngara frogs, *Physalaemus pustulosus*. Anim. Behav. 57:945–956.

SAS INSTITUTE. 1997. JMPIN, version 3.2.1. SAS Institute, Cary, North Carolina.

SCHREIER, P. 1984. Chromatographic studies of biogenesis of plant volatiles. Alfred Hüthig Verlag, Heidelberg.

SEAMAN, F. C., AND V. A. FUNK. 1983. Cladistic analysis of complex natural products: Developing transformation series from sesquiterpene lactone data. Taxon 32:1–27.

SHIMADZU CO. 1993–1996. Class-5000, version 2.0.0. Shimadzu chemical laboratory analysis system and software. Shimadzu Co., Kyoto, Japan.

SMITH, B. H., AND W. M. GETZ. 1994. Non-pheromonal olfactory processing in insects. Annu. Rev. Entomol. 39:351–375.

SPSS. 1999. SPSS for Windows, version 10.0.5. SPSS, Chicago.

STEELE, C. L., J. CROCK, J. BOHLMANN, AND R. CROTEAU. 1998. Sesquiterpene synthases from grand fir (*Abies grandis*): Comparison of constitutive and wound-induced activities, and cDNA isolation, characterization, and bacterial expression of δ-selinene synthase and γ-humulene synthase. J. Biol. Chem. 273:2078–2089.

SWOFFORD, D. L. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer, Sunderland, Massachusetts.

TEMPLETON, A. R. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the humans and apes. Evolution 37:221–244.

TRESSL, R., AND W. ALBRECHT. 1986. Biogenesis of aroma compounds through acyl pathways. Pages 114–133 *in* Biogeneration of aromas (T. H. Parliament and R. Croteau, eds.). American Chemical Society, Washington, D.C.

WANG, J., N. DUDAREVA, S. BHAKTA, R. A. RAGUSO, AND E. PICHERSKY. 1997. Floral scent production in *Clarkia brewerii* (Onagraceae): II. Localization and developmental modulation of the enzyme *s*-adenosyl-L-methionine:(iso)eugenol *o*-methyltransferase and phenylpropanoid emission. Plant Physiol. 114:213–221.

WILEY, R. H. 2000. A new sense of the complexities of bird song. Auk 117:861–868.

WILLIAMS, N. H., AND W. M. WHITTEN. 1999. Molecular phylogeny and floral fragrances of male euglossine bee-pollinated orchids: A study of *Stanhopea* (Orchidaceae). Plant Species Biol. 14:129–136.

WINTER, Y., AND O. VON HELVERSEN. 2001. Bats as pollinators: Foraging energetics and floral adaptations. Pages 148–170 *in* Cognitive ecology of pollination: Animal behavior and floral evolution (L. Chittka and J. D. Thomson, eds.). Cambridge Univ. Press, Cambridge, U.K.

YALPANI, N., J. LEÓN, M. A. LAWTON, AND I. RASKIN. 1993. Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. Plant Physiol. 103:315–321.

ZAR, J. H. 1996. Biostatistical analysis, 3rd edition. Prentice-Hall, Upper Saddle River, New Jersey.

First submitted 10 May 2002; reviews returned 30 September 2002; final acceptance 3 February 2003
Associate Editor: Roberta Mason-Gamer