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ORIGINAL ARTICLE

Evaluating species nonmonophyly as a trait affecting genetic diversity: a case study of three endangered species of *Antirrhinum* L. (Scrophulariaceae)

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Abstract Molecular markers are routinely used to assess levels of diversity within and among populations, particularly with regard to species of conservation concern. However, when interpreting the level and partitioning of diversity observed, an implicit assumption is often made that the populations of the species in question form a monophyletic group. We tested this assumption in three endemics of *Antirrhinum* (*A. charidemi*, *A. subbaeticum*, and *A. valentinum*) using 79 nuclear [internal transcribed spacer (ITS)] and 85 plastid (*psb*A-*trn*H, *trn*T-*trn*L, *trn*K-*mat*K, *trn*S-*trn*G) sequences representing multiple accessions of each of 24 *Antirrhinum* species (single accession of *A. cirrhigerum*). These species share six life history traits implicated in levels of genetic diversity, and have been the subject of previous population genetic studies. Populations of all three species formed monophyletic groups on ITS analysis. In contrast, none of the three species formed monophyletic groups on plastid sequence analysis: populations of *A. charidemi* fall in a monophyletic group including one accession of *A. mollissimum*, populations of *A. subbaeticum* form a polyphyletic group with plastid sequences shared with *A. pulverulentum*, and populations of *A. valentinum* are unresolved within a clade containing six other species. Lack of monophyly using plastid sequences is interpreted as a combination of shared ancestral polymorphism and hybridization in a reticulate evolutionary history of these species. Monophyly in the ITS tree may reflect a more recent sequence homogenization. We draw attention to the evaluation of species monophyly alongside the contribution of other life history traits in the historical interpretation of the level and partitioning of genetic diversity, and its use in recommendations for species conservation programs.

Keywords *Antirrhinum* · Genetic diversity · Hybridization · Life history traits · Phylogeny · Taxonomic species

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Introduction

In what are now considered classic articles, Hamrick et al. (1979, 1992), Loveless and Hamrick (1984), and Hamrick and Godt (1989, 1996) summarized available information on genetic diversity within and among plant populations to establish which life history traits had a profound and significant effect on the level and distribution of diversity observed. Significant effects were described in 8 out of 12 traits examined (Hamrick et al. 1979), and subsequent papers have led to similar conclusions based upon a wide range of currently available molecular techniques (Nybom 2004). However, Hamrick et al. (1992) and Hamrick and Godt (1996) also pointed out that genetic diversity maintained by a species is a function not only of its life history traits [which explain, for example, about 34% of variation detected in woody species (Hamrick et al. 1992)] but must also depend heavily on species ecological and evolutionary history.

In these reviews, the unit of study is almost invariably the taxonomic species (in the sense of Mayden 1997). In

many countries, the taxonomic species is currently the basic unit of assessment for conservation, and is the subject of the majority of conservation legislation and conservation management planning protocols (Isaac et al. 2004; Mace 2004; Garnett and Christidis 2007). However, studies of genetic diversity may be of limited interpretative value if the study species is poorly known, and an implicit assumption is made that the species populations form a natural (monophyletic) group, which means they have a single origin, being more closely related to each other than to any population of a different species (Doyle 1992; Funk and Omland 2003). If the study species is an artificial population assemblage, then the level and distribution of genetic diversity may not reflect population genetic processes within an assumed natural group (Arnold 1997; Rieseberg 1997). If no knowledge of species monophyly is available, then interpretation of the patterns of diversity observed will be incomplete: population genetic structure may primarily reflect the causes of species nonmonophyly rather than population diversity, life traits, and ecological variables (Hamrick et al. 1979). This could seriously impede interpretation of the levels of genetic diversity within taxonomic species.

Species evolutionary history can be inferred with increasingly sophisticated molecular phylogenetic analyses, using complementary loci from up to three different plant genomes (Savolainen and Chase 2003; Hughes et al. 2006). There are several reasons why nonmonophyletic groups are recovered in such reconstructions. These can be of a technical and/or analytical nature (e.g., inadequate phylogenetic signal in the markers used, analysis of unrecognized paralogous genes, imperfect taxonomy, widespread hybridization processes in angiosperms, or detection of previously undetected cryptic taxa) (Goodwillie and Stiller 2001; Treutlein et al. 2003; Álvarez et al. 2005). Having detected such sources of error, suitable phylogenetic analyses are essential to observe the signal of evolutionary events that have impacted on lineage diversification and therefore species monophyly. For instance, it has been documented that hybridization with *Bertya rosmarinifolia* increased levels of genetic diversity in one of the three known populations of the endangered *B. ingramii* (Fatemi and Gross 2009).

The genus *Antirrhinum* L. consists of 25 perennial species primarily distributed throughout the western Mediterranean Basin, with 23 species occurring in the Iberian Peninsula (Sutton 1988; Güemes 2009). Phylogenetic reconstructions have generally included single accessions of each taxon, and have revealed little infrageneric structure (Vargas et al. 2004; Jiménez et al. 2005) with some authors proposing that speciation in *Antirrhinum* is recent, geographically structured, and reticulate (Webb 1971; Jiménez et al. 2005; Vargas et al. 2009). *Antirrhinum* species are perennial herbs or small shrubs having identical chromosome number ($2n = 16$), with most species considered self-incompatible and bee-pollinated (Vargas et al. 2010). Many species are either geographically restricted or narrow endemics, with a total of eight species included in the *Red List of Spanish Vascular Flora* (Moreno 2008). Genetic diversity within *Antirrhinum* has been extensively evaluated using allozyme markers and RAPD (Random Amplified polymorphic DNA) (Table 1), allowing comparative assessment of genetic diversity between species with similar life history traits.

In this article, we used nuclear (ITS) and plastid (*psb*A-*trn*H, *trn*T-*trn*L, *trn*K-*mat*K, *trn*S-*trn*G) gene sequences from multiple individuals of 24 species of *Antirrhinum* (single accession of *A. cirrhigerum*) to investigate whether population genetic structure is related to the level of monophyly detected in three endemic snapdragon species from southeast Iberia (*A. charidemi*, *A. subbaeticum*, and *A. valentinum*). Specifically, we tested whether populations of the three species form monophyletic groups using nuclear ITS and plastid sequences, and whether monophyly gives insights into evolutionary histories not apparent from genetic diversity estimates alone. The three species selected to test the hypothesis of nonmonophyly affecting levels of genetic variation have similar life history traits but show remarkably different levels of allozyme diversity (Tables 1, 2). They share six out of the eight life history traits shown to have a significant effect on genetic diversity (Hamrick et al. 1979; Table 2). These species differ in their mating system: *A. charidemi* and *A. valentinum* are mainly outcrossers due to self-incompatibility, while *A. subbaeticum* is a self-compatible species with a mixed mating system (Carrió et al. 2009). Furthermore, *A. subbaeticum* is not considered a "narrow" endemic as it is represented by two groups of populations separated by ca. 44 km with an extent of occurrence of ca. 200 km^2, although its area of occupancy is <15 km^2, whereas *A. charidemi* and *A. valentinum* are restricted to areas of 15 and 4 km in length, respectively. Differences in allozyme genetic diversity among these taxa have been reported (Table 1): *A. charidemi* had a high degree of intraspecific genetic diversity and little differentiation among populations; *A. subbaeticum* exhibited low levels of intraspecific genetic diversity and high differentiation among populations; and *A. valentinum* had a high level of intraspecific genetic diversity and high population divergence (Mateu-Andrés and Segarra-Moragues 2000; Mateu-Andrés 2004). The three species are considered as endangered in the *Red List of Spanish Vascular Flora* (Moreno 2008). A clear taxonomic delimitation in the three species has been recognized in all revisions of the *Antirrhinum* species (Rothmaler 1956; Sutton 1988; Güemes 2009).

Table 1 Reproductive system and details of population genetic parameters estimates for 16 species of *Antirrhinum*

Taxon	Rep.	Reference	Marker	*N* pops.	H_T	G_{ST}	*D*/*HW*	Reference
A. charidemi	pSI	Carrió et al. (2009)	Allozyme	5	0.103	0.054	4/21	Mateu-Andrés and Segarra-Moragues (2000)
A. cirrhigerum	pSC	Vieira and Charlesworth (2001)	Allozyme	3	0.190	0.132	2/13	Mateu-Andrés and de Paco (2006)
A. graniticum[a]	SI	Vieira and Charlesworth (2001)	Allozyme	3	0.130	0.110	0/14	Mateu-Andrés and Segarra-Moragues (2003)
A. graniticum[a]			Allozyme	3	0.090	0.080	0/14	Mateu-Andrés and Segarra-Moragues (2003)
A. graniticum[a]			Allozyme	10	0.190	0.210	3/14	Mateu-Andrés and Segarra-Moragues (2003)
A. latifolium	SI	Mateu-Andrés and de Paco (2006)	Allozyme	8	0.140	0.093	2/13	Mateu-Andrés and de Paco (2006)
A. linkianum	pSC	Vieira and Charlesworth (2001)	Allozyme	6	0.160	0.176	2/13	Mateu-Andrés and de Paco (2006)
A. litigiosum	SI	Mateu-Andrés and de Paco (2006)	Allozyme	5	0.280	0.148	2/13	Mateu-Andrés and de Paco (2006)
A. lopesianum	SI	Mateu-Andrés (1999)	Allozyme	1	0.193	na	na/14	Mateu-Andrés (1999)
A. majus	SI	Mateu-Andrés and de Paco (2006)	Allozyme	4	0.190	0.100	1/13	Mateu-Andrés and de Paco (2006)
A. microphyllum	SI	Torres et al. (2002)	Allozyme	4	0.520	0.040	na/14	Mateu-Andrés (1999)
A. microphyllum			Allozyme	4	0.469	0.056	2/13	Torres et al. (2003)
A. microphyllum			RAPD	4	0.188	0.076	–	Torres et al. (2003)
A. mollissimum	SI	Mateu-Andrés (1999)	Allozyme	7	0.280	0.110	na/14	Mateu-Andrés (1999)
A. pertegasii	–		Allozyme	4	0.080	0.060	2/14	Mateu-Andrés (2004)
A. pulverulentum	SI	Carrió et al. (unpublished)	Allozyme	5	0.300	0.230	7/14	Mateu-Andrés (2004)
A. siculum	SC	Harrison and Darby (1955)	Allozyme	8	0.030	0.270	2/13	Mateu-Andrés and de Paco (2006)
A. subbaeticum	SC	Carrió et al. (2009)	Allozyme	3	0.070	0.850	0/14	Mateu-Andrés (2004)
A. subbaeticum			RAPD	4	–	0.822	–	Jiménez et al. (2002)
A. tortuosum[b]	SI	Mateu-Andrés and de Paco (2006)	Allozyme	15	0.200	0.081	7/13	Mateu-Andrés and de Paco (2006)
A. valentinum	pSI	Carrió et al. (2009)	Allozyme	5	0.178	0.480	0/21	Mateu-Andrés and Segarra-Moragues (2000)

