

NEW INSIGHTS INTO THE PHYLOGENETIC RELATIONSHIPS,
CHARACTER EVOLUTION, AND PHYTOGEOGRAPHIC PATTERNS OF
CALCEOLARIA (CALCEOLARIACEAE)¹

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Biogeographical patterns and diversification processes in Andean and Patagonian flora are not yet well understood. *Calceolaria* is a highly diversified genus of these areas, representing one of the most specialized plant–pollinator systems because flowers produce nonvolatile oils, a very unusual floral reward. Phylogenetic analyses with molecular (ITS and *matK*) and morphological characters from 103 *Calceolaria* species were conducted to examine relationships, to understand biogeographic patterns, and to detect evolutionary patterns of floral and ecological characters. Total evidence analysis retrieved three major clades, which strongly correspond to the three previously recognized subgenera, although only subgenus *Rosula* was retrieved as a monophyletic group. A single historical event explains the expansion from the southern to central Andes, while different parallel evolutionary lines show a northward expansion from the central to northern Andes across the Huancabamba Deflection, an important geographical barrier in northern Peru. Polyploidy, acquisition of elaiophores, and a nototribic pollination mechanism are key aspects of the evolutionary history of *Calceolaria*. Pollination interactions were more frequently established with *Centris* than with *Chalepogenus* oil-collecting bee species. The repeated loss of the oil gland and shifts to pollen as the only reward suggest an evolutionary tendency from highly to moderately specialized pollination systems.

Key words: Andes; *Calceolaria*; Calceolariaceae; *Centris*; *Chalepogenus*; elaiophore; morphological evolution; nototribic; oil flowers; polyploidy; sternotribic.

Biogeographical patterns and diversification processes in Andean and Patagonian flowering plants, in particular their extraordinarily high rate of speciation are not yet well understood. It has been proposed that diversification of pollinating animals, concurrent with the topographical and environmental changes resulting from the uplift of the Andes, could have promoted diversification of the flora (e.g., Ezcurra, 2002; Kay et al., 2005; Hughes and Eastwood, 2006; Smith and Baum, 2006; Ackermann et al., 2008). In general, these studies were performed in plants that reward pollinators with nectar or pollen, which are likely to attract many different animals. Thus, plants may readily shift among diverse pollinators and changing environmental conditions. The evolutionary history of the Andean plants with unusual floral rewards has remained practically unexplored and yet it could inform us on how much diversification is dependent on the ability of a plant lineage to shift among different kinds of pollinator.

Calceolaria, a highly diversified Andean and Patagonian genus, represents one of the most specialized plant–pollinator

systems because flowers produce nonvolatile oils, a very unusual floral reward that attracts particular solitary oil-collecting bees (Vogel, 1988; Johnson and Steiner, 2000; Sérsic, 2004; Neff and Simpson, 2005). The majority of species in this genus provide oils as reward to pollinators, but a number of shifts in pollination systems have been reported across geographic regions (Molau, 1988; Sérsic, 2004).

Recent molecular studies have placed *Calceolaria* with *Jovellana* and *Porodittia* in the new Austral-Antarctic family, Calceolariaceae (Olmstead and Reeves, 1995; Olmstead et al., 2001). *Calceolaria*, with approximately 250 species ranges from Mexico to Tierra del Fuego, occurs mainly at high altitudes along the Andes but reaches sea level on both the Atlantic and Pacific coasts in Patagonia. Two main centers of diversification have been recognized in *Calceolaria*: at middle latitudes of Chile and at the south of the Huancabamba Deflection in northern Peru (Molau, 1988; Ehrhart, 1997, 2000). This deflection, located at 6°S, is the deepest and widest depression present in the high Andes, representing a major geographical break and a significant barrier for plants and animals (e.g., Simpson, 1975; Fjeldså, 1995; Weigend, 2002). *Jovellana*, with six species, is distributed in Chile (two species) and New Zealand (four species), and the monotypic *Porodittia* has a narrow distribution in the Peruvian Andes. The three genera differ markedly in flower morphology; *Porodittia* is characterized by a three-lobed corolla and three stamens, while *Jovellana* and *Calceolaria* have a bilabiate corolla and two stamens. The distinctive floral trait of *Calceolaria* is an inflated lower lip with an infolded median lobe, bearing a patch of oil-secreting trichomes, the elaiophore (Vogel, 1974; Molau, 1988). Field observations

¹ Manuscript received 11 June 2009; revision accepted 4 September 2009.

