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Molecular evidence for naturalness of genera in the tribe Antirrhineae (Scrophulariaceae) and three independent evolutionary lineages from the New World and the Old

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Abstract. The tribe Antirrhineae consists of 29 genera distributed in the New World and the Old. Phylogenetic analyses of ITS and *ndh*F sequences served to recognize six main lineages: Anarrhinum group (*Anarrhinum*, *Kickxia*); Linaria group (*Linaria*); Maurandya group (*Cymbalaria*, *Asarina*, *Maurandella*, *Rhodochiton*, *Lophospermum*); Schweinfurthia group (*Pseudorontium*, *Schweinfurthia*); Antirrhinum group (*Antirrhinum*, *Pseudomisopates*, *Misopates*, *Acanthorrhinum*, *Howeliella*, *Neogarrhinum*, *Sairocarpus*, *Mohavea*, *Galvezia*); Chaenorrhinum group (*Chaenorrhinum*, *Albraunia*, *Holzneria*). Parsimony (cladistics), distance-based (Neighbor-Joining), and Bayesian inference reveal that: (1) the tribe is a natural group; (2) genera such as *Linaria*, *Schweinfurthia*, *Kickxia*, and *Antirrhinum* also form natural groups; (3) three Antirrhineae lineages containing genera from the New and Old World are the result of three intercontinental disjunctions displaying similar levels of ITS-sequence divergence and differentiation times (Oligocene-Miocene); (4) evolution of flower shapes is not congruent with primitiveness of personate flowers; (5) both polyploidy and dysploidy appear to be responsible for most variation in chromosome number in the six main lineages. Nuclear and chloroplast evidence also supports the split of American and Mediterranean species of *Antirrhinum* into different genera, a result that should be contemplated in the interest of a more natural (monophyletic) taxonomy. Nucleotide additivity causes poor resolution in the ITS analysis of 22 species of Mediterranean *Antirrhinum* and lead us to interpret extensive hybridization in the Iberian Peninsula.

Key words: Scrophulariaceae, Antirrhineae, *Antirrhinum*, ITS, *ndh*F, phylogeny, Northern Hemisphere biogeography, character evolution.

For a long time, the family Scrophulariaceae has been an undisputed dicot family of about 270 genera and 5,100 species (Mabberley 1997), despite it is characterized by a great morphological diversity in life forms (from annual herbs to trees); nutrition adaptations (from autotrophic to totally parasitic); physiologic mechanisms (C3 and C4 metabolic routes); leaf phyllotaxis (leaves alternate, opposite, or whorled); leaf venation (pinnately or palmately veined); inflorescence architecture (flowers solitary, cymes or racemes); flower symmetry (actinomorphic or zygomorphic); number of calyx lobes (2, 4, 5), corolla lobes (3, 4, 5), stamens (2, 4, 5), staminoides (0–3),

carpels (2–3), stigmas (1, 2); fruit types (dry or fleshy); and fruit aperture (schizocarps or capsules dehiscent or indehiscent) (Watson and Dallwitz 2000). One of the most remarkable results in molecular systematics of angiosperms is the disintegration of the family Scrophulariaceae. Phylogenetic analysis using chloroplast DNA sequences suggests that the Scrophulariaceae comprises at least five lineages of parasitic and non-parasitic scrophs (Olmstead and Reeves 1995, Olmstead et al. 2001). In addition, the placement of some of its genera within clades involving well-known families, such as Plantaginaceae, Callitrichaceae, Haloragidaceae, Orobanchaceae, and Globulariaceae, indicates that a profound family rearrangement is needed (Olmstead et al. 2001). Practical nomenclatural recommendations have been proposed at the familial level to name monophyletic assemblages of genera (e. g. Antirrhinaceae, Reeves and Olmstead 1998; Veronicaceae, Olmstead et al. 2001). However, profound incongruence between morphological characters based primarily on flower morphology and phylogenetic results based on molecular markers indicates need for (i) further investigation using additional and independent characters, and (ii) a full sample of all genera to imply a consistent familial classification (Brummitt 2002).

The tribe Antirrhineae is a group of Scrophulariaceae bearing tubular flowers with a basal appendix (spur, gibbous or saccate), poricidal capsule dehiscence (Rothmaler 1943), and unique iridoid glycosides (Kooiman 1970, Sutton 1988). The finding of a significant diversity in flower morphologies, chromosome numbers, and geographic distributions has promoted the recognition of an increasing number of genera within Antirrhineae over time (nine in Rouy 1909; 11 in Rothmaler 1943; 27 in Sutton 1988). Flower types range from actinomorphic to zygomorphic corollas closed by a palatee (personate flower), including peloric flowers as observed and described first time by Linnaeus (1749) in *Linaria*. Groupings of species of Antirrhineae by chromosome numbers (ranging from $2n = 12$ to $2n = 56$), supported circumscription of New and Old World genera, coupled with pollen (Elisens 1986), seed (Elisens and Tomb 1983), and macromorphological (Sutton 1988) characters. The tribe is mostly distributed in the Northern Hemisphere, but a few species also occur in South America. Floristic implications of disjunct distributions in *Linaria* and *Antirrhinum* were discussed in the past (Hong 1983) and multiple hypotheses of intercontinental relationships in Antirrhineae have been historically posed (Rothmaler 1943, Elisens and Tomb 1983, Hong 1983, Raven and Axelrod 1995).

Snapdragons display personate flowers with an inferior gibbous corolla in a group of species from the New World and the Old. This remarkable flower type is profoundly related to pollinators and has been used to circumscribe species of the genus *Antirrhinum*. Although a synthetic taxonomy has been adopted by some authors for this genus (Thompson 1988, Freeman and Scogin 1999), a most comprehensive revision of the Antirrhineae (Sutton 1988) recognizes species with personate flowers from the New World in three genera (*Sairocarpus*, *Neogaerrhinum*, and *Howeliella*) based on flower and fruit characters. Some other species from the Old World with personate flowers have also been included in different genera (*Kickxia* and *Misopates*) by European authors in the past (see Webb 1972, Sutton 1988). Accordingly, we adopt an analytical taxonomic treatment in which the genus *Antirrhinum s. str.* consists of ca. approx. 25 Old World species (Sutton 1988, Güemes, unpublished data) distributed in the Mediterranean Basin.

In a family such as the traditional Scrophulariaceae, where assemblages of unnatural groups appear to be common, the question remains about the circumscription of the tribe Antirrhinae and the recognition of monophyletic groups. Analysis of DNA chloroplast (mostly uniparental) sequences (*ndh*F), based on a sample of 16 genera of Antirrhineae, suggested four natural groups (Ghebrehiwet et al. 2000). To gain a better understanding of

phylogenetic relationships within the tribe, a mostly analytical classification of 29 genera and c. 320 species (Table 1) is herein followed (Sutton 1988), with the inclusion of *Nanorrhinum* (Ghebrehiwet 2000) and the recently described monotypic *Pseudomisopates* (Güemes 1997). Nuclear ribosomal ITS (Internal Transcribed Spacer) sequences (biparental) have proven to be a powerful tool in phylogenetics and systematics of closely related genera of angiosperms families, such as Asteraceae (Noyes and Rieseberg 1999, Fiz et al. 2002) and Araliaceae, (Valcárcel et al. 2003). We considered an extended sample of 22 genera and the use of 69 ITS sequences to investigate phylogenetic relationships of Antirrhineae taxa based on nuclear sequences. Additionally, the genus *Antirrhinum* was analyzed in detail performing phylogenetic analysis of ITS sequences and morphological characters of most of its species. The particular objectives of this study are to: (1) evaluate congruence between new nuclear (ITS) and previous chloroplast (*ndh*F) sequences; (2) describe monophyletic groups in Antirrhineae; (3) infer naturalness of three key genera (*Antirrhinum*, *Linaria*, *Schweinfurthia*); (4) interpret character evolution of personate flowers and chromosome numbers; (5) investigate the origin of New and Old World lineages.