Rep reproductive system: *SI* self-incompatible, *SC* self-compatible, *pSC* partially self-compatible, *pSI* partially self-incompatible. *Marker* genetic markers used in original study, *N pops.* number of populations studied in original study, H_T total diversity at species level, G_{ST} allele frequency differences among populations averaged across populations, *D*/*HW* number of loci that deviate from Hardy–Weinberg equilibrium in at least one population (compared with the number of loci interpreted)

[a] Treated as *A. graniticum* Rothm. ssp. *ambiguum* (Lange) Mateu & Segarra, *A. graniticum* Rothm. ssp. *brachycalyx* D.A. Sutton, and *A. graniticum* Rothm. ssp. *graniticum* in Mateu-Andrés and Segarra-Moragues (2003), respectively

[b] Genetic diversity data of *A. tortuosum* included *A. australe* populations, according to Mateu-Andrés and de Paco (2006), therefore estimates for the former species may be artificially elevated and may account for the high number of loci deviating from Hardy–Weinberg equilibrium

Materials and methods

Sampling

A total of 85 accessions representing 24 *Antirrhinum* species were sampled for phylogenetic reconstructions (Table 3). A rare species, *A. martenii* (Font Quer) Rothm., was not included in the study, because only the type material is available (Rothmaler 1956). Sampling strategy was based on morphological studies (Güemes 2009) and a criterion of collecting at the locus classicus of each species (see also Vargas et al. 2009). The number of individuals sampled of each species ranged from two to eight (single accession of *A. cirrhigerum*), being from geographically separate populations (Fig. 1; Table 3). Four individuals of the related genera *Chaenorhinum*, *Gambelia*, *Misopates*, and *Pseudomisopates* (*C. crassifolium*, *G. speciosa*, *M. orontium* and *P. rivas-martinezii*) were used as outgroup samples, according to a previous phylogenetic study (Vargas et al. 2004, 2009).

Table 2 Details of 12 life history traits (Hamrick et al. 1979) for the three studied species (*Antirrhinum charidemi*, *A. subbaeticum*, and *A. valentinum*)

		A. charidemi	*A. subbaeticum*	*A. valentinum*
1	**Taxonomic status**	Dicots[a]	Dicots[a]	Dicots[a]
2	**Geographic range**	**Narrow**[a]	**Regional**[a]	**Narrow**[a]
3	**Generation length**	Perennial[a]	Perennial[a]	Perennial[a]
4	Mode of reproduction	Sexual[b]	Sexual[b]	Sexual[b]
5	**Mating system**	**Outcrosser**[b]	**Mixed mater**[b]	**Outcrosser**[b]
6	**Pollination mechanism**	Entomophilous[b]	Entomophilous[b]	Entomophilous[b]
7	**Fecundity**	10^2–10^3 seeds/plant[c]	10^2–10^3 seeds/plant[c]	10^2–10^3 seeds/plant[c]
8	Seed dispersal mechanism	Boleoanemochory[d]	Boleoanemochory[e]	Boleoanemochory[d]
9	**Chromosome number**	$2n = 16$[f]	$2n = 16$[g]	$2n = 16$[h]
10	**Successional stage**	Late[d]	Late[e]	Late[d]
11	Habitat type	Mesic[d]	Mesic[e]	Mesic[d]
12	Cultivated status	Noncultivated[d]	Noncultivated[e]	Noncultivated[d]

The eight life history traits identified by Hamrick et al. (1979) as significantly affecting the level and distribution of genetic variation, and those that differ among the studied species, are shown in bold

[a] Güemes (2009)

[b] Carrió et al. (2009)

[c] Carrió et al. (unpublished)

[d] Sutton (1988)

[e] Sánchez-Gómez et al. (2003)

[f] Diosdado et al. (1994)

[g] Coy et al. (1997)

[h] Boscaiu et al. (1997)

DNA extraction, gene amplification, and sequencing

DNA was extracted from 20–25 mg leaf material using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. DNA was amplified via polymerase chain reaction (PCR) on a Perkin Elmer PCR System 9700 thermal cycler. We obtained and analyzed 89 *psb*A-*trn*H, 89 *trn*T-*trn*L, 2 *trn*K-*mat*K, and 6 *trn*S-*trn*G sequences (Table 3). GenBank accession numbers are given in Table 3. The *psb*A-*trn*H intergenic spacer was amplified using the primers of Hamilton (1999), and the *trn*T-*trn*L intergenic spacer using primers a and b of Taberlet et al. (1991). Reactions included 1 µl dimethyl sulfoxide (DMSO) at 99.9% in each 25 µl reaction. The thermocycling profile consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 2 min (50°C for *trn*T-*trn*L, 58°C for *psb*A-*trn*H), and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min and storage at 4°C. Amplified products were purified using spin filter columns (MoBio Laboratories) following the manufacturer's protocol, and directly sequenced with dye terminators (Big Dye Terminator v. 2.0, Applied Biosystems) using an Applied Biosystems Prism Model 3700 automated sequencer. Procedures used for DNA sequencing of the *trn*K-*mat*K spacer and the *trn*S-*trn*G spacer are given in Vargas et al. (2009).

Data analysis

Two matrices were constructed to perform phylogenetic analyses: one with 89 sequences (85 of *Antirrhinum*, 4 of the outgroup) of each plastid region (*psb*A-*trn*H, *trn*T-*trn*L, *trn*K-*mat*K, *trn*S-*trn*G), and the other one with 81 sequences (79 of *Antirrhinum*, 2 of the outgroup) of the nuclear ribosomal ITS region (Table 3). We obtained 87 *trn*K-*mat*K, 83 *trn*S-*trn*G, and all ITS sequences from two previous phylogenetic analyses (Vargas et al. 2004, 2009; Table 3). Sequences were corrected and aligned manually using BioEdit Sequence Alignment Editor 7.0.9 (Hall 1999). Alignments were adjusted manually to minimize the number of gaps following the logic of Kelchner (2000).

Phylogenetic analyses were performed using maximum parsimony (MP) as implemented in PAUP* (Swofford 2002) and Bayesian inference (BI) as implemented in MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003). MrModeltest v.2.1 was used to determine appropriate models of sequence evolution for each dataset (Posada and Crandall 1998; Nylander 2004) via bottom-up strategy of hierarchical likelihood ratio test and Akaike information

Table 3 List of studied material including sample number (in brackets after species name), locality, voucher (herbarium initial or collector number), and GenBank accession numbers (ITS/*psb*A-*trn*H/*trn*T-*trn*L/*trn*K-*mat*K/*trn*S-*trn*G)

Taxon	Locality	Voucher	GenBank accession no. (ITS/*psb*A-*trn*H/*trn*T-*trn*L/*trn*K-*mat*K/*trn*S-*trn*G)				
Antirrhinum L.							
A. australe Rothm. (1)	Spain: Albacete, Yeste	JG4077	EU677194[a]	HM152881	HM152792	EU717968[a]	EU673477[a]
A. australe Rothm. (2)	Spain: Cádiz, Benaocaz	ECA49	EU677195[a]	HM152882	HM152793	EU717969[a]	EU673478[a]
A. australe Rothm. (3)	Spain: Granada, Castril	VAL140895	AY731273[a]	HM152883	HM152794	EU717970[a]	EU673479[a]
A. braun-blanquetii Rothm. (1)	Spain: Asturias, Cuetu L'Abeyera	JRV5333	EU677196[a]	HM152884	HM152795	EU717971[a]	EU673480[a]
A. braun-blanquetii Rothm. (2)	Spain: León, Oblanca	JRV5177	EU677197[a]	HM152885	HM152796	EU717972[a]	EU673481[a]
A. charidemi Lange (1)	Spain: Almería, cerro de La Lobera	132PV05(1)	EU677198[a]	HM152886	HM152797	EU717973[a]	EU673482[a]
A. charidemi Lange (2)	Spain: Almería, barranco del Sabinar	VAL37158	AY731282[a]	HM152887	HM152798	HM152972	HM152970
A. charidemi Lange (3)	Spain: Almería, cerro de Santa Cruz	136PV05(2)	EU677199[a]	HM152888	HM152799	EY717975[a]	EU673484[a]
A. charidemi Lange (4)	Spain: Almería, Vela Blanca	137PV05(5)	FJ487611[a]	HM152889	HM152800	EU717979[a]	EU673488[a]
A. cirrhigerum Welw. ex Ficalho (1)	Morocco: Doukkala-Abda, El Jadida	VAL111299	EU677200[a]	HM152890	HM152801	EU717980[a]	EU673489[a]
A. controversum Pau (1)	Spain: Albacete, Villa de Ves	VAL145152	AY731272[a]	HM152891	HM152802	HM152974[a]	EU673490[a]
A. controversum Pau (2)	Spain: Alicante, Jalón	BB47	EU677201[a]	HM152892	HM152803	EU717981[a]	EU673491[a]
A. controversum Pau (3)	Spain: Almería, Berja	ECA37	EU677202[a]	HM152893	HM152804	EU717982[a]	EU673492[a]
A. controversum Pau (4)	Spain: Granada, Bérchules	BB15b	EU677203[a]	HM152894	HM152805	HM152975[a]	EU673493[a]
A. controversum Pau (5)	Spain: Valencia, Bolomor	JG4001	EU677204[a]	HM152895	HM152806	EU71783[a]	EU673494[a]
A. controversum Pau (6)	Spain: Valencia, Carcagente	BB29	EU677205[a]	HM152896	HM152807	EU717984[a]	EU673495[a]
A. controversum Pau (7)	Spain: Valencia, Chella	JG4067	EU677206[a]	HM152897	HM152808	EU717985[a]	EU673496[a]
A. controversum Pau (8)	Spain: Valencia, Colom	BB2	EU677207[a]	HM152898	HM152809	EU717986[a]	EU673497[a]
A. graniticum Rothm. (1)	Spain: Madrid, Fuentidueña del Tajo	JG4009	AY731283[a]	HM152899	HM152810	EU717987[a]	EU673498[a]
A. graniticum Rothm. (2)	Spain: Soria, Caltojar	JG4101	EU677208[a]	HM152900	HM152811	EU717988[a]	EU673499[a]
A. graniticum Rothm. (3)	Spain: Huelva, Aracena	ECA54	EU677209[a]	HM152901	HM152812	EU717989[a]	EU673500[a]
A. grosii Font Quer (1)	Spain: Ávila, El Trampal	ECA77	AY731281[a]	HM152902	HM152813	EU717990[a]	EU673501[a]
A. grosii Font Quer (2)	Spain: Ávila, Guisando	276PV06	EU677210[a]	HM152903	HM152814	EU717991[a]	EU673502[a]
A. hispanicum Chav. (1)	Spain: Granada, Juviles	BB14	FJ487614[a]	HM152904	HM152815	EU717992[a]	EU673503[a]
A. hispanicum Chav. (2)	Spain: Granada, Veleta	120PV99	AY731286[a]	HM152905	HM152816	EU717993[a]	EU673504[a]
A. hispanicum Chav. (3)	Spain: Granada, Vélez de Benaudalla	ECA40	EU677211[a]	HM152906	HM152817	EU717994[a]	EU673505[a]
A. latifolium Mill. (1)	Spain: Girona, Collada de Toses	JG4142	EU677212[a]	HM152907	HM152818	EU717995[a]	EU673506[a]
A. latifolium Mill. (2)	Spain: Lérida, Bagà	VAL144658	AY731274[a]	HM152908	HM152819	EU717996[a]	EU673507[a]
A. latifolium Mill. (3)	Spain: Lérida, Martinet	JG4139	–	HM152909	HM152820	EU717997[a]	EU673508[a]
A. latifolium Mill. (4)	Italy: Piamonte, Cuneo	MS781	EU677213[a]	HM152911	HM152822	HM152976[a]	EU673509[a]
A. linkianum Boiss. (1)	Spain: La Coruña, Cedeira	SO (s.n.)	EU677214[a]	HM152912	HM152823	EU717998[a]	EU673510[a]
A. linkianum Boiss. (2)	Portugal: Peniche, Cabo Carvoeiro	IS (ALQ3435)	EU677215[a]	HM152913	HM152824	EU717999[a]	EU673511[a]
A. linkianum Boiss. (3)	Portugal: Trafaria, Almada	IS(ALQ4877)	–	HM152914	HM152825	EU718000[a]	EU673512[a]
A. litigiosum Pau (1)	Spain: Teruel, Griegos	ECA74	EU677216[a]	HM152915	HM152826	EU718001[a]	EU673513[a]