The authors thank Bianca Delfosse for editing the English version of the manuscript and two anonymous reviewers for their constructive criticism and comments. A.C. thanks RLB for RLP-P05 fellowship and the Doctorate in Biology, University of Córdoba. A.A.C. and A.N.S. acknowledge CONICET as fellow researchers and A.C. as its fellowship holder. The study was supported by CONICET (PIP 5174), FONCYT (PICT 01-10952 & 01 33755) and SeCyT UNC). The program TNT is freely available, thanks to a subsidy from the Willi Hennig Society.

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TABLE 1. Subgenera, sections, and geographic distribution of the *Calceolaria* species included in this study following Molau's (1988) classification. Type species are boldfaced.

Subgenera	Section	Species	Distribution	Subgenera	Section	Species	Distribution
<i>Calceolaria</i>	<i>Anacyrta</i>	<i>C. comosa</i>	Co, sE, nP	<i>Calceolaria</i>	<i>Salicifolia</i>	<i>C. stricta</i>	sE
		<i>C. glauca</i>	cP			<i>C. tetragona</i>	nP
		<i>C. rugulosa</i>	cP		<i>Symplocophylla</i>	<i>C. connatifolia</i>	nP
		<i>C. sericea</i>	nE			<i>C. semiconnata</i>	sE
		<i>C. variifolia</i>	sE, nP		<i>Teucrifoliae</i>	<i>C. helianthemoides</i>	sE
	<i>C. virgata</i>	sE, sP	<i>C. lavandulifolia</i>			sE	
	<i>Calceolaria</i>	<i>C. chelidonoides</i>	A, B, Co, Ch, E, G, M, P		<i>C. santolinoides</i>	nA, sB	
		<i>C. mandoniana</i>	nB, sP		<i>C. scabra</i>	cP	
		<i>C. rivularis</i>	nA, cB, sP		<i>C. teucroides</i>	nA, sB	
	<i>Englerina</i>	<i>C. tripartita</i>	E, M, sP		<i>Thamnobia</i>	<i>C. gossypina</i>	nE
		<i>C. boliviana</i>	nB, sP			<i>C. rosmarinifolia</i>	sE
	<i>Ericoides</i>	<i>C. engleriana</i>	sP		<i>Urticopsis</i>	<i>C. spruceana</i>	cE
		<i>C. barbata</i>	nP			<i>C. ballotifolia</i>	nP
		<i>C. ericoides</i>	E, nP		<i>C. bicrenata</i>	nP	
	<i>Integerrimae</i>	<i>C. linearis</i>	ncP		<i>C. elatior</i>	nA, sB	
		<i>C. pinifolia</i>	A, Ch		<i>C. flexuosa</i>	cP	
	<i>Lehmanniana</i>	<i>C. thyrsoiflora</i>	Ch		<i>C. lamifolia</i>	sCo, nE	
		<i>C. cypripediiflora</i>	sP		<i>C. maculata</i>	nP	
		<i>C. lehmanniana</i>	sCo, nE		<i>C. penlandii</i>	sCo, nE	