Materials and methods

Plant material and sequencing. A total of 61 accessions were chosen for sequencing the Internal Transcribed Spacers (ITS) and representing the morphological diversity and geographical distribution of the genera of Antirrhineae (Table 1). Total genomic DNA was extracted from silica-dried material collected in the field and in a few cases from herbarium specimens. Approximately 0.5 g of dried leaf tissue was ground into a fine powder and incubated in 1 mL of 2x CTAB (hexadecyltrimethylammonium bromide) (Doyle and Doyle 1987, as modified by Loockerman and Jansen 1996), or extracted using the Dneasy-kit (QIAGEN Inc.). DNA was amplified using the PCR (Polymerase Chain Reaction) and external primers 17SE and 26SE described by Sun et al. (1994). PCR conditions followed Baldwin (1992), modified for symmetric amplifications (with equimolar primer concentrations) and implemented in a Perkin-Elmer PCR System 9700 thermal cycler. Amplified products were cleaned using spin filter columns (PCR Clean-up kit, MoBio Laboratories, California) following the protocols provided by the manufacturer. Purified products were then directly sequenced using dye terminators (Big Dye Terminator v 2.0, Applied Biosystems, California). For cycle sequencing on forward and reverse strands the primers ITS4 and ITS5 (White et al. 1990) and the following conditions were used: 95°C for 2 min followed by 25 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Polyacrylamide gel electrophoresis of sequencing products were conducted by using a Perkin-Elmer/Applied Biosystems model 377 automated sequencer. Sequence data were entered in a contig file and edited using the program Seqed (Applied Biosystems). IUPAC symbols were used to represent nucleotide ambiguities. Additionally, several PCR products were agarose-gel purified and ligated into the vector provided with the p-GEM-T (Promega) cloning kit. Plasmid DNA from individual recombinant colonies was isolated according to a miniprep protocol (High Pure Plasmid Isolationkit, Roche Diagnostics). For sequencing, cloned ITS products were reacted with BigDye Terminator Cycle Sequencing Ready Reaction (Perkin-Elmer, Applied Biosystems) using the ITS4 and ITS5 primers.

Molecular analyses. Phylogenetic reconstructions were performed in 61 species of the Antirrhineae as the ingroup, plus *Halleria*, *Chelone*, *Tetranema*, *Isoplexis*, *Plantago*, *Globularia*, and *Veronica* as the outgroup, based on previous *ndh*F sequences and phylogenies (Ghebrehiwet et al. 2000, Olmstead et al. 2001). To investigate relationships among species of *Antirrhinum*, we chose *Asarina procumbens* and *Cymbalaria muralis* as outgroup species based on our ITS phylogeny of Antirrhineae (Fig. 1). Phylogenetic reconstructions were obtained by using a matrix of ITS sequences aligned with Clustal X 1.62b (Thompson et al. 1997), manual adjustment of sequences, and implementing PAUP*4.0b10 (Swofford 2002). Maximum parsimony (MP) analyses were conducted using Fitch parsimony with equal weighting of all characters. Heuristic searches were replicated 100 times with random taxon-addition sequences, Tree Bisection-Reconnection (TBR) branch

Table 1. Accessions for the ITS-sequence study of Antirrhineae, including natural distribution of taxa, locality of wild populations, collection voucher, and GenBank accession numbers

Taxon	Natural distribution	Locality	Voucher	GenBank accession number
Acanthorrhinum Rothm. (1 sp.)	N Africa			
Acanthorrhinum ramosissimum (Cosson & Durieu) Rothm.	N Africa	Morocco: road from Ouarzazate to Zagora.	VAL 41469	AY731261
Albraunia Speta (3 spp.)	SW Asia			
Albraunia foveopilosa Speta	SW Iran	Iran: Khuzistan, Baghmalek-Haftgel	TARI 38909	AY731250
Anarrhinum Desf. (8 spp.)	Europe, N Africa, and SW Asia			
Anarrhinum corsicum Jordan & Fourr.	Corse	Corse	Podlech 47340 (A)	AF513881
Anarrhinum bellidifolium (L.) Willd.	W Europe	Natural Botanical Garden of Dublin	VAL 145150	AY731263
Antirrhinum L. (25 spp.)	Western Mediterranean region			
Antirrhinum australe Rothm.	SW Spain	Spain: Granada, Castril	VAL 36940	AY731273
Antirrhinum barrelieri Boreau	S & SE Spain-N Morocco	Spain: Albacete, Villa de Ves	VAL 145152	AY731272
Antirrhinum braun-blanquetii Rotm.	NW Spain-NE Portugal	Spain: Palencia, Cervera de Pisuerga	VAL 35121	AY731269
Antirrhinum charidemi Lange	SE Spain	Spain: Almería, Cabo de Gata	VAL 37158	AY731282
Antirrhinum graniticum Rothm.	C Spain-E Portugal	Spain: Madrid, Fuentidueña del Tajo	VAL 99540	AY731283
Antirrhinum grosii Font Quer	CW Spain	Spain: Ávila, Sierra de Gredos	VAL 37049	AY731281
Antirrhinum hispanicum Chav.	S Spain	Spain: Granada, Sierra Nevada	Vargas 120-99	AY731286
Antirrhinum latifolium Miller	N Spain-N Italy	Spain: Lérida, Bapà	VAL 144658	AY731274
Antirrhinum linkianum Boiss. & Reuter	W Portugal	Portugal: Sintra	VAL 144655	AY731278
Antirrhinum litigiosum Pau	E Spain	Spain: Valencia, Serra	VAL 144656	AY731271
Antirrhinum litigiosum Pau	E Spain	Spain: Zaragoza, Nuévalos	VAL 31598 (cloned)	AY731277
Antirrhinum majus L.	N Spain- S France	cultivated	Oyama s/n	AF513888
Antirrhinum majus L.	N Spain- S France	Spain: Lérida, Vall d'Aran	VAL 144657	AY731280
Antirrhinum meonanthum Hoffmanns. & Link	W Spain-E Portugal	Spain: Avila, El Tremedal	Vargas 149-99	AY731284
Antirrhinum microphyllum Rothm.	CE Spain	Spain: Guadalajara, Entrepeñas	VAL 40051	AY731267
Antirrhinum molle L.	N Spain	Spain: Huesca, Sopeira	VAL 35176	AY731268

Table 1 (Continued)

Antirrhinum mollissimum Rothm.	SE Spain	Spain: Almería, Sierra de Gádor	VAL 37143	AY731275
Antirrhinum pulverulentum Lázaro	CE Spain	Spain: Zaragoza, Nuévalos	VAL 31592	AY731279
Antirrhinum sempervirens Lapeyr.	N Spain-S France (Pyrenees)	Spain: Huesca, Escalar de Panticosa	VAL 145148	AY731270
Antirrhinum siculum Millar	CE Mediterranean region	Italy: Sicilia, Messine	VAL 119899	AY731276
Antirrhinum subbaeticum Güemes	SE Spain	Spain: Albacete, Bogarra	VAL 25645	AY731287
Antirrhinum tortuosum Boscex Vent.	CE Mediterranean region	Italy: Ancona, Sirolo	VAL 39871	AY731285
Antirrhinum valentinum Font Quer	E Spain	Spain: Valencia, La Safor	VAL 39799	AY731266
Asarina Miller (1 sp.)	NE Spain-S France			
Asarina procumbens Miller	NE Spain-S France	Botanischer Garten Berlin-Dahlem	VAL 145146	AF513879
Chaenorrhinum (DC.) Reichb. (21 spp.)	Europe and Mediterranean region			
Chaenorrhinum minus (L.) Lange	Europe-SW Asia	Unknown	McNeils 96-336 (GH)	AF513875
Chaenorrhinum tenellum (Cav.) Lange	E Spain	Spain: Valencia, Moixent	VAL 37839	AY731251
Chelone L. (6 spp.)	N America			
Chelone oblicua Mix	N America	Unknown	Wolfe et al., 2002	AF375164
Cymbalaria Hill (9 spp.)	Europe, Mediterranean region, and SW Asia			
Cymbalaria muralis P. Gaertner & al.	S Europe	Switerland	Nyffeler s.n.	AF513883
Galvezia Dombey ex Juss. (4 spp.)	S America			
Galvezia fruticosa J. F. Gmelin	Perú	Botanischer Garten Berlin-Dahlem	VAL 145156	AY731252
Globularia L. (22 spp.)	Europe, Mediterranean region, and SW Asia			
Globularia salicina Lam.	Canary Islands	Cultivated	Albach and Chase, 2001	AF313039
Halleria L. (4 spp.)	Africa			
Halleria lucida L.	S Africa	Unknown	Wolfe et al., 2002	AF375149
Holzneria Speta (2 spp.)	SW Asia			
Holzneria spicata (Korovin) Speta	SW Asia	Iran: Khorasan, Tobart-e Sefid	TARI 23577	AY731258
Howelliella Rothm. (1 spp.)	N America (California)			
Howelliella ovata (Eastw.) Rothm.	SW North America	California	Thompson 434 (GH)	AF513899
Isoplexis (Lindley) Loudon (3 spp.)	Macaronesia			
Isoplexis canariensis (L.) Loud.	Canary Islands	Cultivated	Albach and Chase, 2001	AF313033

Table 1 (Continued)