Table 3 continued

Taxon	Locality	Voucher	GenBank accession no. (ITS/*psb*A-*trn*H/*trn*T-*trn*L/*trn*K-*mat*K/*trn*S-*trn*G)				
A. litigiosum Pau (2)	Spain: Valencia, Serra	ECA44	AY731271[a]	HM152916	HM152827	EU718002[a]	EU673514[a]
A. lopesianum Rothm. (1)	Portugal: Bragança, Alfaião	FA & SB (s.n.)	–	HM152917	HM152828	EU718003[a]	EU673515[a]
A. lopesianum Rothm. (2)	Portugal: Vimioso, Carçao	FA & SB (s.n.)	EU677217[a]	HM152918	HM152829	EU718004[a]	EU673516[a]
A. lopesianum Rothm. (3)	Spain: Salamanca, Corporario	FA & SB (s.n.)	EU677218[a]	HM152919	HM152830	EU718005[a]	EU673517[a]
A. majus L. (1)	Spain: Barcelona, Grèixer	JG4150	FJ487615[a]	HM152920	HM152831	EU718006[a]	EU673518[a]
A. majus L. (2)	Spain: Huesca, Panticosa	JG4108	FJ648325[a]	HM152921	HM152832	EU718007[a]	EU673519[a]
A. majus L. (3)	Spain: Lérida, Valle d'Arán	JG26/8/99	AY731280[a]	HM152922	HM152833	EU718008[a]	EU673520[a]
A. majus L. (4)	France: Hérault, St. Chinian	230PV06	FJ487616[a]	HM152923	HM152834	EU718009[a]	EU673521[a]
A. majus L. (5)	France: Pyrénéés Orientales, Salses	VAL39727	FJ487613[a]	HM152924	HM152835	EU718010[a]	EU673522[a]
A. majus L. (6)	Spain: Gerona, La Molina	273PV06	EU677219[a]	HM152925	HM152836	HM152977[a]	EU673523[a]
A. meonanthum Hoffmans. & Link (1)	Spain: Ávila, Gredos	149PV99	AY731284[a]	HM152926	HM152837	EU718011[a]	EU673530[a]
A. meonanthum Hoffmans. & Link (2)	Spain: Soria, Río Lobos	JG4098	EU677220[a]	HM152927	HM152838	EU718012[a]	EU673531[a]
A. microphyllum Rothm. (1)	Spain: Cuenca, Buendía	JG4024	EU677221[a]	HM152928	HM152839	EU718013[a]	EU673532[a]
A. microphyllum Rothm. (2)	Spain: Guadalajara, Bolarque	JG4021	EU677222[a]	HM152929	HM152840	EU718014[a]	EU673533[a]
A. microphyllum Rothm. (3)	Spain: Guadalajara, Sacedón	JG4023	EU677223[a]	HM152930	HM152841	EU718015[a]	EU673534[a]
A. molle L. (1)	Spain: Barcelona, Rigurèixer	JG4143	FJ487612[a]	HM152931	HM152842	EU718016[a]	EU673524[a]
A. molle L. (2)	Spain: Huesca, Sopeira	JG4117	AY731268[a]	HM152932	HM152843	EU718017[a]	EU673525[a]
A. mollissimum Rothm. (1)	Spain: Almería, Benizalón	ECA29	EU677224[a]	HM152933	HM152844	EU718018[a]	EU673526[a]
A. mollissimum Rothm. (2)	Spain: Almería, Caballar	ECA32	EU677225[a]	HM152934	HM152845	EU718019[a]	EU673527[a]
A. pertegasii Rothm. (1)	Spain: Castellón, Cova Fosca	JG4092	EU677226[a]	HM152935	HM152846	EU718020[a]	EU673528[a]
A. pertegasii Rothm. (2)	Spain: Castellón, Solà d'en Brull	JG4091	EU677227[a]	HM152936	HM152847	EU718021[a]	EU673529[a]
A. pulverulentum Lázaro Ibiza (1)	Spain: Cuenca, Hoz de Beteta	ECA28	EU677229[a]	HM152937	HM152848	EU718022[a]	EU673535[a]
A. pulverulentum Lázaro Ibiza (2)	Spain: Guadalajara, Durón	JG4027	EU677230[a]	HM152938	HM152849	EU718023[a]	EU673536[a]
A. pulverulentum Lázaro Ibiza (3)	Spain: Guadalajara, La Pelegrina	JG4035	EU677231[a]	HM152939	HM152850	EU718024[a]	EU673537[a]
A. pulverulentum Lázaro Ibiza (4)	Spain: Guadalajara, Peralejos Truchas	ECA26	EU677232[a]	HM152940	HM152851	EU718025[a]	EU673538[a]
A. pulverulentum Lázaro Ibiza (5)	Spain: Teruel, Tramacastilla	ECA71	EU677233[a]	HM152941	HM152852	EU718026[a]	EU673539[a]
A. pulverulentum Lázaro Ibiza (6)	Spain: Guadalajara, Alcorlo	JG4028	EU677234[a]	HM152942	HM152853	EU718027[a]	EU673540[a]
A. sempervirens Lapeyr. (1)	Spain: Huesca, Bielsa	JG4114	EU677235[a]	HM152943	HM152854	EU718029[a]	EU673542[a]
A. sempervirens Lapeyr. (2)	Spain: Huesca, Plan	JG4116	EU677236[a]	HM152944	HM152855	EU718030[a]	EU673543[a]
A. sempervirens Lapeyr. (3)	Spain: Huesca, Panticosa	JG4107	AY731270[a]	HM152945	HM152856	EU718031[a]	EU673544[a]
A. siculum Mill. (1)	Italy: Sicily, Catania	GB66/06	EU677237[a]	HM152946	HM152857	EU718032[a]	EU673545[a]
A. siculum Mill. (2)	Italy: Sicily, Messine	VAL119899	AY731276[a]	HM152947	HM152858	EU718033[a]	EU673546[a]
A. siculum Mill. (3)	Italy: Sicily, Siracusa	VAL178308	FJ648327[a]	HM152948	HM152859	EU718034[a]	EU673547[a]
A. siculum Mill. (4)	Morocco: Oriental, Zegzel	192PV00	EU677238[a]	HM152949	HM152860	HM152978[a]	EU673548[a]
A. subbaeticum Güemes, Mateu & Sánchez Gómez (1)	Spain: Albacete, El Batán	JG4081	AY731287[a]	HM152950	HM152861	EU718035[a]	EU673549[a]
A. subbaeticum Güemes, Mateu & Sánchez Gómez (2)	Spain: Albacete, Los Vizcaínos	JG4084	EU677239[a]	HM152951	HM152862	EU718036[a]	EU673550[a]