		<i>C. martinezzi</i>	cE		<i>Cheiloncos</i>	<i>Micranthera</i>	<i>C. dichotoma</i>
<i>C. oblonga</i>		cP	<i>C. tucumana</i>	nA, sB			
<i>Lobatae</i>	<i>C. pedunculata</i>	nE	<i>Rugosae</i>	<i>C. utricularioides</i>	cnP		
	<i>C. vulpina</i>	cP		<i>C. cavanillesii</i>	sA, scCh		
	<i>C. lobata</i>	nB, cP		<i>C. dentata</i>	sA, scCh		
	<i>C. obtusa</i>	sE		<i>C. integrifolia</i>	sA, scCh		
<i>Parvifoliae</i>	<i>C. phaceliifolia</i>	nP	<i>C. petioalaris</i>	cnCh			
	<i>C. myriophylla</i>	P	<i>C. plectranthifolia</i>	nA, sB			
	<i>C. parvifolia</i>	A, B, P	<i>C. polifolia</i>	cCh			
<i>Perfoliatae</i>	<i>C. sparsiflora</i>	B, P	<i>C. volckmanii</i>	sA, sCh			
	<i>C. calycina</i>	sE, nP	<i>C. sp. nova 1</i>	sA			
	<i>C. dilatata</i>	cE	<i>Rosula</i>	<i>Bellidifolia</i>	<i>C. brunellifolia</i>	cA	
	<i>C. lanata</i>	cE			<i>C. glacialis</i>	nA, sB	
	<i>C. pavonii</i>	sE, ncP	<i>C. mendocina</i>	sA			
	<i>C. perfoliata</i>	cCo, CR, Pa, V	<i>C. parviflora</i>	cA			
	<i>C. purpurascens</i>	nE	<i>C. umbellata</i>	nA, sB			
<i>C. tomentosa</i>	cnP	<i>C. corymbosa</i>	sA, sCh				
<i>Polyclada</i>	<i>C. atahualpae</i>	cB, cP	<i>Corymbosae</i>	spp. floccosa			
	<i>C. brachiata</i>	nE		<i>C. corymbosa</i>	sA, sCh		
	<i>C. inflexa</i>	cP		spp. montana			
	<i>C. jujuensis</i>	nA, sB		<i>C. crenatiflora</i>	sA, sCh		
	<i>C. polyclada</i>	nA, sB		<i>C. filicaulis</i>	sA, sCh		
<i>Revolutae</i>	<i>C. vaccinioides</i>	nB, sP	<i>C. germainii</i>	sA, sCh			
	<i>C. buchtieniana</i>	cB, sP	<i>C. tenella</i>	sA, sCh			
	<i>C. revoluta</i>	sP	<i>Kremastocheilos</i>	<i>C. biflora</i>	sA, sCh		
<i>C. rupestris</i>	cP	<i>C. luxurians</i>		cA, cCh			
<i>Salicifolia</i>	<i>C. dentifolia</i>	nP	<i>C. lagumae-</i>	sA			
	<i>C. fusca</i>	sCo, nE	<i>blancae</i>				
	<i>C. gaultherioides</i>	nP	<i>C. lanceolata</i>	sA			
	<i>C. microbefaria</i>	Co, V	<i>C. polyrhiza</i>	sA, sCh			
	<i>C. moyobambae</i>	nP	<i>C. prichardii</i>	sA			
	<i>C. nivalis</i>	cE, cP	<i>C. uniflora</i>	sA, sCh			
	<i>C. phaeotricha</i>	sE	<i>C. pennellii</i>	sCh			
	<i>C. salicifolia</i>	cnP	<i>C. sp. nova 2</i>	sA			