Taxon	Natural distribution	Locality	Voucher	GenBank accession number
Kickxia Dumort. (9 spp.)	Europe, N Africa, and W Asia			
Kickxia elatine (L.) Dumort.	Eurasia and N Africa	Spain: Barcelona, Sant Pere de Ribes-Sitges	VAL 41793	AY731265
Kickxia spuria (L.) Dumort.	Eurasia and N Africa	Spain: Valencia, Chera	VAL 37098	AY731264
Linaria Dumort. (150 spp.)	Africa, Asia, and Europe			
Linaria alpina (L.) Miller	C & S Europe	Spain: Huesca, Bujaruelo	VAL 145147	AY731243
Linaria chalepensis (L.) Chaz.	SW Europe	Chipre: Larnaka, Caje Kiti	MA 495681	AY731245
Linaria hirta (L.) Moench	S Iberian Peninsula and N Africa	Spain: Zaragoza, Malenquilla	MA 532669	AY731244
Linaria micrantha (Cav.) Hoffmanns. & Link	Mediterranean region and SW Asia	Spain: Alicante, Vall Gallinera	MA 562630	AY731242
Linaria repens (L.) Millar	W Europe	Spain: Cantabria, Toranzo	MA 595943	AY731246
Linaria spartea (L.) Chaz.	SW Europe	Spain: Ciudad Real, Solera del Pino	MA 596688	AY731247
Linaria triornithophora (L.) Willd.	W Iberian Peninsula	Spain: León, Palacios del Sil	MA 617622	AY731248
Linaria vulgaris Miller	Europe	Escaped from cultivation in Havard campus	Oyama RK 23 (A)	AF513874
Lophospermum D. Don (5 spp.)	N and C America			
Lophospermum erubescens D. Don	Mexico	Botanischer Garten Berlin-Dahlem	VAL 145154	AY731249
Maurandella (A. Gray) Rothm. (1 spp.)	SW North America			
Maurandella antirrhiniflora (Willd.) Rothm.	E Mexico	Mexico	Hill 18323 (GH)	AF513878
Misopates Raf. (7 spp.)	Asia, Europe, and N Africa			
Misopates calycinum (Vent.) Rothm.	SW Iberian Peninsula and N Africa	Spain: Canary Islands, Lanzarote	ORT s/n	AY731259
Misopates orontium (L.) Raf.	Mediterranean region	Spain: Valencia, Serra	VAL 145155	AY731260
Mohavea A. Gray (2 spp.)	N America			
Mohavea confertiflora (A. DC.) A. A. Heller	SW North America	California	Hileman L (s.n.)	AF513891
Neogaerrhinum Rothm. (2 spp.)	SW North America			
Neogaerrhinum strictum (Hooker & Arnott) Rothm.	SW North America	California	Thompson 306 (GH)	AF513904

Table 1 (Continued)

Plantago L. (270 spp.)	Cosmopolitan			
Plantago major L.	Eurasia	Unknown	Rondstead et al., 2002	AY101861
Pseudomisopates Güemes (1 spp.)	SW Europe			
Pseudomisopates rivas-martinezii (Sánchez Mata Güemes)	CW Spain	Spain: Avila, Sierra de Gredos, Conventos creek	Vargas 377-99	AY731262
Pseudorontium (A. Gray) Rothm. (1 spp.)	SW North America			
Pseudorontium cyathiferum (Bentham) Rothm.	SW North America	California	Van Devender 92–268 (AZ)	AF513884
Rhodochiton Zucc. ex Otto & Dieta. (3 spp.)	C America			
Rhodochiton atrosanguineum (Zucc.) Rothm.	C Mexico	Bergius Botanical Garden	VAL 145153	AF513876
Sairocarpus D.A. Sutton (13 spp.)	N America			
Sairocarpus nuttallianus D. A. Sutton	SW North America	California	Oyama RK 27 (A)	AF513895
Schweinfurthia A. Braun (6 spp.)	Africa and SW Asia			
Schweinfurthia pterosperma (A. Rich.) A. Braun	NE Africa and SW Asia	Unknown	Thulin 8205	AF513882
Schweinfurthia imbricata A. G. Miller & al.	E Oman	Oman: Wadi Bed	E 99215	AY731254
Schweinfurthia latifolia Baker ex Oliver	Yemen	Yemen: Hadramout, Wadi 'Aidid	E 99214	AY731255
Schweinfurthia papilionacea (L.) Boiss.	SW Asia	Oman: Near Muscat	E 46435	AY731253
Schweinfurthia pedicellata (T. Anderson) Balf. fil.	NE Africa and SW Asia	Socotra: Ras Bashorah	E 99213	AY731256
Schweinfurthia spinosa A. G. Miller & al.	Oman	Oman: Dhofar, Manston to Mudhai	E 99203	AY731257
Tetranema Benth (2 supp.)	C America			
Tetranema mexicanum Benth ex Lindl.	Mexico and Guatemala	Unknown	Wolfe et al.	AF 375151
Veronica L. (180 spp.)	Cosmopolitan			
Veronica sp.	unknown	Cultivated	Oyama RK (s.n.)	AF513873

Note. Herbarium acronyms following voucher numbers as in the Index Herbariorum Part (http://www.nybg.org/bsci/ih).

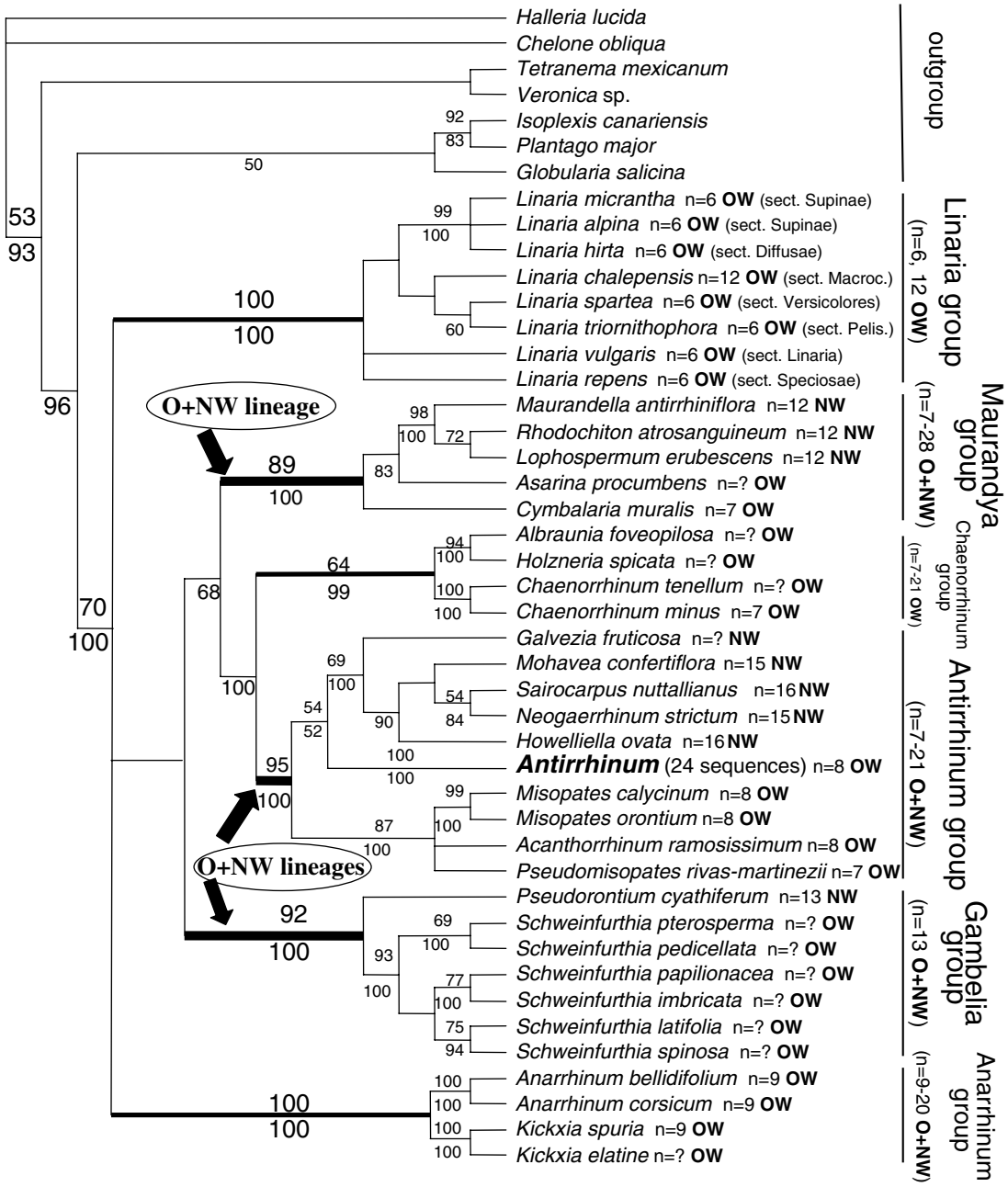

Fig. 1. Strict consensus tree of 2,515 most parsimonious trees of 1,623 steps from the analysis of the 68 ITS sequences of the Antirrhineae (CI: 0.46; RI: 0.68), with seven Scrophulariaceae genera serving as outgroup taxa based on Olmstead et al. (2001). Full bootstrapping and Bayesian posterior probabilities values over 50% are shown above and below branches, respectively. Species names as in Table 1, followed by gametophytic chromosome numbers (n), and abbreviation of distribution ranges: Old World (OW), New World (NW), and both (O + NW). Thick branches and vertical groupings indicate the six main Antirrhineae lineages