Table 3 continued

Taxon	Locality	Voucher	GenBank accession no. (ITS/*psb*A-*trn*H/*trn*T-*trn*L/*trn*K-*mat*K/*trn*S-*trn*G)				
A. subbaeticum Güemes, Mateu & Sánchez Gómez (3)	Spain: Murcia, Benízar	JG4068	EU677240[a]	HM152952	HM152863	EU718037[a]	EU673551[a]
A. subbaeticum Güemes, Mateu & Sánchez Gómez (4)	Spain: Murcia, Hondares	BB227	EU677241[a]	HM152953	HM152864	EU718038[a]	EU673552[a]
A. tortuosum Bosc ex Vent. (1)	Italy: Sardinia, Cagliari	GB136/06	FJ487617[a]	HM152954	HM152865	EU718039[a]	EU673553[a]
A. tortuosum Bosc ex Vent. (2)	Morocco: West Rif, Talembot	IS(ALQ3441)	EU677242[a]	HM152955	HM152866	EU718040[a]	EU673554[a]
A. tortuosum Bosc ex Vent. (3)	Turkey: Sulcuk, Efeso	GB316/06	FJ487618[a]	HM152956	HM152867	EU718041[a]	EU673555[a]
A. tortuosum Bosc ex Vent. (4)	Morocco: Taza-Al Hoceima, Taza	188PV06	FJ487619[a]	HM152957	HM152868	EU718042[a]	EU673556[a]
A. tortuosum Bosc ex Vent. (5)	Italy: Latina, Norma	VAL142945	–	HM152958	HM152869	HM152973	HM152971
A. tortuosum Bosc ex Vent (6)	Turkey: Bursa Ili, Gemlik	164PV06	FJ648326[a]	HM152910	HM152821	EU718045[a]	EU673560[a]
A. tortuosum Bosc ex Vent. (7)	Morocco: Taza-Al Hoceima, Tazzeka	MA643294	–	HM152959	HM152870	EU718043[a]	EU673558[a]
A. tortuosum Bosc ex Vent. (8)	Morocco: Tadla-Azilal, Ighir	MA746269	–	HM152960	HM152871	EU718044[a]	EU673559[a]
A. valentinum Font Quer (1)	Spain: Valencia, Bolomor	BB229	EU677243[a]	HM152961	HM152872	EU718046[a]	EU673561[a]
A. valentinum Font Quer (2)	Spain: Valencia, Buixcarró	JG4002	EU677244[a]	HM152962	HM152873	EU718047[a]	EU673562[a]
A. valentinum Font Quer (3)	Spain: Valencia, Font del Cirer	BB8	EU677245[a]	HM152963	HM152874	EU718048[a]	EU673563[a]
A. valentinum Font Quer (4)	Spain: Valencia, La Drova	JG4004	EU677246[a]	HM152964	HM152875	EU718049[a]	EU673564[a]
A. valentinum Font Quer (5)	Spain: Valencia, Peña Colom	BB1	AY39799[a]	HM152965	HM152876	EU718050[a]	EU673565[a]
Chaenorhinum (DC.) Rchb.							
C. crassifolium (Cav.) Lange	Spain: Huesca, Sopeira	JG (s.n.)	–	HM152877	HM152788	EU718052[a]	HM152966
Gambelia Nutt.							
Gambelia speciosa Nutt.	Botanischer Garten Berlin-Dahlen	VAL45156	–	HM152878	HM152789	EU718054[a]	HM152967
Misopates Raf.							
M. orontium (L.) Raf.	Spain: Valencia, Serra	VAL145155	AY731260[a]	HM152879	HM152790	EU718055[a]	HM152968
Pseudomisopates Güemes							
P. rivas-martinezii (Sánchez Mata) Güemes	Spain: Ávila, Sierra de Gredos	377PV99	AY731262[a]	HM152880	HM152791	EU718057[a]	HM152969

[a] GenBank accession numbers published in Vargas et al. (2004) and Vargas et al. (2009)

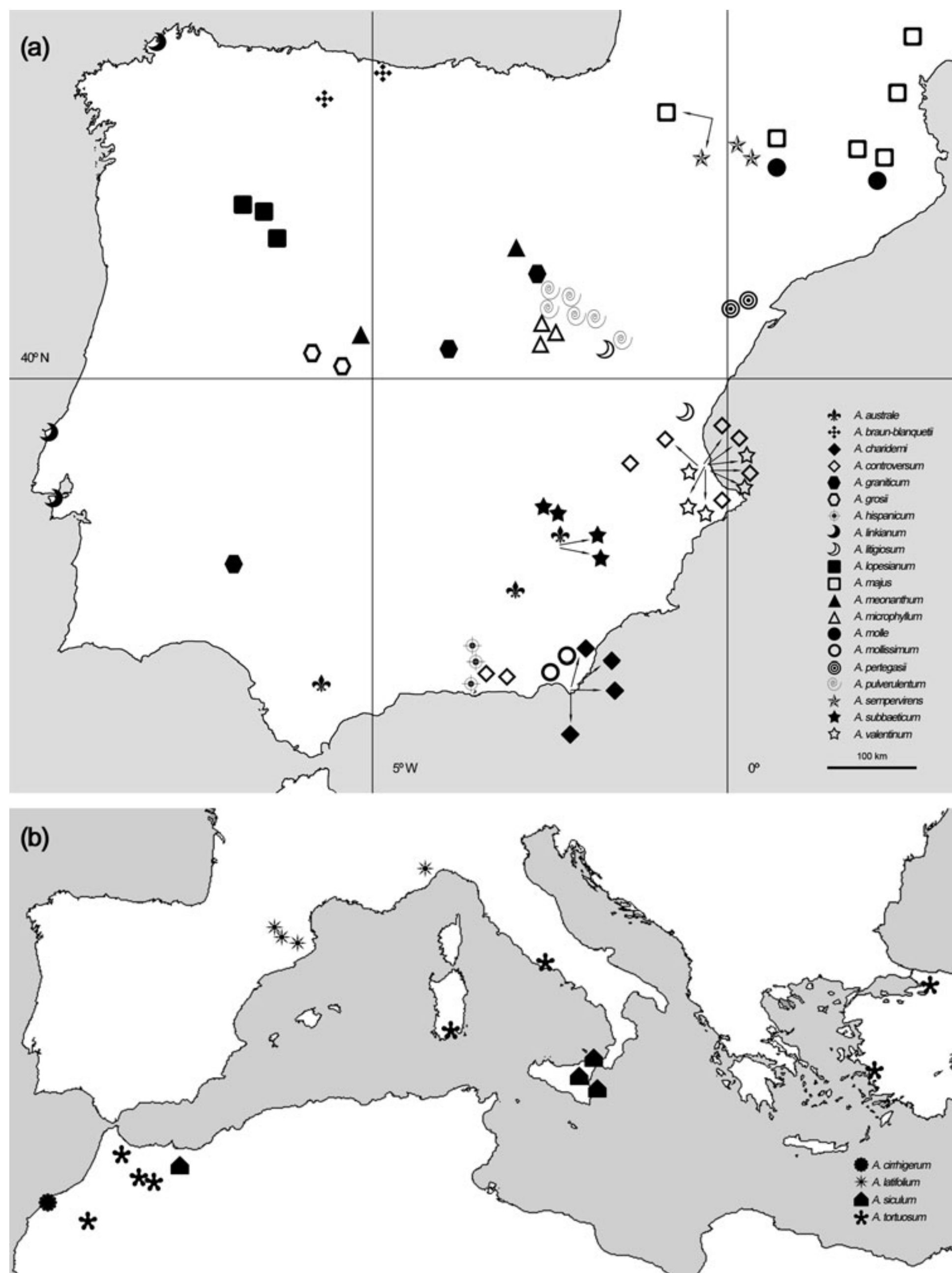


Fig. 1 Geographical distribution of the 85 populations of the 24 *Antirrhinum* species sampled for the molecular data. **a** Populations mainly sampled in the Iberian Peninsula. **b** Populations mainly sampled outside the Iberian Peninsula

criterion (AIC; Akaike 1979). When different evolutionary models were obtained by different criteria, each dataset was analyzed under both models. The analyses based on different models displayed the same topologies differing only in support values, thus the tree topologies and clade supports presented are those obtained from applying the evolutionary model selected by AIC. Pairwise sequence divergence for both nuclear (ITS) and plastid (*psb*A-*trn*H/*trn*T-*trn*L/*trn*T-*trn*L/*trn*S-*trn*G) sequences were calculated under the common model retrieved by Mr. Modeltest, using the program PAUP* (Swofford 2002).

For MP analysis, heuristic search was conducted with 100 times random-addition sequences, tree-bisection reconnection (TBR) branch swapping, and the "MulTrees" and "Steepest Descent" options in effect. All trees collected were combined and used as starting trees, with "MulTrees" on and no tree limit (these trees were then swapped to completion) and "Sub-tree-Pruning-Regrafting" (SPR; Salamin et al. 2003). Internal support was assessed using 1,000 replicates with simple taxon addition and SPR branch swapping, but permitting only ten trees per replicate to be held (Chase et al. 2003).

For BI both data matrices were run for 3,000,000 generations [four Markov chain Monte Carlo (MCMC), chain temperature = 0.2; sample frequency = 100]. A 50% majority rule consensus tree was calculated from the

pooled sample using the "sumt" command to yield the final Bayesian estimate of phylogeny.

As previous studies have identified different copies of ITS within individual accessions and interpreted these as evidence of the failure of concerted evolution after hybridization in the evolutionary history of recently evolved lineages (Fuertes Aguilar et al. 1999), the number and position of ITS additivities were assessed.

Results

Characteristics of nuclear (ITS) and plastid (*psb*A-*trn*H, *trn*T-*trn*L, *trn*K-*mat*K, *trn*S-*trn*G) sequences

Detailed information on the nuclear and plastid sequences obtained is given in Table 4. Within *Antirrhinum*, ITS sequence divergence ranges from 0.00% to 3.90%. *psb*A-*trn*H, *trn*T-*trn*L, *trn*K-*mat*K, and *trn*S-*trn*G sequence divergence varied from 0.00% to 3.71%. Mean sequence divergence within *A. charidemi*, *A. subbaeticum*, and *A. valentinum* was 0.00%, 0.96 ± 0.37%, and 0.82 ± 0.53% for ITS, and 0.02 ± 0.00%, 0.40 ± 0.29%, and 0.14 ± 0.04% for the combined plastid regions, respectively.

Phylogenetic analysis

The BI and MP analysis of ITS sequences yielded similar topologies, with BI displaying higher support values (Fig. 2). The BI analysis using GTR + I + G as the simplest model of DNA evolution reached equilibrium after 350,000 generations. Six species displayed either monophyly or identity of ITS sequences: the four accessions of *A. charidemi* (identical sequences), the four accessions of *A. subbaeticum*, with 88% posterior probability (PP) and 57% bootstrap value (BS), and the five accessions of *A. valentinum*, with 100% PP and 68% BS; well-supported monophyletic groups were also retrieved for *A. braun-blanquetii* (99% PP, 81% BS) and *A. sempervirens* (97% PP, 61% BS); the two accessions of *A. mollissimum* formed a monophyletic group with 95% PP but low BS support (<50% BS). Within *A. subbaeticum* two subgroups were identified: one was well supported (100% PP, 93% BS), while the other was weakly supported (100% PP, <50% BS).