Notes: A = Argentina, B = Bolivia, c = central, Co = Colombia, Ch = Chile, CR = Costa Rica, E = Ecuador, G = Guatemala, M = Mexico, n = northern, Pa = Panama, P = Peru, s = southern, V = Venezuela.

have demonstrated that the elaiophore is associated with oil-collecting bees (Vogel, 1974; Molau, 1988; Sérsic, 2004), and the remarkable diversification of *Calceolaria*, in comparison with *Jovellana* and *Porodittia*, needs to be examined in the context of its close association with these highly specialized pollinators (Molau, 1988).

The majority of *Calceolaria* species are shrubby, but there are also perennial and annual herbs, and the genus is characterized by a noteworthy floral variation (Molau, 1988; Ehrhart,

2000). Most of this variation is defined by the shape and size of the corolla lobes, the position of the elaiophore or lack thereof, stamen morphology, and sometimes, by the shape of the calyx. Analyzed from a historical perspective, gains and losses of the elaiophore in *Calceolaria*, as well as variation in its structure could prove important, mainly because of its close association with a number of pollinators. The pollination system for a number of *Calceolaria* species has been determined. For example, species lacking elaiophores are either autogamous or pollinated

by a broad assemblage of pollen-collecting bees (Vogel, 1974; Sérsic, 2004). Moreover, a unique association with birds has been reported for two *Calceolaria* species of southern Patagonia (Sérsic and Cocucci, 1996a; Roitman et al., 2002) where the elaiophore-bearing lobe of the flowers has functionally differentiated trichomes modified into a juicy fruiting body. Most *Calceolaria* species have nototribic flowers (i.e., pollen is deposited on the bee's dorsum or bird's front), but a few have sternotribic flowers (i.e., pollen is deposited on the bee's sternum). The great number of species in *Calceolaria* as well as the variation in floral reward, shape, and size; in the relative position of the elaiophore-bearing lobe; in the filament and style length; and in the branching pattern of flowering shoots (i.e., synflorescence) make the genus an interesting model of evolution for understanding the role that pollinators have played in diversification processes (Molau, 1988; Sérsic, 1991; Sérsic and Cocucci, 1996a; Ehrhart, 2000; Johnson and Steiner, 2000; Sérsic, 2004).

Calceolaria has been subdivided into three subgenera with 24 sections (Molau, 1988) (Table 1). The vast majority of the sections are in subgenus *Calceolaria*, mainly distributed in tropical regions, while subgenera *Cheiloncos* and *Rosula* are mainly distributed in temperate South America. Subgenus *Calceolaria* includes shrubs, subshrubs, and herbs that have stamens with filaments shorter than anthers. Subgenus *Cheiloncos* also includes herbs and subshrubs, but their stamens have filaments that are several times longer than their anthers. Subgenus *Rosula* includes rosulate or subrosulate herbs that have stamen filaments about as long as the anthers and includes the two strictly extratropical sections in the genus: *Corymbosae* and *Kremastocheilos* (Molau, 1988).

Andersson (2006) made the first attempt to determine the phylogenetic relationships of *Calceolaria* based on molecular characters (ITS and *matK*) with representative species mostly from the northern part of its distribution. However, the resulting phylogenetic hypotheses did not resolve character evolution, geographical patterns, or the relationships between the temperate and tropical species in *Calceolaria*. Results were partly a consequence of the scarce sampling of southern temperate representatives of the genus and the lack of morphological data. Therefore, in the current study, the sampling of *Calceolaria* species was extended to 103 taxa, and, in addition to molecular data (ITS and *matK*), morphological characters were considered in the phylogenetic analyses.

The goal of this study was to determine the phylogenetic relationships among *Calceolaria* species using a total evidence approach (i.e., combining morphological and molecular data). Phylogenetic hypotheses were used to analyze geographic patterns, chromosome number, and shifts in the pollination systems within *Calceolaria*.

MATERIALS AND METHODS

Taxon sampling—Of the approximately 250 species in *Calceolaria*, 103 were sampled. Representative taxa from 21 of the 24 generic sections were included in the analyses. Morphological variation and distribution patterns were taken into account to select the taxa. *Porodittia triandra*, the two South American species of *Jovellana*, and two species of *Kohleria* (Gesneriaceae) were used as outgroups (Olmstead et al., 2001; Andersson 2006). Vouchers for morphological and molecular analyses and GenBank accessions are listed in Appendix 1. The taxonomy of *Calceolaria* follows Molau (1988) and Ehrhart (2000) except for the recognition of *C. mendocina*, *C. lanceolata*, and *C. prichardii*, which Ehrhart (2000) placed under the polymorphic *C. polyrhiza*.

Morphological characters—Thirty-six morphological characters were coded (Appendix 2). Character states were observed in specimens collected for this project and when necessary complemented with information from the literature (Molau, 1988; Correa, 1999; Ehrhart, 2000). The two continuous characters (25 and 30) were coded based on the gap-weighting (GW) method proposed by Thiele (1993) because it has been shown to recover the highest number of informative characters, with a strong phylogenetic signal (García-Cruz and Sosa, 2006).