swapping, and with the options MULPARS and STEEPEST DESCENT in effect. Support for monophyletic groups was assessed by both "fast" bootstrapping and jacknifing (10,000 resamplings of the data) and "full" bootstrapping and jacknifing (100 resamplings) using the heuristic search

strategy as indicated above (see Mort et al. 2000 for discussion). The coding of gaps as a fifth base (indels), or any other coding strategy was not considered following the logic on alignment impact in Fiz et al. (2002). Accordingly, indels in the Antirrhineae matrix were treated as missing data. Alternatively, we addressed an approach by excluding from the Antirrhineae matrix two fragment regions at sites 102–152 (ITS-1) and 450–490 (ITS-2) to prevent equivocal alignments. A second matrix of *Antirrhinum* sequences plus those from the outgroup (*Asarina*, *Cymbalaria*) was analyzed. As a result of the alignment, there were two consecutive missing nucleotides in *Antirrhinum* (at sites 243–244) coded as three different character states: two gaps, one gap, and zero gaps. This coding strategy is particular convenient to include mutation events when there are no alignment conflicts (Simmons and Ochoterena 2000).

In addition, phylogenetic reconstructions of ITS sequences of Antirrhineae were also performed by using two different approaches: distance-based (Neighbor Joining) and Bayesian inference. To determine the simplest model of sequence evolution that best fits the sequence data, the Hierarchical Likelihood Ratio Test (hLRT) and the Akaike Information Criterion (AIC) were implemented by using MrModeltest 1.1b (Nylander 2000), which is a simplified version of Modeltest 3.06 (Posada and Crandall 1998). Among the 24 models of nucleotide substitution, GTR + G + I was selected. A Bayesian Inference for the ITS matrix was conducted using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2003). MrBayes was performed sampling for one million generations (with four MC chains, chain temperature 0.2; sample frequency was 100; burn-in 50,000). A 50% majority rule tree was obtained. For an ITS-sequence distance-based analysis, we used PAUP* 4.0b10, the GTR + G + I model, and the Neighbor-Joining method (Saitou and Nei 1987).

We used Felsenstein's tree-wide likelihood-ratio (LR) test for rate-constancy of molecular evolution across lineages of ITS trees (Baldwin and Sanderson 1998). Single exemplar sequences of each genus were used in the LR tests. Most parsimonious ITS trees were described in PAUP* 4.0b10 with and without a molecular-clock constraint. Differences in the log-likelihoods of clock-constrained and clock-unconstrained trees were assessed.

Morphological analysis. We examined characters considered taxonomically important in previous revisions of *Antirrhinum* (Wettstein 1891, Rothmaler 1956, Sutton 1988) to obtain data for cladistic analyses. Morphological characters were examined both in the field and from herbarium material (BCF, E, JACA, MA, SALA, SALAF, SEV, TARI, VAL). We eliminated characters that would not be phylogenetically informative and those that we could not reliably code for character-states. Thus, features concerning habit, hair length, leaf size, flower number and color, inflorescence arrangement, corolla hairiness, and capsule texture were excluded from the analyses. We identified 11 morphological characters that were potentially informative for the ingroup for species based on study of field specimens, herbarium material, and published literature. Ten of the 11 characters are qualitative, of which four were taken from vegetative parts (Table 2).

Table 2. Morphological characters used in the phylogenetic analyses of *Antirrhinum*

Character	Character state description
1 Glandular hairs in the lowermost part of the stem	0, absence; 1, presence
2 Eglandular hairs in the lowermost part of the stem	0, absence; 1, presence
3 Branches of stems twining	0, absence; 1, presence
4 Shape of upper bracts	0, wide like leaves; 1,
5 Calyx teeth	narrower than leaves
6 Corolla length	0, acute; 1, subacute to obtuse
7 Orientation of upper corolla lobes	0, > 30 mm; 1, < 30 mm
8 Shape of capsule	0, patent; 1, upwards
9 Eglandular hairs on the capsule	0, subglobose; 1, ovoid
10 Glandular hairs on the capsule	0, absence; 1, presence
11 Ornamentation of seeds	0, absence; 1, presence
	0, reticulate; 1, cristate

Table 3. States of the characters indicated in Table 2 for each of the 25 species of *Antirrhinum*, and infrageneric classification of the taxa examined following Rothmaler (1956)

	Character										
	1	2	3	4	5	6	7	8	9	10	11
Asarina procumbens	1	0	0	0	0	0	0	0	0	0	0
Cymbalaria muralis	0	0	0	0	0	1	0	0	0	0	1
Antirrhinum subsect. ***Kickxiella***											
A. sempervirens	0	1	0	0	0	1	0	0	1	0	1
A. pulverulentum	0	1	0	0	0	1	0	0	1	0	1
A. pertegasii	0	1	0	0	0	1	0	0	1	1	1
A. microphyllum	0	1	0	0	0	1	0	0	1	0	1
A. valentinum	1	1	0	0	1	1	1	0	0	1	1
A. subbaeticum	0	1	0	0	0	1	1	0	1	0	0
A. martenii	0	1	0	0	1	1	0	?	?	?	?
A. charidemi	0	1	0	0	1	1	0	0	1	1	0
A. molle	1	1	0	0	0	0	0	1	0	1	0
A. lopesianum	0	1	0	0	0	1	0	0	1	1	0
A. mollissimum	1	1	0	0	1	1	0	1	0	1	0
A. hispanicum	1	0	0	0	1	1	0	1	0	1	1
A. grosii	1	0	0	0	0	0	0	0	0	1	0
Antirrhinum subsect. ***Streptosepalum***											
A. meonanthum	1	1	0	1	0	1	0	1	0	1	0
A. braun-blanquetii	1	1	0	1	0	0	0	1	0	1	0
Antirrhinum subsect. ***Antirrhinum***											
A. australe	1	0	1	1	1	0	0	1	0	1	0
A. graniticum	1	0	0	1	1	0	0	1	0	1	0
A. latifolium	1	0	0	1	1	0	0	1	0	1	0
A. majus	1	0	0	1	1	0	0	1	0	1	0
A. linkianum	1	0	0	1	1	0	0	1	0	1	0
A. cirrhigerum	1	0	1	1	1	0	0	1	0	1	1
A. tortuosum	0	0	1	1	1	0	0	1	0	1	0
A. litigiosum	1	0	1	1	1	0	0	1	0	1	1
A. barrelieri	0	1	1	1	0	1	0	1	0	1	0
A. siculum	0	0	0	1	0	1	0	1	0	1	1

Patterns of historical biogeography and character evolution (life forms, chromosome variation, personate flower) were explored using the character-evolution reconstruction function of MacClade 3.01 (Maddison and Maddison 1992) on the most parsimonious trees. Both ACCTRAN (maximizing the proportion of the homoplasy that is accounted by parallelism) and DELTRAN (maximazing that by reversals) resolutions were considered and analyzed.

Results

Variation in ITS sequences. ITS sequence length in genera of Antirrhineae ranges between 578 (*Galvezia fruticosa*) and 619 bp (*Schweinfurthia imbricata*): 208–235 bp for ITS-1, 166 bp for 5.8S, and 203–225 bp for ITS-2. We chose the alignment obtained by CLUSTAL-based and manual adjustment of sequences minimizing alignment impact on

characters. In any case, tree topologies were mostly congruent irrespective of analyzing different Antirrhineae matrices (see below). Three-hundred and thirty-four variable and 245 parsimony-informative characters were found within Antirrhineae. The number of variable/parsimony-informative characters was distributed as follows: 162/126 bp for ITS-1, 11/4 bp for 5.8S, and 161/115 bp for ITS-2. Length variation among species of *Antirrhinum* ranges between 596 bp and 598 bp. The number of variable/parsimony-informative sites was 74/15, distributed as follows: 35/8 in ITS-1, 5/2 in 5.8S, and 34/5 in ITS-2. Corrected pair-wise sequence divergence (Kimura-2-parameter model; Kimura 1980) between accessions of the ITS region ranges from 0.00 to 38.5 % within Antirrhineae and from 0.00 to 4.37 % within *Antirrhinum* species. Nucleotide additivity for direct ITS sequences at some polymorphic sites, i. e. double peaks in the chromatograms, were observed in 14 species of *Antirrhinum*: *A. australe*, *A. barrelieri*, *A. graniticum*, *A. grosii*, *A. hispanicum*, *A. latifolium*, *A. litigiosum*, *A. majus*, *A. microphyllum*, *A. molle*, *A. siculum*, *A. subbaeticum*, and *A. tortuosum*.