Nucleotide additivity in ITS (i.e., double peaks in the chromatograms) was present in 56 of the 79 accessions of *Antirrhinum*. These additivities occurred at 44 of the 55 parsimony-informative sites. No additivities were detected in the four accessions of *A. charidemi*, while two of the four accessions of *A. subbaeticum* showed a single additivity, and three of the five accessions of *A. valentinum* showed eight, two, and three additivities sites, respectively (Table 5).

A majority rule consensus tree for plastid sequences obtained from BI under the GTR (*psb*A-*trn*H, *trn*K-*mat*K), GTR + I + G (*trn*T-*trn*L), and GTR + G (*trn*S-*trn*G) model is shown in Fig. 3. The BI analysis reached equilibrium after 500,000 generations. Plastid sequence data do not clearly support monophyly of any of the species of *Antirrhinum*. However, all accessions of *A. charidemi* were located in a well-supported clade (100% PP, 60% BS) that also included a single accession of *A. mollissimum*. In *A. subbaeticum*, accessions 3 and 4 were clustered together with 100% PP and 87% BS, but were unresolved with regard to other accessions. The other two accessions (1 and 2) of *A. subbaeticum* were identical to a single accession (4) of *A. pulverulentum*, and were located in a well-supported clade (100% PP, 98% BS). In *A. valentinum*, two well-supported clades were identified: one formed by accessions 1 and 5 with 100% PP and 86% BS, and the other one containing accesions 2 and 3 with 99% PP and 67% BS. Accession 4 was not resolved into strongly supported groups. The plastid sequence analysis therefore revealed lack of monophyly for populations of the three species.

Discussion

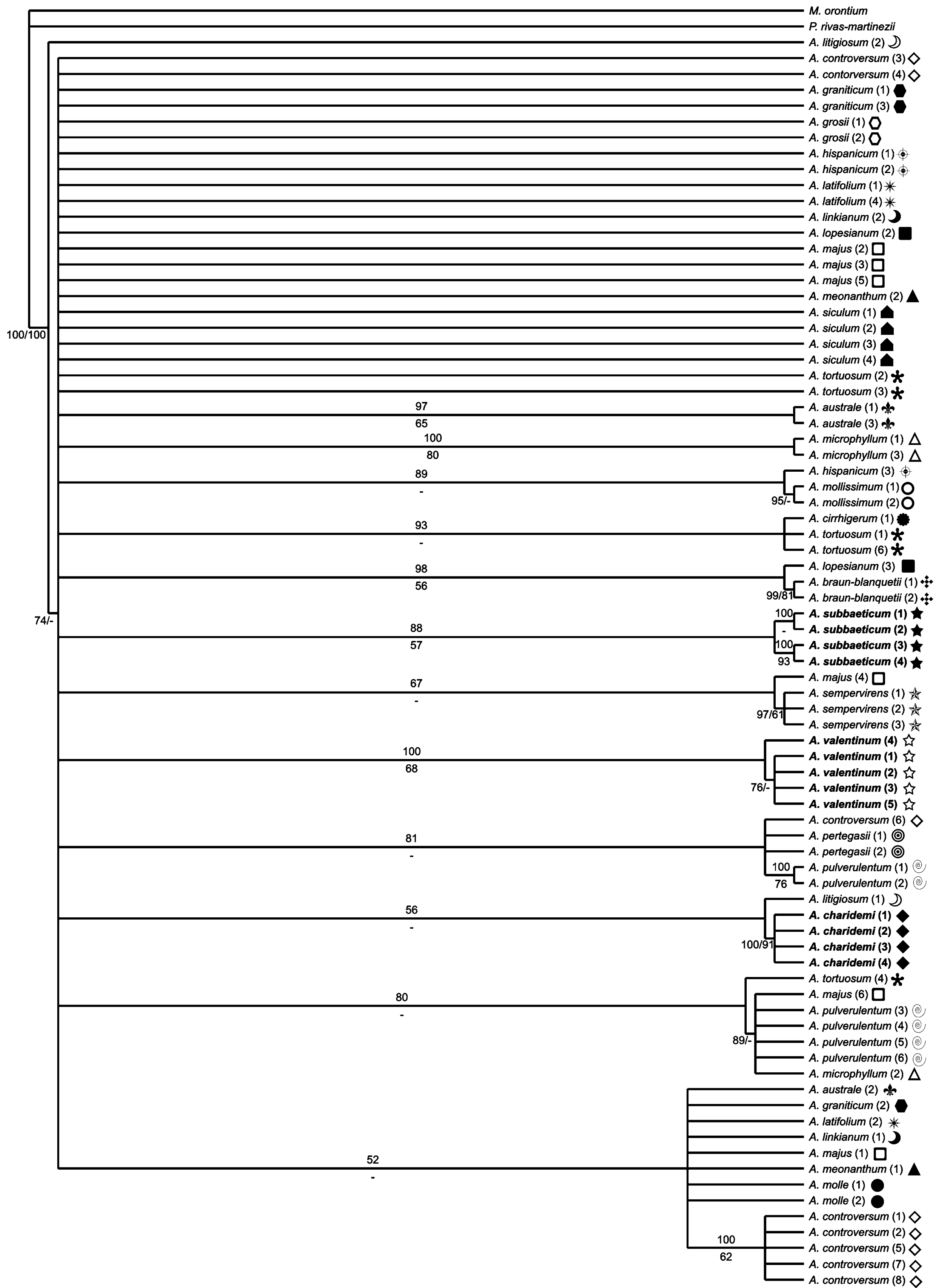
Testing monophyly at the species level

ITS accessions of the three study species of *Antirrhinum* formed three monophyletic groups (Fig. 2), which supports

Table 4 Sequence characteristics obtained from the analysis of ITS, *psb*A-*trn*H, *trn*T-*trn*L, *trn*K-*mat*K, and *trn*S-*trn*G in *Antirrhinum*

	ITS	*psb*A-*trn*H	*trn*T-*trn*L	*trn*K-*mat*K	*trn*S-*trn*G
Length range (bp)	578–595	228–299	613–624	1,255–1,267	662–665
Aligned length (bp)	599	311	636	1,267	667
No. variable/informative characters	109/55	37/31	30/20	28/49	19/39
Informative indels (no. bp)	2 (1–16)	12 (1–12)	7 (1–7)	1 (12)	0
Mean G + C content	60.6%	28.6%	27.2%	34.2%	32.0%
Simplest model[a]	GTR + I + G	GTR	GTR + I + G	GTR	GTR + G

[a] Akaike information criterion (Akaike 1979)


M. orontium
P. rivas-martinezii
A. litigiosum (2)
A. controversum (3)
A. contorversum (4)
A. graniticum (1)
A. graniticum (3)
A. grosii (1)
A. grosii (2)
A. hispanicum (1)
A. hispanicum (2)
A. latifolium (1)
A. latifolium (4)
A. linkianum (2)
A. lopesianum (2)
A. majus (2)
A. majus (3)
A. majus (5)
A. meonanthum (2)
A. siculum (1)
A. siculum (2)
A. siculum (3)
A. siculum (4)
A. tortuosum (2)
A. tortuosum (3)
100/100
97
65
A. australe (1)
A. australe (3)
100
80
A. microphyllum (1)
A. microphyllum (3)
89
-
A. hispanicum (3)
A. mollissimum (1)
A. mollissimum (2)
95/-
93
-
A. cirrhigerum (1)
A. tortuosum (1)
A. tortuosum (6)
98
56
A. lopesianum (3)
A. braun-blanquetii (1)
A. braun-blanquetii (2)
99/81
74/-
88
57
100
-
100
93
A. subbaeticum (1)
A. subbaeticum (2)
A. subbaeticum (3)
A. subbaeticum (4)
67
-
A. majus (4)
A. sempervirens (1)
A. sempervirens (2)
A. sempervirens (3)
97/61
100
68
A. valentinum (4)
A. valentinum (1)
A. valentinum (2)
A. valentinum (3)
A. valentinum (5)
76/-
81
-
A. controversum (6)
A. pertegasii (1)
A. pertegasii (2)
100
76
A. pulverulentum (1)
A. pulverulentum (2)
56
-
A. litigiosum (1)
A. charidemi (1)
A. charidemi (2)
A. charidemi (3)
A. charidemi (4)
100/91
80
-
A. tortuosum (4)
A. majus (6)
A. pulverulentum (3)
A. pulverulentum (4)
A. pulverulentum (5)
A. pulverulentum (6)
A. microphyllum (2)
89/-
52
-
A. australe (2)
A. graniticum (2)
A. latifolium (2)
A. linkianum (1)
A. majus (1)
A. meonanthum (1)
A. molle (1)
A. molle (2)
100
62
A. controversum (1)
A. controversum (2)
A. controversum (5)
A. controversum (7)
A. controversum (8)

◀ **Fig. 2** Phylogenetic analysis of ITS sequences of *Antirrhinum* species based on the 50% majority consensus tree from BI analysis. *Above branches* posterior probabilities, *below branches* bootstrap support >50% of the strict consensus tree of 89,540 MP trees [confidence interval (CI) 0.56; retention index (RI) 0.73; 263 steps]. Incongruence between the BI tree and the MP strict consensus tree or bootstrap support <50% is indicated with a *hyphen* below branches. Population numbers are given in *brackets* after species name (see Table 3). Species symbol follow each accession (see Fig. 1). *A. charidemi*, *A. subbaeticum*, and *A. valentinum* are shown in **bold**

the morphological and taxonomical boundaries of these species. In contrast, analyses of the plastid sequences did not reveal monophyly for any of the study species, potentially reflecting different evolutionary histories. Several processes have been proposed as potential mechanisms for retrieving nonmonophyletic groups at the species level estimated for nuclear and plastid phylogenies. Particularly in plants, lack of congruence between nuclear and plastid data sets and the fact that identical plastid sequences are shared among unrelated species suggest that lineage sorting, hybridization, or a combination of both processes may be responsible (Hardig et al. 2000; Linder and Rieseberg 2004).