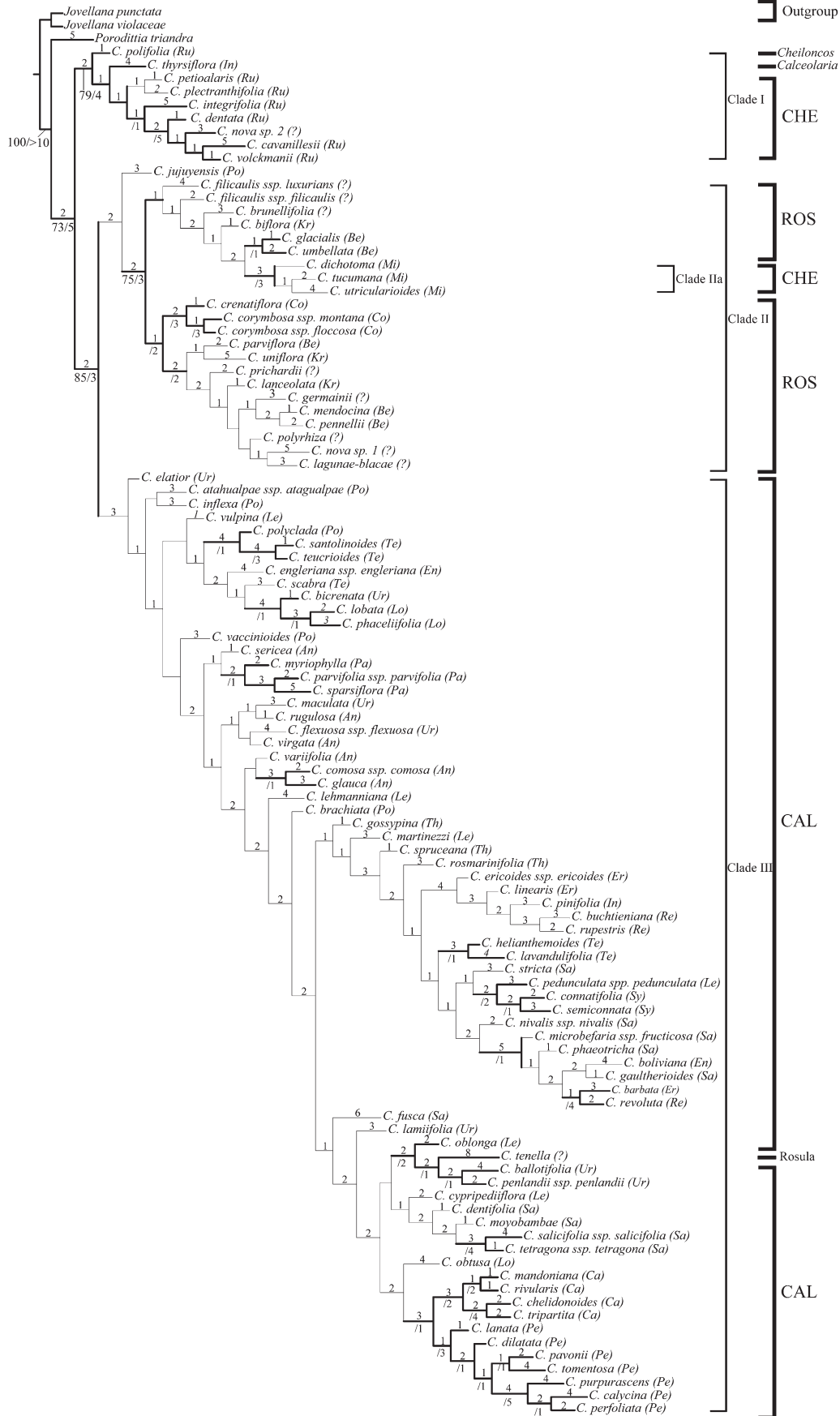
Molecular data—DNA was extracted using the CTAB method (Doyle and Doyle, 1987) and purified using QIAquick columns (Qiagen, Carlsbad, California, USA) following protocols provided by the manufacturer. A *matK* segment of approximately 840 bp containing the highest amount of variation was amplified and sequenced using the primers 323f and 1189r based on Andersson (2006). The entire ITS region including the 5.8S gene was amplified and sequenced using primers ITS5 and ITS4 and two internal primers, ITS2 and ITS3 (White et al., 1990). Amplified, double-stranded DNA fragments were purified using QIAquick columns and sequenced using *Taq* BigDye Terminator Cycle Sequencing Kits (Perkin Elmer Applied Biosystems, Foster City, California, USA) on an ABI 310 automated DNA sequencer (Perkin Elmer Applied Biosystems). Morphological and molecular data matrices are given in Appendix S1 (see Supplemental Data with the online version of this article).