Analyses of Antirrhineae ITS sequences. Results from the cladistic (MP) analysis using the matrix of ITS sequences of Antirrhineae aligned with Clustal X basically did not differ from those obtained when this matrix was manually realigned. Similar resolution and support was also obtained when ITS sites between 102–152 (ITS-1) and 450–490 (ITS-2) were excluded from the analyses to prevent any biased result from equivocal alignments. Cladistic analysis using the 61 ITS sequences and *Halleria*, *Chelone*, *Tetranema*, *Isoplexis*, *Plantago*, *Globularia*, and *Veronica* as outgroup taxa yielded 191,400 trees of 1076 steps (CI = 0.53 and RI = 0.46, including uninformative characters) before running out of memory (results not shown). There was no resolution for the 24 sequences of *Antirrhinum* (results not shown), which form a monophyletic group (100 % bootstrap support). To improve resolution among genera, we analyzed all Antirrhineae sequences and only one of *Antirrhinum* (*A. majus*), yielding 2,515 most parsimonious trees (CI = 0.46 and RI = 0.68, including uninformative characters) and similar topological resolution (Fig. 1). The 61 ITS sequences of Antirrhineae are shown to be monophyletic (70 % bootstrap support). In this ITS phylogeny, the Antirrhineae group is derived with respect to these genera, whereas the single accession of Antirrhineae (*Antirrhinum majus*) in the *ndh*F phylogeny was placed in a relatively basal position (Olmstead et al. 2001). The Antirrhineae clade contains six well-supported subclades (Fig. 1): (1) *Linaria* (Linaria group, 100 % boostrap support); (2) *Anarrhinum-Kickxia* (Anarrhinum group; 100% bootstrap support); (3) *Maurandella-Rhodochiton-Lophospermum-Asarina-Cymbalaria* (Maurandya group, 89% bootstrap support); (4) *Chaenorrhinum-Holzneria-Albraunia* (Chaenorrhinum group, 64% bootstrap support); (5) *Pseudomisopates-Acanthorrhinum-Misopates-Antirrhinum-Galvezia-Howeliella-Mohavea-Sairocarpus-Neogaerrhinum* (Antirrhinum group, 95% bootstrap support); (6) *Pseudorontium-Schweinfurthia* (Schweinfurthia group; 92% bootstrap support). The *Antirrhinum* group is formed by two subgroups: *Antirrhinum-Galvezia-Howeliella-Mohavea-Neogaerrhinum-Sairocarpus* (54% bootstrap support) and *Misopates-Acanthorrhinum-Pseudomisopates* (87% bootstrap support). The *Maurandya* group is resolved into a pectinate topology, in which a basal branch of *Cymbalaria* comes out first, then *Asarina*, *Maurandella*, and finally *Rhodochiton* and *Lophospermum*.

Long branches in the Neighbor-Joining (NJ) trees using both alignments (results not shown) and the GTR + G + I were congruent with the major, well-supported clades of the parsimony-based (MP) analyses. Bayesian analysis under GTR + G + I model also produced identical tree topology as that found in the NJ and MP analyses. Internal branches were supported by high Bayesian posterior probabilities (P). Computing values over P = 95 % were concordant with those found

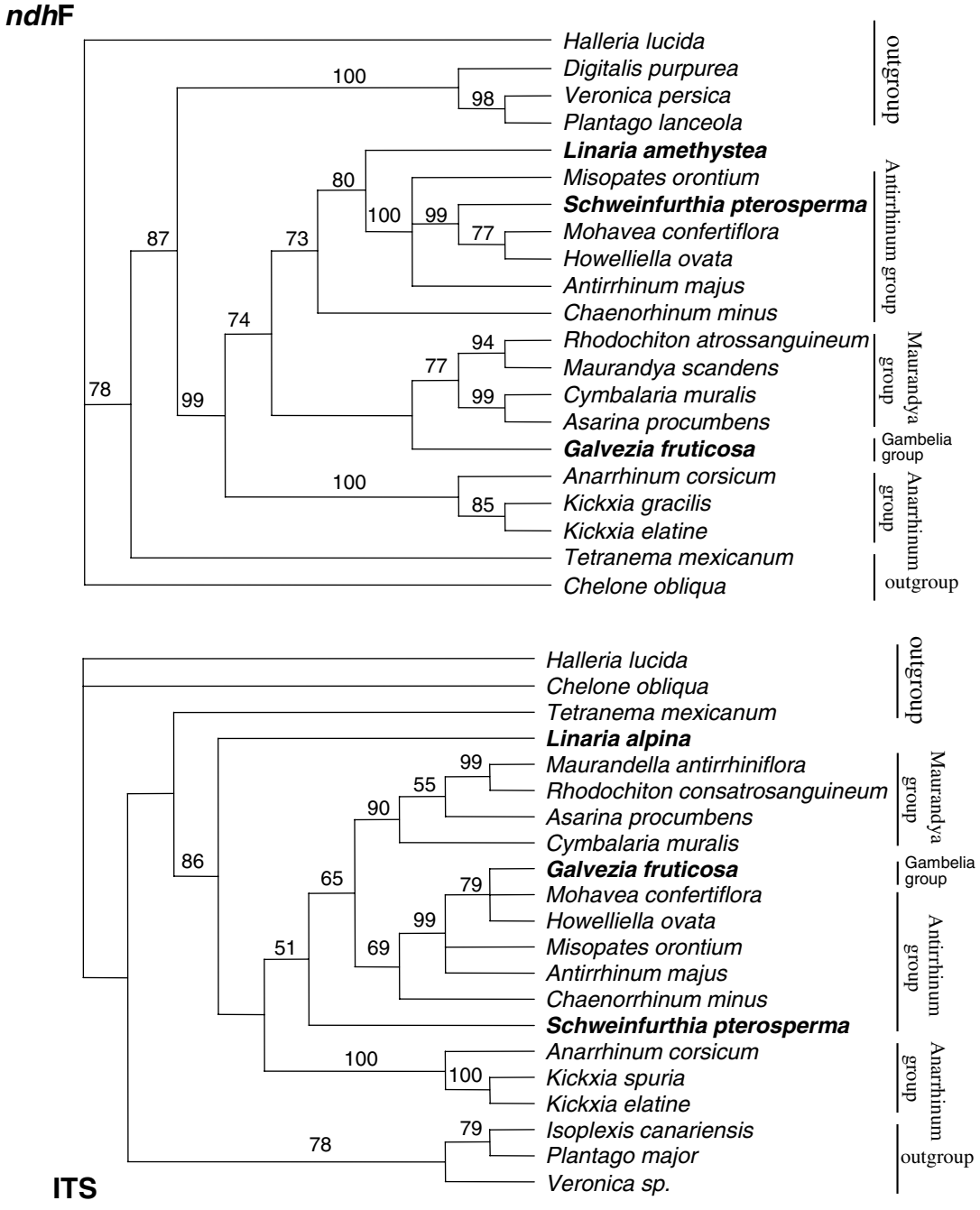

Fig. 2. Comparison of phylogenetic relationships obtained from heuristic analysis of *ndh*F (above; sequences taken from Olmstead et al. 2001, Ghebrehiwet et al. 2000) and ITS (below; the present study). The two analyses involved almost the same set of 14 genera of Antirrhineae plus *Digitalis*, *Isoplexis Veronica*, *Plantago*, *Chelone*, *Tetranema*, and *Halleria* (outgroup). The *ndh*F strict consensus tree of two most parsimonious trees was of 1,283 steps (CI: 0.69, including uninformative characters; RI: 0.59). The ITS strict consensus tree of three most parsimonious trees was of 1,052 steps (CI: 0.59, including uninformative characters; RI: 0.54). Full bootstrapping values over 50% are shown above. Names in bold indicate genera placed at different positions in both analyses