In *A. charidemi*, a shared history for populations is suggested by plastid sequences, as they occur in the same clade and have high similarity. The occurrence of a single accession of *A. mollissimum* in this clade may reflect hybridization between these species, as they are geographically proximal (Fig. 1). A more complex evolutionary history is interpreted in the case of *A. subbaeticum*. This species clearly consists of two related lineages, as recognized not only in ITS sequence analysis but also in allozyme (Mateu-Andrés 2004) and RAPD (Jiménez et al. 2002) analyses. These populations also show some evidence of the effects of inbreeding, with very low allozyme and RAPD diversity. However, our plastid reconstruction reveals topological incongruence with the ITS tree, including a close relationship between two populations of *A. subbaeticum* and one of *A. pulverulentum* (identical plastid sequences). The low number of ITS additivities in *A. subbaeticum* accessions (Table 5) does not give support for recent hybridization events, and the fact that a high number of characters (17–18) separate the plastid sequences of the two lineages is difficult to explain by mechanisms such as lineage sorting. Nevertheless, these two mechanisms (hybridization versus lineage sorting) have been historically invoked as responsible for topological incongruence between genome phylogenies (Doyle 1992; Rosenberg 2002; Degnan and Rosenberg 2009). As recommended (Maddison and Knowles 2006), a sample increase of populations and DNA regions, by considering those of Vargas et al. (2009), and the use of coalescence methods do not alleviate the problem of lineage sorting. Thus, ancient hybridization between these populations of *A. subbaeticum* and *A. pulverulentum* (see also Fig. 3) or a related ancestral taxon is a more parsimonious explanation for the distribution of these plastid sequences resulting in a nonmonophyletic group for plastid sequences of *A. subbaeticum* and a weakly supported ITS group. In *A. valentinum*, despite strongly supported monophyly for ITS sequences, plastid sequences were unresolved within a clade containing six other species that are widespread in southeast Iberia due to a low number of informative characters rather than character incongruence. This makes it difficult to distinguish unequivocally between the effects of lineage sorting and hybridization as an explanation for nonmonophyly. However, evidence from intra-individual ITS variation supports recent or contemporary hybridization in *A. valentinum*: three of the five accessions of this species contain ten ITS additivities (Table 5).

Hybridization has been historically suggested as significant in *Antirrhinum* evolution because: (1) plants display intermediate morphological characters in nature (Rothmaler 1956; Webb 1971), (2) reproductive barriers between any species pair are weak in nature and under experimental conditions (Baur 1932; Rothmaler 1956; Sutton 1988; Xue et al. 1996), (3) high number of ITS additivities is common in overlapping geographical areas of many species (Vargas et al. 2009), and (4) additivity patterns are well documented through analysis of ITS clones (Vargas et al. 2004; Vargas et al. unpublished) and fingerprints (Jiménez et al. 2005). Moreover, nuclear and plastid phylogenies are incongruent, and plastid haplotype variation reflects geography rather than species taxonomy (Vargas et al. 2009). This scenario is not unexpected in genera that have undergone recent diversification since the onset of the Mediterranean climate 2–3 Ma, as reported for *Antirrhinum* (Vargas et al. 2009), which additionally has some species still involved in hybrid zones in the Pyrenees (Whibley et al. 2006).

The question remains as to whether lack of single ancestry characterizes *Antirrhinum* species or is a common pattern in angiosperms. Syring et al. (2007) reviewed 16 studies from four representative journals covering diverse taxonomic groups. Of the 460 taxa considered, 170 (37%) could be evaluated for monophyly due to multiple accessions being sampled in phylogenetic reconstructions, and of these 53% were nonmonophyletic. As the review of Syring et al. (2007) was small and considered only articles that included multiple accessions of at least some taxa, and that these taxa were a priori closely related, we have compiled similar but more broad-ranging data from three further sources [articles cited in Gitzendanner and Soltis (2000), 378 taxa cited in the *Genetical Flora of the British Isles* by Squirrell et al. (2006), and 172 articles published in six leading journals between 2000 and 2007]. Studies

Table 5 Polymorphic sites in ITS sequences of *A. charidemi*, *A. subbaeticum*, and *A. valentinum*

Taxon	017	019	021	037	040	041	045	080	081	085	094	095	109	113	125	136	145	163	173
A. charidemi (1)	C	C	C	C	C	G	G	G	A	T	G	-	C	C	C	C	A	C	C
A. charidemi (2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. charidemi (3)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. charidemi (4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. subbaeticum (1)	.	.	.	.	.	.	.	T	C	G	C	G	.	.	.	.	.	T	.
A. subbaeticum (2)	.	.	.	.	.	.	.	T	C	G	C	G	.	.	.	.	.	T	.
A. subbaeticum (3)	.	T	.	.	.	.	.	T	C	G	G	–	.	.	.	.	G	T	.
A. subbaeticum (4)	A	T	G	.	.	.	.	T	C	A/G	G	–	.	.	.	.	.	.	.
A. valentinum (1)	.	.	.	.	T	.	.	.	.	G	G	–	.	T	G	A	.	T	T
A. valentinum (2)	.	.	.	.	.	A	.	.	.	G	G	–	.	.	.	A	.	T	.
A. valentinum (3)	.	.	.	C/G	C/T	.	A/G	.	.	G	G	–	.	.	.	A	.	T	.
A. valentinum (4)	.	.	.	.	C/T	.	A/G	.	.	G	G	–	.	.	.	A	.	T	.
A. valentinum (5)	.	.	.	C/G	T	.	A	.	.	G	G	–	C/T	.	.	A	.	T	.

Taxon	174	186	213	226	356	364	378	412	438	446	448	449	534	549	551	552	556	583	589
A. charidemi (1)	C	C	C	C	A	C	C	G	C	A	C	G	C	T	C	C	C	C	T
A. charidemi (2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. charidemi (3)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. charidemi (4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. subbaeticum (1)	.	T	.	.	.	T	.	A	.	.	T	.	.	G	T	.	.	T	T
A. subbaeticum (2)	.	T	.	.	.	.	.	A	.	.	T	.	.	G	T	.	.	T	.
A. subbaeticum (3)	T	T	.	C/T	.	.	.	A	.	.	T	.	T	G	T	.	.	T	T
A. subbaeticum (4)	T	T	.	.	.	.	.	A	.	.	T	.	T	G	T	.	A	.	T
A. valentinum (1)	T	T	.	.	.	.	.	A	.	G	T	.	.	A	T	T	A	.	T
A. valentinum (2)	T	T	.	.	.	.	.	A	.	G	T	A	.	A	T	.	A	.	T
A. valentinum (3)	T	T	C/T	.	A/G	.	.	A	.	A/G	T	A/G	.	A/G	T	.	A	.	T
A. valentinum (4)	T	T	T	.	G	.	.	A	T	.	T	.	.	G	T	.	A	.	T
A. valentinum (5)	T	T	.	.	.	.	C/T	A	.	G	T	A	.	A	T	.	A	.	T

Numbers refer to the aligned sequences. Population numbers are given in brackets after species name (see Table 3)

examining plant species for population genetic diversity were examined on a taxon-by-taxon basis to assess whether each taxon could be assessed for monophyly based upon published phylogenetic reconstructions, and how many of these species were monophyletic. Data from 270 articles identified 634 species (328 genera, 117 families), of which 109 (16.6%) could be assessed for monophyly due to a generic phylogeny containing multiple accessions of the study taxon being available. Of those taxa for which monophyletic status could be deduced from the published phylogeny ($N = 92$), 57 (62%) were monophyletic for all markers assayed. This brief review demonstrates that species nonmonophyly is rarely considered for specific taxa assessed for population and conservation genetic purposes, due primarily to the fact that multiple-accession generic phylogenies are not available for the taxa under study. When nonmonophyly is assessed, it appears to be taxonomically widespread [detected here in 19 families representing conifers (1), monocots (5), and dicots (13)] and perhaps more common than generally recognized in both plant (38%; this study) and animal taxa [23% nonmonophyly in mitochondrial DNA (mtDNA); Funk and Omland (2003)]. In support of the widespread occurrence of plant species nonmonophyly, a recent DNA barcoding study by Fazekas et al. (2008) determined that 31–39% of plant species assessed were nonmonophyletic using a combination of plastid loci. All these results reflect that lack of monophyly is a more common pattern than generally recognized in angiosperms and can dramatically influence assessments of genetic diversity.

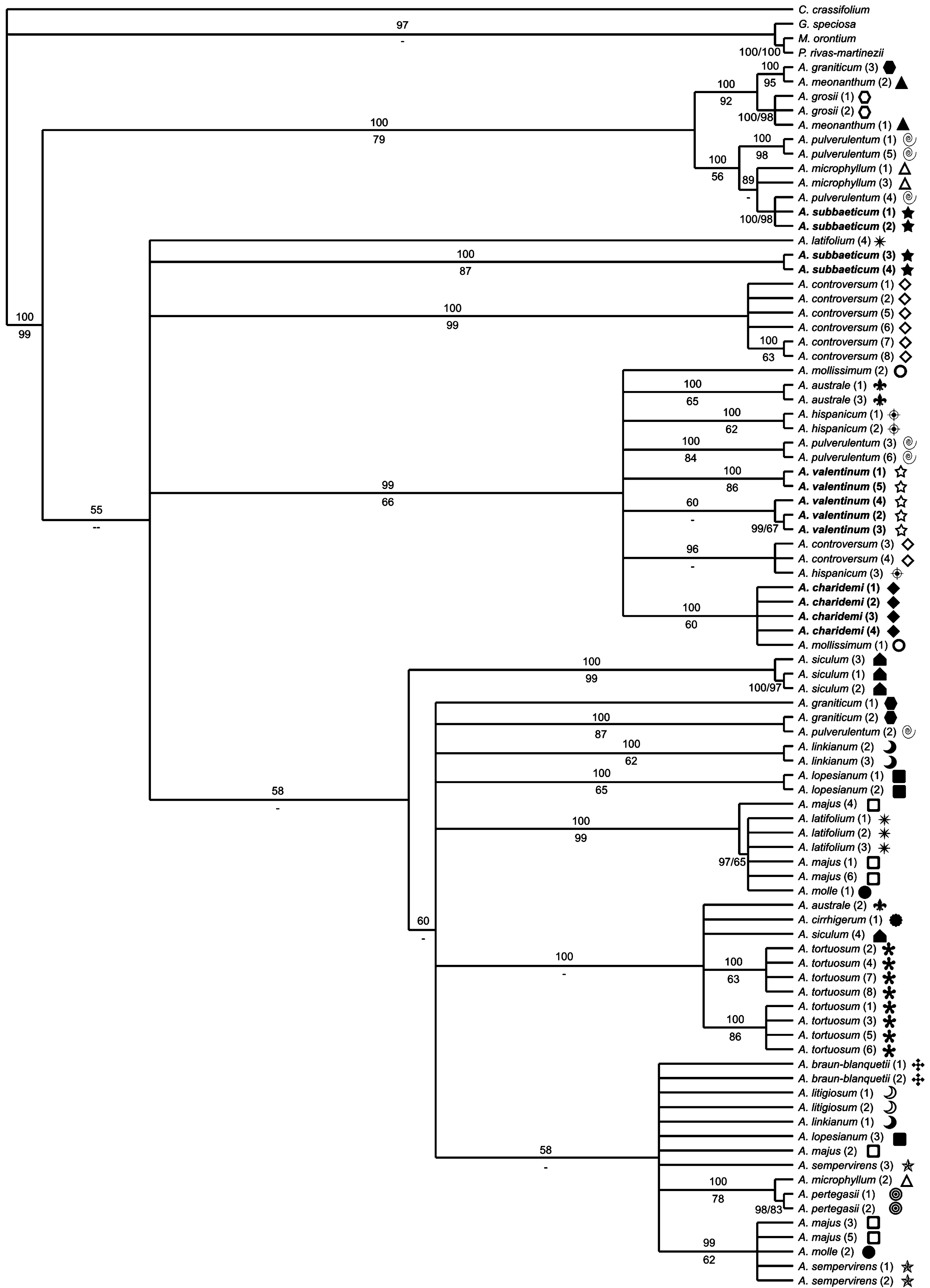
Life history traits, monophyly, and genetic diversity