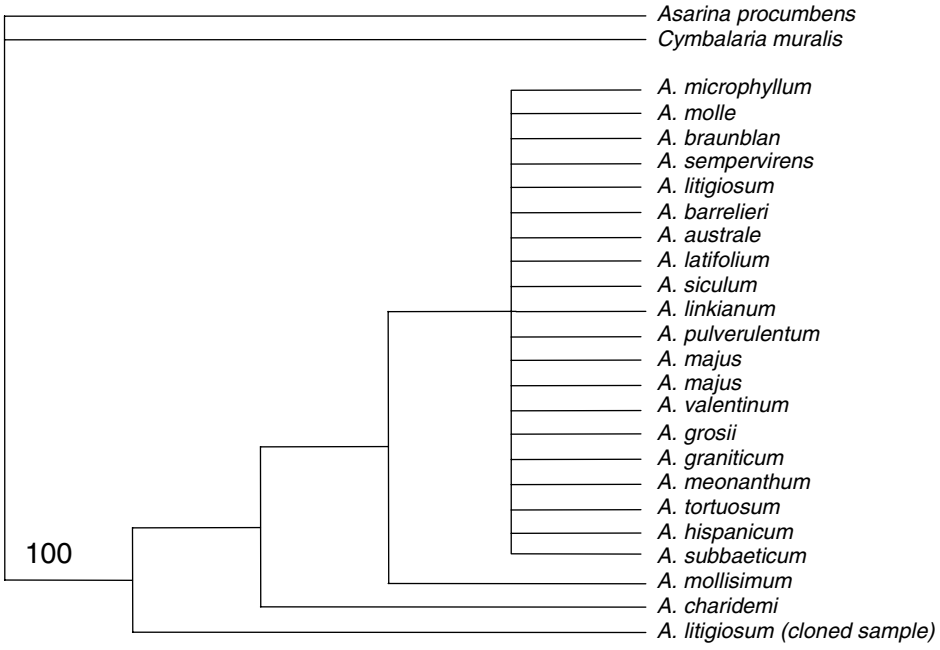

Fig. 3. Strict consensus tree of 190,400 most parsimonious trees (213 steps) obtained from analysis of 23 ITS sequences of *Antirrhinum*, with *Asarina procumbens* and *Cymbalaria muralis* serving as outgroup taxa (CI: 0.86, including uninformative characters; RI: 0.73)

after bootstrapping heuristic searches (Fig. 1), even though with tendency to reach higher values.

Analyses of Antirrhineae *ndh*F and ITS sequences. A *ndh*F matrix of sequences from 16 Antirrhineae genera was compiled by retrieving sequences from the GenBank (Ghebrehiwet et al. 2000, Olmstead et al. 2001). Cladistic analysis using 21 *ndh*F sequences, including *Halleria*, *Chelone*, *Digitalis*, *Plantago*, *Tetranema*, and *Veronica* as outgroup, yielded 2 trees of 1,655 steps with CI = 0.62 and RI = 0.58 (results not shown). The use of a most exclusive outgroup (*Halleria*), chosen from the analysis of the Scrophulariaceae (Olmstead et al. 2001), did not affect the resolution of the ingroup shown in Ghebrehiwet et al. (2000).

To allow comparison and combination of chloroplast and nuclear sequences, we analyzed a subset of *ndh*F and ITS sequences of 14 Antirrhineae genera primarily in common with a previous analysis (Ghebrehiwet et al. 2000) (Fig. 2). Although the topology is different in both clades, we recognize 11 genera in congruent clades in both the *ndh*F and ITS trees: the *Anarrhinum* group (*Anarrhinum-Kickxia*), the *Maurandya* group (*Rhodochiton-Maurandya-Maurandella-Asarina-Cymbalaria*), and the *Antirrhinum* group (*Antirrhinum-Misopates-Mohavea-Howeliella-Chaenorrhinum*). However, placement of three genera is profoundly discordant: *Linaria* is basal in the ITS tree, but sister to the Antirrhinum group in the *ndh*F tree; *Schweinfurthia* is placed at different ITS and *ndh*F subclades in the Antirrhinum group; and *Galvezia* is associated to two different ITS and *ndh*F groups (Fig. 2). To explore the significance of phylogenetic incongruences, we extended the sample of *Linaria* and *Schweinfurthia*. As a result, one species of *Linaria* representing each of the seven sections and the six species of the genus *Schweinfurthia* were ITS sequenced and analyzed. Circumscription of genera does not affect our phylogenetic reconstruction because the accessions of both genera form well-supported monophyletic groups (Fig. 1).

Analyses of *Antirrhinum* ITS sequences. The strict consensus tree of 190,400 equally most parsimonious trees of 213 steps (CI: 0.86; RI: 0.73) from the analysis of *Antirrhinum* yielded low resolution (Fig. 3). Consensus-tree topologies display polytomies mostly as a result of: (1) insufficient number of informative characters; (2) character incongruence across accessions; (3) more than one character state (additivity) at the same informative sites. As stated above, nucleotide additivity for direct ITS sequencing in 26 accessions of *Antirrhinum* was observed in forward and reverse chromatograms. Although it could not be disclosed whether in some cases double-peak patterns may be the result of sequencing artifacts, equimolar proportions of alternative nucleotide peaks in many accessions suggested the presence of different ITS copies. This view is supported by the facts that only two alternative nucleotides at the same site were present across species, most ambiguous nucleotides were at the 16 parsimony-informative positions, and cloning the ITS products of *Antirrhinum litigiosum* revealed alternative nucleotides at additive sites.

Analysis of morphology in *Antirrhinum*. A morphological matrix of 25 taxa x 11 characters was analyzed using the above heuristic parameters. Cladistic analyses of morphological data revealed 2,889 most parsimonious trees of 31 steps from 88 islands with a consistency index of 0.35 and a retention index of 0.79. The strict consensus tree displayed no resolution.

Discussion

Circumscription of Antirrhineae. The Antirrhineae has been considered a well-defined tribe of Scrophulariaceae based on the single occurrence of an iridoid glycoside (antirrhinoside) (Kooiman 1970), remarkable tendency toward tube-zygomorphic flowers, initial development of endosperm haustoria, and presence of poricidal capsules, which is a unique character in Scrophulariaceae (Chavanes 1833, Bentham 1846, Wettstein 1891). Generic circumscription of the tribe has been more or less consistent since Rothmaler (1943), excluding three genera (*Colpis*, *Diclis*, *Nemesia*) with septicidal capsules. Previous phylogenetic studies using chloroplast markers supported the tribe Antirrhineae (dePamphilis et al. 1994, Ghebrehiwet et al. 2000). Our phylogenetic reconstruction, using an unrelated marker (nrITS sequences), 61 ingroup sequences, and the closest relatives taken from a phylogeny of the family (Olmstead et al. 2001), also indicates that Antirrhineae is a natural group (Fig. 1). The 22 of the 29 genera currently recognized within Antirrhineae are grouped together in a single clade (70 % bootstrap) of Old and New World species.

Naturalness of genera. Although we have sequenced a relatively low number (61) of species belonging to the tribe Antirrhineae (ca. 326 species, Sutton 1988), phylogenetic analyses of a selected sample, which includes all or most species of certain genera (*Acanthorrhinum*, *Antirrhinum*, *Asarina*, *Maurandella*, *Pseudomisopates*, *Pseudorontium*, *Howeliella*, and *Schweinfurthia*) and representatives of most sections of another 14 genera, depict fundamental lineages in Antirrhineae. Apart from the five monotypic genera (Table 1), the ITS phylogeny suggests that the following genera are monophyletic: *Misopates*, *Schweinfurthia, Antirrhinum*, *Kickxia*, and *Linaria*. Therefore, a single origin is not only suggested for small genera with less than 10 species, but also for large genera such as *Antirrhinum* (25) and *Linaria* (ca. 150). Despite conspicuous morphological differences in habit (annual herbs to suffrutescent perennials), stems (spiny or not), and seed ornamentation, the six *Schweinfurthia* species form a natural group (92 % bootstrap). Our results strongly support segregation of Old World species of *Antirrhinum*, as described by Sutton (1988) from New World species, because they form a monophyletic group (100 % bootstrap) independent to American species. Although the sample of *Kickxia* is obviously scarce (2 sequences), monophyly of this genus is well supported. Our ITS tree (Fig. 1)

clearly supports its independence from *Linaria* (100 % bootstrap), with which it has often been associated due to the presence of a floral spur (Bentham 1846, Wettstein 1891). Lack of relatedness between *Kickxia* and *Linaria* was previously documented in the *ndh*F phylogeny (Ghebrehiwt et al. 2000). Our sample of *Linaria*, which includes representatives of the seven sections recognized by Sutton (1988), leads us to suggest common ancestry for this complex. Therefore, occurrence of most accessions from the same genera in well-supported clades indicates that previous taxonomic treatments of Antirrhineae genera (Rothmaler 1943, Sutton 1988), which are based on morphological characters, mostly reflect natural groups of species.

Spetiation patterns in *Antirrhinum*. Interpretations of phylogenetic relationships in *Antirrhinum* were discussed by Rothmaler (1956), who speculated that speciation occurred primarily in the Iberian Peninsula as a result of dryness since the Tertiary. This author suggested that during Ice Ages plants with characters found in subsect. *Kickxiella* (Table 3) colonized mountains of the Iberian Peninsula. When the climate became warmer and drier, fragmented distributional areas were created and the genus evolved into two lineages (subsect. *Antirrhinum* and *Streptosepalum*), which display morphological adaptations to dry environments. Our ITS phylogenetic reconstructions yielded poor resolution, preventing us from establishment of relationships of major groups of species despite a reasonable number of informative characters (16). The ITS region belongs to a multigene family affected by the process known as concerted evolution (Arnheim 1983). This mechanism causes potentially asymmetric homogenization of sequences of the ribosomal DNA tandem repeats in a biased fashion (Nieto Feliner et al. 2001). Alternatively, lack of concerted evolution may explain in some cases occurrence of polymorphic repeats when obtaining direct PCR amplifications from total DNA (Fuertes Aguilar et al. 1999). Additivity in most ITS sequences of *Antirrhinum* suggests failure in full concerted evolution and extensive hybridization among species of the three subsections. Our morphological phylogeny, based on 11 characters, also displays poor resolution due to a pattern of character incongruence as manifested by low consistency and retention indexes (CI: 0.35; RI: 0.79). Therefore, homoplasy found in morphological characters and variation in ITS sequences give a phylogenetic signal that may be the result of hybridization at the same chromosome level of $n = 8$ (homoploid hybridization) across time. Secondary contacts may have been common when recurrent wetter environments occurred in interstadial periods (Rothmaler 1956). There are weak interspecific genetic barriers, according to the high number of hybrids described (Rothmaler 1943, Thompson 1988) and the easiness to obtain consecutive generations in artificial crossings (Thompson 1988, Xue et al. 1996). As compared with another angiosperm groups (Richardson et al. 2001), maximum ITS sequence divergence (4.9 % between *A. mollissimum* and *A. valentinum*) indicates that differentiation and reticulation in *Antirrhinum* may have pre-dated the Pliocene, timing in agreement with Rothmaler's speculations.

Phylogenetic relationships within Antirrhineae. Five groups (subtribes) of Antirrhineae were defined by Rothmaler (1943, 1956): Linariinae, with 11 genera (*Asarina*, *Cymbalaria*, *Kickxia*, *Linaria*, *Chaenorrhinum*, *Antirrhinum*, *Neogaerrhinum*, *Acanthorrhinum*, *Schweinfurthia*, *Pseudorontium*, *Misopates*); Maurandyinae, with five genera (*Rhodochiton*, *Lophospermum*, *Epixiphium*, *Maurandya*, and *Maurandella*); Gambeliinae, with three genera (*Galvezia*, *Saccularia*, and *Gambelia*); Mohaveinae, with only *Mohavea*; and Anarrhinae with only *Anarrhinum*. This classification has recently been tested using phylogenetic analyses of *ndh*F sequences from 16 genera (Ghebrehiwt et al. 2000). Four of these groups (*Anarrhinae*, *Maurandyinae*, *Gambeliinae*, and *Mohaveinae*) are recognized in the *ndh*F phylogeny, even though generic composition of the clades defining these groups is

profoundly different. Also, in disagreement with the subtribal classification proposed by Rothmaler (1943, 1956), our ITS tree (Fig. 1) of 22 genera of Antirrhineae is resolved into six well-defined lineages: (1) Linaria group; (2) Anarrhinum group; (3) Maurandya group; (4) Chaenorrhinum group; (5) Antirrhinum group; (6) Schweinfurthia group. The 11 genera considered by Rothmaler (1943) in the Linarinae are intermingled in each clade of both *ndh*F and ITS phylogenies (Figs. 1, 2). Resolution in the ITS tree is, in part, congruent with those of the *ndh*F tree; however, *Linaria*, *Schweinfurthia*, and *Galvezia* are found in different placements (Fig. 2). Fundamental discordance between ITS and *ndh*F phylogenies may be due to the independent origin of similar characters (homoplasy), lineage sorting or reticulation (Neigel and Avise 1986, Doyle 1992, Avise 1994). The sources of data available to date from Antirrhineae do not allow ruling out any of the three hypotheses. *Linaria* forms an independent ITS clade (100 % bootstrap) (Fig. 1), whereas the Schweinfurthia group includes *Schweinfurthia* together with *Pseudorontium* (92% bootstrap). The Maurandya group agrees with Rothmaler's Maurandyinae, except for *Mohavea*, which is included in the *Antirrhinum* group in both the *ndh*F and ITS trees. In the two phylogenetic reconstructions *Asarina* and *Cymbalaria* are resolved into this well-defined group (89% bootstrap), despite they were considered in the subtribe Linariinae by Rothmaler. An independent lineage consists of *Galvezia* and *Gambelia* in the *ndh*F tree of Ghebrehiwet et al. (2000); both genera were included in Rothmaler's Gambeliinae. Impossibility of obtaining an ITS sequence for *Gambelia* and the incongruent placement of its sister group (*Galvezia*) in the *ndh*F and ITS trees (Fig. 2) suggest that this group should be revisited with an extended sample. The two monotypic subtribes (Mohaveinae and Anarrhinae) do not form two major, independent lineages because *Mohavea* forms part of the Antirrhinum group and *Anarrhinum* is closely related to *Kickxia* in the two-marker phylogenetic reconstructions.

Elisen's and Tomb's (1983) and Rothmaler's (1943) trees hypothesized for Antirrhineae are not fully in agreement with the *ndh*F and ITS phylogenies (Figs. 1, 2). The former authors considered that the New World genera evolved from a single lineage containing *Antirrhinum* sect. *Saerorhinum* as the "most related representative of the phylad that gave rise to the sections . . . ." i.e. American genera. In contrast, Rothmaler considered that the American *Antirrhinum* s. l. contained different genera and the New World harbors unrelated lineages of Antirrhineae, as demonstrated in the present paper. His speculations on the phylogenetic relationships of Antirrhineae are also concordant with our molecular results in some other respects: basal placement of *Anarrhinum*; close relationships among *Mohavea-Misopates-Antirrhinum* and *Maurandya-Maurandella-Rhodochyton-Lophospermum*; and acquisition of annual forms multiple times as adaptation to dry environments. Annual forms are, in fact, found in the six main ITS lineages of Antirrhineae (Fig. 1). However, many other relationships and the circumscription of his "Gruppen" are not supported by either chloroplast or nuclear phylogenies.

Profound morphological diversity of the *Antirrhinum* group is manifested by description of new genera in the last decades. Segregation of the former *Antirrhinum* species (Sutton 1988) into American genera is herein recommended based on the consistent results between the *ndh*F and ITS phylogenies. The 14 species of New World *Antirrhinum* recognized by Munz (1959) and Thompson (1988) were circumscribed within *Sairocarpus* (11 species) (Sutton 1988), *Howelliella* (1 species), and *Neogarrhinum* (2 species) (Rothmaler 1943). These three genera are closely related in the ITS tree (Fig. 1). They are also in the same clade in a *trn*L phylogeny (Freeman and Scogin 1999), together with *Mohavea*. One more monotypic genus (*Pseudomisopates*) from the Old World, described after Sutton's monography (Güemes 1997), forms part of a subclade (87% bootstrap) together with *Acanthorrhinum* and *Misopates*.

Character evolution in Antirrhineae. The typical flower closed by a palatae (personate flower) of Antirrhineae has been a classical objective for genetic studies (see Schwarz-Sommer et al. 2003 for revision), but a clear evolutionary pattern has not been described yet. The use of *Halleria* as outgroup in our *ndh*F and ITS analyses resulted in tree topologies where the tribe Antirrhineae has a terminal position with respect to the other Scrophulariaceae (Figs. 1, 2). In contrast, the single accession of Antirrhineae *s. str.* (*Antirrhinum*) has not a terminal position both in the *rbc*L + *ndh*F phylogeny (Reeves and Olmstead 1998) and in the comprehensive *ndh*F phylogeny of Scrophulariaceae (Olmstead et al. 2001). A sample effect should be contemplated for the incongruence between independent analyses using the same chloroplast marker (*ndh*F), having our subset a significant lower number of accessions (Fig. 2). However, our ITS analysis includes most species of *Antirrhinum* (22) and 22 of the 29 genera of Antirrhineae (Fig. 1). A derived placement of the Antirrhineae, as suggested in our study of almost 100 sequences from two different genomes (chloroplast and nuclear), is not congruent with previous *ndh*F results in which plants with personate flowers are placed at a mid position (Olmstead et al. 2001). *Antirrhinum majus* has also a mostly derived position in a phylogeny of Scrophulariaceae using sequences of the chloroplast *trn*L intron (Freeman and Scogin 1999), a result fully in agreement with our *ndh*F (Fig. 2) and ITS phylogenies (Fig. 1). Therefore, the question remains whether the direction of character transformations of floral traits can be from actinomorphy to zygomorphy, with an increase in the complexity of flower structures. The evolution of this type of flower appears to be very complex because neither phylogenies based on neutral markers nor analyses of genes related to floral expression (Schwarz-Sommer et al. 2003) are conclusive at this respect.