Hamrick et al. (1979) indicated that significant differences in levels and patterns of genetic diversity existed between species with different life history traits. Accordingly, we should expect to find a comparable level and distribution of genetic variation between species with similar life history traits where taxa are closely related. However, previous allozyme and RAPD analysis revealed markedly different levels of genetic diversity within and among populations of the three species evaluated herein (Mateu-Andrés and Segarra-Moragues 2000; Mateu-Andrés 2004) despite the similarity of their life history traits (Table 2). Genetic diversity differences may therefore reflect additional sources of variation in the evolutionary history of the species concerned.

For outcrossing perennial species, the expectation is that species with lower overall diversity (H_T) will have higher differentiation among populations (G_{ST}), and vice versa. This trend is broadly apparent in *Antirrhinum* species (Table 1), with some notable exceptions. Narrow endemic species such as *A. charidemi* and *A. microphyllum* both harbor relatively high levels of diversity with a corresponding low estimate of population differentiation, whereas *A. siculum* shows a low level of diversity and high population differentiation (Mateu-Andrés 1999; Mateu-Andrés and Segarra-Moragues 2000; Torres et al. 2003; Mateu-Andrés and de Paco 2006). The latter species is self-compatible, whereas the former two are self-incompatible, therefore the differences may be explained by variation in breeding system. Exceptions to this trend include *A. pertegasii*, which has both low diversity and little differentiation among populations, and *A. pulverulentum* and *A. valentinum*, which both have medium to elevated overall diversity and the highest levels of differentiation among population in *Antirrhinum* (Table 1). Diversity estimates for the majority of *Antirrhinum* species fall between these extreme examples.

In addition, outcrossing species tend to show higher levels of within-population variation and lower levels of differentiation among populations (Hamrick and Godt 1996), so the high level of differentiation ($G_{ST} = 0.480$) in *A. valentinum* is intriguing. This level of diversity is not apparent from ITS sequences as it is in *A. subbaeticum*. Other species in the same plastid clade have comparable levels of H_T (*A. mollissimum* 0.280; *A. pulverulentum* 0.300), but lower estimates of G_{ST} (*A. mollissimum* 0.110; *A. pulverulentum* 0.230) compared with *A. valentinum*. It has been suggested that *A. valentinum* maintains high genetic diversity as a consequence of a large population size in the past (currently 1,332 individuals) due to the fact that the species was common just 50 years ago (Mateu-Andrés and Segarra-Moragues 2000). However, this short time period would not allow development of high G_{ST} estimates due to fragmentation, suggesting that gene flow among populations is restricted by an alternate mechanism. This may also account for a reduction in interspecific gene flow explaining why *A. valentinum* is the only species represented in this plastid clade to attain monophyly for ITS sequences. Levels of genetic diversity can be shaped by natural hybridization, wherein hybrids may exhibit elevated levels of genetic diversity resulting from the mixing of parental genomes (Rieseberg and Wendel 1993; Arnold 1997). High diversity in species of this clade may be the result of historical introgression among divergent lineages (see Doyle1992).

Antirrhinum charidemi and *A. valentinum* share the eight life history characteristics with effect on genetic variation (Table 2). The differences estimated for population genetic parameters between these species can be largely explained through differences in gene flow among populations (four separated populations in *A. valentinum* versus continuous distribution of populations in *A. charidemi*) and the potential effects of introgression in *A. valentinum*. While genetic diversity in *A. subbaeticum*


C. crassifolium
97
-
G. speciosa
M. orontium
100/100
P. rivas-martinezii
100
A. graniticum (3)
100
95
A. meonanthum (2)
92
A. grosii (1)
100
79
A. grosii (2)
100/98
A. meonanthum (1)
100
A. pulverulentum (1)
98
A. pulverulentum (5)
100
A. microphyllum (1)
56
89
A. microphyllum (3)
-
A. pulverulentum (4)
A. subbaeticum (1)
100/98
A. subbaeticum (2)
A. latifolium (4)
100
A. subbaeticum (3)
87
A. subbaeticum (4)
A. controversum (1)
A. controversum (2)
100
A. controversum (5)
100
99
A. controversum (6)
99
100
A. controversum (7)
63
A. controversum (8)
A. mollissimum (2)
100
A. australe (1)
65
A. australe (3)
100
A. hispanicum (1)
62
A. hispanicum (2)
100
A. pulverulentum (3)
84
A. pulverulentum (6)
100
A. valentinum (1)
99
66
86
A. valentinum (5)
60
A. valentinum (4)
55
--
-
A. valentinum (2)
99/67
A. valentinum (3)
A. controversum (3)
96
A. controversum (4)
-
A. hispanicum (3)
A. charidemi (1)
A. charidemi (2)
100
A. charidemi (3)
60
A. charidemi (4)
A. mollissimum (1)
100
A. siculum (3)
99
A. siculum (1)
100/97
A. siculum (2)
A. graniticum (1)
100
A. graniticum (2)
87
A. pulverulentum (2)
100
A. linkianum (2)
62
A. linkianum (3)
100
A. lopesianum (1)
65
A. lopesianum (2)
58
-
A. majus (4)
100
A. latifolium (1)
99
A. latifolium (2)
A. latifolium (3)
97/65
A. majus (1)
A. majus (6)
A. molle (1)
A. australe (2)
60
A. cirrhigerum (1)
-
A. siculum (4)
A. tortuosum (2)
100
100
A. tortuosum (4)
-
63
A. tortuosum (7)
A. tortuosum (8)
A. tortuosum (1)
100
A. tortuosum (3)
86
A. tortuosum (5)
A. tortuosum (6)
A. braun-blanquetii (1)
A. braun-blanquetii (2)
A. litigiosum (1)
A. litigiosum (2)
A. linkianum (1)
A. lopesianum (3)
58
A. majus (2)
-
A. sempervirens (3)
100
A. microphyllum (2)
78
A. pertegasii (1)
98/83
A. pertegasii (2)
A. majus (3)
A. majus (5)
99
A. molle (2)
62
A. sempervirens (1)
A. sempervirens (2)

◀ **Fig. 3** Phylogenetic analysis of combined *psb*A-*trn*H/*trn*T-*trn*L/*trn*K-*mat*K/*trn*S-*trn*G sequences of *Antirrhinum* species based on the 50% majority consensus tree from BI analysis. *Above branches* posterior probabilities, *below branches* bootstrap support >50% of the strict consensus tree of 54,670 MP trees (CI 0.88; RI 0.91; 428 steps). Incongruence between the BI tree and the MP strict consensus tree or bootstrap support <50% is indicated with a *hyphen* below branches. Population numbers are given in *brackets* after species name (see Table 3). Species symbol follow each accession (see Fig. 1). *A. charidemi*, *A. subbaeticum*, and *A. valentinum* are shown in **bold**

is undoubtedly influenced by its different reproductive strategy and geographic range, the extreme plastid subdivision suggests that historical processes have also played a role in shaping current diversity.

In summary, the impact of reticulation involved in species formation should be included in the list of factors responsible for influencing levels of genetic diversity. Indeed, lack of monophyly and incongruence between plastid and nuclear phylogenies provide an essential framework to detect reticulation processes. The 12 life history traits described by Hamrick et al. (1979) are not necessarily the most significant for assessing levels of genetic diversity within species. Careful attention should be paid to monophyly to elucidate the potential impact of hybridization on estimates of genetic variation.