Gametophytic chromosome numbers in Antirrhineae range from $n = 6$ to $n = 28$. Despite no chromosome number has been reported yet for many species of Antirrhineae, a considerable number of chromosome countings has already been found within this range ($n = 6$, 7, 8, 9, 12, 13, 14, 15, 16, 18, 20, 21, 24, 28) (Sutton 1989). Chromosome numbers concordant with multiple chromosome complements within a single genus has been interpreted as polyploidy in *Chaenorrhinum*, *Cymbalaria*, *Kickxia*, *Linaria*, and *Nuttallanthus*. Additionally, dysploidy has been hypothesized for all European genera of the Antirrhineae (Sutton 1988), even though a successive series of chromosome numbers has only been found in the North American *Sairocarpus* ($n = 15$, 16). This diversity of cytological data has not been tested in a phylogenetic framework to date. The six natural groups (lineages) of the Antirrhineae suggested by both the ITS and *ndh*F phylogenies (Figs. 1, 2) contain more than one chromosome number. Several chromosome numbers are found in unrelated lineages: $n = 7$ in species of the Maurandya and Antirrhinum groups; $n = 12$ in species of the Linaria and Maurandya groups. However, a few numbers occur in a single lineage: $n = 9$ in the Anarrhinum group; $n = 13$ in the Schweinfurthia group; $n = 15$ in the Antirrhinum group. The occurrence of no clear pattern in the evolution of chromosome numbers is also manifested by the polyploid series of $n = 7$, 14, 21 found in single genera of both the Maurandya group (*Cymbalaria*) and Antirrhinum group (*Chaenorrhinum*). Multiple events involved in chromosome variation within each lineage are consistent with MacClade reconstructions of ancestral chromosome numbers. ITS and *ndh*F trees suggest that variation of chromosome numbers in New and Old World lineages is the result of multiple, independent events of dysploidy and polyploidy. We believe that previous phylogenetic speculations based on morphology and a series of chromosome numbers (6, 8, 12, 15) misled Elisens and Tomb (1983) in considering that the New World taxa formed a phylogenetically coherent assemblage isolated from the Old World lineages.

Biogeographic implications. The use of phylogenetic relationships, as prerequisite for historical biogeography, served to interpret intercontinental disjunctions between eastern Asia and North America (Xiang et al. 1998, Wen 1999). The principle of historical biogeography underlining congruence between phylogenetic topologies and geographic distributions is used to infer shared biogeographic histories (Xiang et al. 1998). Three of the six major groups of Antirrhineae (*Maurandya*, *Antirrhinum*, and *Schweinfurthia*) based on ITS sequences contain genera of both the Old World and the New. The same is true for these three groups recognized in Ghebrehiwet et al. (2000) using *ndh*F sequences. Cladistic reconstructions of Antirrhineae based on tree topologies and primitive-derived character evolution reveal that American and Eurasian genera diverged from common ancestors at least three times. The most remarkable biogeographic results are: (1) nine American genera form three independent monophyletic subgroups; (2) ten Old World genera are closely related to these nine New World genera. In the ITS tree (Fig. 1), two lineages display Old World genera at a basalmost placement, suggesting a biogeographic pattern in which at least eight of nine American genera are the result of colonization from Eurasia. Phylogenetic reconstructions of *ndh*F sequences (Ghebrehiwet et al. 2000) also support a derived origin of the American genera of Antirrhineae from Eurasian ancestors.

Pseudocongruence (Cunningham and Collins 1994), i. e. concordance in the phylogenetic reconstruction as a result of colonization at different geological times, has been documented between plant groups sharing similar disjunction distributions in eastern Asia, eastern North America, and western North America (Xiang et al. 1998). Analysis of divergence times using molecular clocks serves to estimate geological times for the split of New and Old World lineages. Under certain assumptions, divergence times may be estimated from molecular data (Fleisher et al. 1998), even though rate calibrations are not free of caveats and uncertainties (Hillis et al. 1996). We failed in finding a constant rate of molecular evolution across ITS lineages of Antirrhineae. Heterogeneous rates of molecular evolution appears to be the rule rather than the exception in angiosperms (Sanderson 2002). In any case, comparison of sequence divergences may be useful to exclude alternative hypotheses in historical biogeography of Antirrhineae. A time frame was calculated by considering maximum ages of both ITS sequence diversification and geological events. Kimura-2-parameter pairwise distance between American and Eurasian genera ranges are dissimilar within the three ITS lineages, but maximum sequence divergences reach similar values: 9.0–10.4 % in the Maurandya group; 7.1–11.1 % in the Schweinfurthia group; and 3.2–9.2 % in the Antirrhinum group. The most extreme values of calibrated rates (substitution/site/year) for 13 angiosperm groups range from 1.72×10^{-9} to 9.0×10^{-9} (s/s/y) (Richardson et al. 2001). Maximum divergence times, by applying the most conservative ITS sequence divergence rate (1.72×10^{-9}), indicate that the splits into New and Old World post-date the Oligocene: ~30.6 Myr in the Maurandya group; ~32.5 Myr in the Schweinfurthia group; and ~27.1 Myr in the Antirrhinum group. Therefore, the Antirrhineae disjunction between Eurasia and America may have originated at similar geological times for the three lineages in a biogeographic congruence fashion (Cunningham and Collins 1994). This would represent the first documented case of congruence within a single plant group (Antirrhineae). Three major hypotheses of colonization routes are suggested to account for congruence of historical biogeography of Antirrhineae: (1) although the connection between Europe and America through Greenland (Thulean route) appears to be broken in the Early Eocene and never reestablished (Tiffney 1985), functional land bridges for colonization may have existed piecemeal until the mid-Miocene (Gronlie 1979) or throughout most the Tertiary (Strauch 1970); (2) the Bering land bridge was used for exchange of plants between Asia

and western North America throughout the Tertiary (Tiffney 1985), being proposed as the only route for 10 genera of Scrophulariaceae (Hong 1983); (3) three long-distance colonization events of three independent Antirrhineae lineages. Three intercontinental dispersal events followed by colonization do not appear to be plausible for plants with mechanisms not related to long-distance dispersal (seeds in dehiscent capsules) (Sutton 1988), particularly assuming that the three dispersions may have occurred at similar geological times.

Approximately 30 genera of angiosperms display disjunct distributions in eastern Asia and North America (Xiang et al. 1998). These floristic disjunctions were mainly a result of fragmentation from mid-Miocene to recent (Xiang et al. 2000). Fourteen genera of Scrophulariaceae have distributional patterns shared by North America and Eurasia, of which 10 may be the result of dispersion through the Bering land bridge (Hong 1983). The tribe Antirrhineae was pointed out to represent a complex case (Hong 1983). Antirrhineae is one of the few Madrean-Tethyan elements primarily distributed in western America and western Mediterranean Basin (Raven and Axelrod 1995). Our results suggest closely related connections between both continents for Antirrhineae, as manifested by the high number (three) of common ancestors within a single taxonomic tribe. Distributional patterns of 18 Antirrhineae genera involved in these three America-Eurasia disjunctions lead us to assume a low likelihood of a Pacific track because significant absence of species in eastern China, Korea, and Japan (Donoghue et al. 2001). In contrast, most of Antirrhineae genera and species are distributed in North America, the Mediterranean region, Europe, and southwestern Asia; this distributional assignation suggests migration through an Atlantic track.

Although many eastern Asian-eastern North American disjunctions occurred in the Pliocene, some others such as *Liriodendron* and *Pachysandra* split into two intercontinental groups in the late- and mid-Miocene (Xiang et al. 2000). Assessment of ITS sequence evolution of Antirrhineae divergence using the average pairwise Kimura-2-Parameter distance from 13 angiosperm groups (Richardson et al. 2001) help hypothesize three vicariant events between 9 and 11 Myr (mid-Miocene). Extensive plant exchange through an Atlantic pathway in the Miocene (Donoghue et al. 2001) does not disagree with our estimation of vicariance times for Antirrhineae.

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