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References

Akaike H (1979) A new look at the statistical model identification. IEEE Trans Automat Contr AC-19:716–723

Álvarez I, Cronn R, Wendel JF (2005) Phylogeny of the New World diploid cottons (*Gossypium* L., Malvaceae) based on sequences of three low copy nuclear genes. Plant Sys Evol 252:199–214

Arnold ML (1997) Natural hybridization and evolution. Oxford University Press, New York

Baur E (1932) Artumgrenzung und Artbildung in der Gattung *Antirrhinum*, Sektion *Antirrhinastrum*. Z Indukt Abstamm Vererbungsl 63:256–302

Boscaiu M, Riera J, Estrellés E, Güemes J (1997) Números cromosomáticos de plantas occidentales, 751–776. An Jard Bot Madr 55:430–431

Carrió E, Jiménez JF, Sánchez-Gómez P, Güemes J (2009) Reproductive biology and conservation implications of three snapdragon species (*Antirrhinum*, Plantaginaceae). Biol Conserv 142:1854–1863

Chase MW, Knapp S, Cox AV, Clarkson JJ, Butsko Y, Joseph J, Savolainen V, Parokonny AS (2003) Molecular systematics, GISH and the origin of hybrid taxa in Nicotiana (Solanaceae). Ann Bot (Lond) 92:107–127

Coy E, Tamayo MJ, Carrillo AF, Sánchez-Gómez P, Güemes J (1997) Números cromosomáticos de plantas occidentales, 746–750. An Jard Bot Madr 55:430

Degnan JH, Rosenberg NA (2009) Gene tree discordance, phylogenetic inference and the multispecies coalescent. Trends Ecol Evol 24:332–340

Diosdado JC, Vioque J, Juan R, Pastor J (1994) IOPB chromosome data 7. Int Org Pl Biosyst Newslett 22:3–4

Doyle JJ (1992) Gene trees and species trees—molecular systematics as one—character taxonomy. Syst Bot 17:144–163

Fatemi M, Gross CL (2009) Life on the edge—high levels of genetic diversity in a cliff population of Bertya ingramii are attributed to *B. rosmarinifolia* (Euphorbiaceae). Biol Conserv 142:1461–1468

Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG, Husband BC, Percy DM, Hajibabaei M, Barrett SCH (2008) Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. PLoS One 3(7):e2802

Fuertes Aguilar J, Rosselló JA, Nieto Feliner G (1999) Nuclear ribosomal DNA (nrDNA) concerted evolution in natural and artificial hybrids of *Armeria*. Mol Ecol 8:1341–1346

Funk DJ, Omland KE (2003) Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annu Rev Ecol Syst 34:397–423

Garnett ST, Christidis L (2007) Implications of changing species definitions for conservation purposes. Bird Conserv Int 17:187–195

Gitzendanner MA, Soltis PS (2000) Patterns of genetic variation in rare and widespread plant congeners. Am J Bot 87:783–792

Goodwillie C, Stiller JW (2001) Evidence for polyphyly in a species of *Linanthus* (Polemoniaceae): convergent evolution in self-fertilizing taxa. Syst Bot 26:273–282

Güemes J (2009) *Antirrhinum* L. In: Castroviejo S, Herrero A, Benedí C, Rico E, Güemes J (eds) Flora Iberica.13. CSIC, Madrid, pp 134–166

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hamilton M (1999) Four primers pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Mol Ecol 8:521–523

Hamrick JL, Godt MJW (1989) Allozymic diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL, Weir BS (eds) Plant population genetics, breeding and germplasm resources. Sinauer, Sunderland, pp 43–63

Hamrick JL, Godt MJW (1996) Effects of life history traits on genetic diversity in plant species. Philos Trans R Soc Lond B Biol Sci 351:1291–1298

Hamrick JL, Linhart YB, Mitton JB (1979) Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. Annu Rev Ecol Syst 10:173–200

Hamrick JL, Godt MJW, Sherman-Boyles SL (1992) Factors influencing levels of genetic diversity in woody plant species. New Forests 6:95–124

Hardig TM, Soltis PS, Soltis DE (2000) Diversification of the North America shrub genus *Ceanothus* (Rhamnaceae): conflicting phylogenies from nuclear DNA and chloroplast DNA. Am J Bot 87:108–123

Harrison BJ, Darby LA (1955) Unilateral hybridization. Nature 176:982

Hughes CE, Eastwood RJ, Bailey CD (2006) From famine to feast? Selecting nuclear DNA sequence loci for plant species-level phylogeny reconstruction. Philos Trans R Soc Lond B Biol Sci 361:211–225

Isaac NJB, Mallet J, Mace GM (2004) Taxonomic inflation: its influence on macroecology and conservation. Trends Ecol Evol 19:464–469

Jiménez JF, Sánchez-Gómez P, Güemes J, Werner O, Rosselló JA (2002) Genetic variability in a narrow endemic snapdragon (*Antirrhinum subbaeticum*, Scrophulariaceae) using RAPD markers. Heredity 89:387–393

Jiménez JF, Sánchez-Gómez P, Güemes J, Rosselló JA (2005) Phylogeny of snapdragon species (*Antirrhinum*; Scrophulariaceae) using non-coding cpDNA sequences. Isr J Plant Sci 53:47–54

Kelchner SA (2000) The evolution of non-coding chloroplast DNA and its application in plant systematics. Ann Mo Bot Gard 87:482–498

Linder CR, Rieseberg LH (2004) Reconstructing patterns of reticulate evolution in plants. Am J Bot 91:1700–1708

Loveless MD, Hamrick JL (1984) Ecological determinats of genetic tructure in plant populations. Annu Rev Ecol Syst 15:65–95

Mace GM (2004) The role of taxonomy in species conservation. Philos Trans R Soc Lond B Biol Sci 359:711–719

Maddison W, Knowles L (2006) Inferring phylogenetic despite incomplete lineage sorting. Syst Biol 55:21–30

Mateu-Andrés I (1999) Allozymic variation and divergence in three species of *Antirrhinum* L. (Scrophulariaceae–Antirrhineae). Bot J Linn Soc 131:187–199

Mateu-Andrés I (2004) Low levels of allozyme variability in the threatened species *Antirrhinum subbaeticum* and *A. pertegasii* (Scrophulariaceae): implications for conservation of the species. Ann Bot (Lond) 94:797–804

Mateu-Andrés I, de Paco L (2006) Genetic diversity and the reproductive system in related species of *Antirrhinum*. Ann Bot (Lond) 98:1053–1060

Mateu-Andrés I, Segarra-Moragues JG (2000) Population subdivision and genetic diversity in two narrow endemics of *Antirrhinum* L. Mol Ecol 9:2081–2087

Mateu-Andrés I, Segarra-Moragues JG (2003) Patterns of genetic diversity in related taxa of *Antirrhinum* L. assessed using allozymes. Biol J Linn Soc 79:299–307

Mayden R (1997) A hierarchy of species concepts: the denouement in the saga of the species problem. In: Claridge M, Dawah H, Wilson M (eds) Species: the units of biodiversity. Chapman and Hall, London, pp 381–424

Moreno JC (2008) Lista roja 2008 de la flora vascular española. Dirección General de Medio Natural y Política Forestal (Ministerio de Medio Ambiente, y Medio Rural y Marino, y Sociedad Española de Biología de la Conservación de Plantas), Madrid

Nybom H (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol Ecol 13:1143–1155

Nylander JAA (2004) MrModeltest 2.1. Program distributed by the author. Evolutionary Biology Centre, Uppsala University

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818

Rieseberg LH (1997) Hybrid origins of plant species. Annu Rev Ecol Syst 28:359–389

Rieseberg LH, Wendel J (1993) Introgression and its consequences in plants. In: Harrison R (ed) Hybrid zones and the evolutionary process. Oxford University Press, New York, pp 70–114

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Rosenberg NA (2002) The probability of topological concordance of genes tress and species trees. Theor Popul Biol 61:225–247

Rothmaler W (1956) Taxonomische Monographie der Gattung *Antirrhinum*. Feddes Repert 136:1–24

Salamin N, Chase MW, Hodkinson TR, Savolainen V (2003) Assesing internal support with large phylogenetic DNA matrices. Mol Phylogenet Evol 27:528–539

Sánchez-Gómez P, Jiménez JF, Carrión MA, Güemes J (2003) *Antirrhinum subbaeticum* Güemes, Mateu and Sánchez-Gómez. In: Bañares A, Blanca G, Güemes J, Moreno JC, Ortiz S (eds) Atlas y libro rojo de la flora vascular amenazada de España. Dirección General de Conservación de la Naturaleza, Madrid, pp 588–589

Savolainen V, Chase MW (2003) A decade of progress in plant molecular phylogenetics. Trends Genet 19:717–724

Squirrell J, Gornall RJ, French GC, Pullan M, Hollingsworth PM (2006) Genetical Flora of the British Isles database. http://rbg-web2.rbge.org.uk/geneticflora. Accessed 01 Sept 2009

Sutton DA (1988) A revision of the tribe Antirrhineae. Oxford University Press, London

Swofford DL (2002) PAUP*: phylogenetic analysis using parsimony and other methods, version 4.0b. Sinauer, Sunderland

Syring J, Farrell K, Businský R, Cronn R, Liston A (2007) Widespread genealogical nonmonophyly in species of *Pinus* Subgenus *Strobus*. Syst Biol 56:163–181

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chroloplast DNA. Plant Mol Biol 17:1105–1109

Torres E, Iriondo JM, Pérez C (2002) Vulnerability and determinants of reproductive success in the narrow endemic *Antirrhinum microphyllum* (Scrophulariaceae). Am J Bot 89:1171–1179

Torres E, Iriondo JM, Pérez C (2003) Genetic structure of an endangered plant, *Antirrhinum microphyllum* (Scrophulariaceae): allozyme and RAPD analysis. Am J Bot 90:85–92

Treutlein J, Smith GF, van Wyk B-E, Wink M (2003) Evidence for the polyphyly of *Haworthia* (Asphodelaceae, subfamily Alooideae; Asparagales) inferred from nucleotide sequences of rbcL, matK, ITS1 and genomic fingerprinting with ISSR-PCR. Plant Biol 5:513–521

Vargas P, Rosselló JA, Oyama R, Güemes J (2004) Molecular evidence for naturalness of genera in the tribe Antirrhineae (Scrophulariaceae) and three independent evolutionary lineages from the New World and the Old. Plant Syst Evol 249:151–172

Vargas P, Carrió E, Guzmán B, Amat E, Güemes J (2009) *Antirrhinum* (Scrophulariaceae) speciation since the Pliocene based on plastid and nuclear DNA polymorphisms. J Biogeogr 142:1854–1863

Vardas P, Ornosa C, Ortiz-Sánchez FJ, Arroyo J (2010) Is the ocluded corolla of Antirrhinum bee-specialized? J Nat Hist 44:1427–1443

Vieira CP, Charlesworth D (2001) Low diversity and divergence in the *fil1* gene family of *Antirrhinum* (Scrophulariaceae). J Mol Evol 52:171–181

Webb DA (1971) Taxonomic notes on *Antirrhinum* L. Bot J Linn Soc 64:271–275

Whibley AC, Langlade NB, Andalo C, Hanna AI, Bangham A, Thébaud C, Coen E (2006) Evolutionary paths underlying flower color variation in *Antirrhinum*. Science 313:963–966

Xue Y, Carpenter R, Dickinson HG, Coen ES (1996) Origin of allelic diversity in *Antirrhinum* S locus RNases. Plant Cell 8:805–814