Sequence alignment—The reverse and forward sequences for both *matK* and ITS were assembled and edited using the program Sequencher 4.1 (Gene Codes, Ann Arbor, Michigan, USA). The matrices were initially aligned in the program Clustal_X (Higgins and Sharp, 1988; Thompson et al., 1997) and later edited by eye with the alignment program Se-Al v.2.0a11 (Rambaut, 2002). Insertion/deletion mutations (indels) in the ITS matrix were coded manually following the simple method proposed by Simmons and Ochoterena (2000).

Cladistic analyses—Exploratory phylogenetic analyses were conducted separately with each data partition (ITS, *matK*, and morphology). Results from the ITS and *matK* analyses did not show any major topological conflict and provided a higher resolution when analyzed together than separately (see Results, *Phylogenetic analyses*). Consequently, molecular data sets were combined (ITS+*matK*) in a single analysis. In contrast, the morphological data set showed topological incongruence with the molecular partition, which was confirmed using the incongruence length difference (ILD) test of Farris et al. (1994) as implemented in the program WinClada (Nixon, 2002) (see Results, *Phylogenetic analyses*). However molecular and morphological evidence was combined applying a "total evidence" criterion (Kluge, 1989, 1998; Nixon and Carpenter, 1996; DeSalle and Brower, 1997). Results with the morphological matrix and with the molecular matrix (ITS+*matK*) are also presented.

Parsimony searches were conducted with the program TNT (Goloboff et al., 2003) using only potentially informative characters. "Traditional" heuristic searches were carried out with a total of 1000 random addition sequences and submitted to tree-bisection-reconnection (TBR) branch swapping, holding 50 trees, followed by a more extensive TBR holding 50000 trees. The most parsimonious trees (MPT) found were collected and a strict consensus was calculated using the "Nelsen" option in TNT (Goloboff et al., 2003). Support was estimated by jackknife as implemented in TNT, resampling 1000 times with TBR set to 100 replications holding 20 trees, followed by a more extensive TBR holding 5000 trees, and saving the consensus for each resampling matrix. Bremer support (Bremer, 1994) was calculated using the "absolute supports" option in TNT (Goloboff et al., 2003) on 10000 trees held in memory only for the morphological data set.

For the molecular and the combined molecular-morphological data matrix, Bayesian inference was conducted to estimate additional clade support. Models for the molecular data were evaluated using the program ModelTest v. 3.06 (Posada and Crandall, 1998) to find the best fit according to the Akaike information criterion (Akaike, 1974). The GTR+I+ Γ model (Γ shape = 0.8437, $p[\text{inv}] = 0.4629$) was found to be the most appropriate. For morphological data, we used the Mk (Markov k) model of Lewis (2001) as recommended in Nylander et al. (2004). The Mk model is a generalized JC69 model, which assumes that lineages are always in one of k possible states ($k \geq 2$), with no state considered plesiomorphic or apomorphic a priori (Lewis, 2001). Four Markov chains starting with a random tree were run simultaneously in two independent runs for 30000000 generations using the program MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001). Trees were sampled every 10000 generations. Convergence diagnostics for log likelihood values and standard deviations of split frequencies were assessed visually using StatView v. 4.5 software (Abacus



Concepts, Berkeley, California, USA). Trees prior to stationarity were excluded, and the remaining trees were used to calculate the Bayesian posterior probabilities (PP).

Character evolution and pollination systems—With the software Mesquite v.2.0 (Maddison and Maddison, 2007), the patterns of evolution for nine of the 36 morphological characters included in the analyses related to habit (1), reproduction (35), floral morphology (17, 25, 27, 30, 33) and pollination mechanism (29, 34) were visualized in one of the most parsimonious trees (chosen at random) derived from the total evidence analysis.

To elucidate the evolutionary patterns of the relationship between *Calceolaria* and its pollinators, we first determined the pollinators for every species based on previous studies (Molau, 1988; Rasmussen, 1999; Rasmussen and Olesen, 2000; Sérsic, 2004; A. Cosacov, A. Sérsic and A. A. Cocucci, unpublished manuscript). Chromosome numbers were taken from Molau (1988) and Ehrhart (2000). These characters were optimized in the same most parsimonious tree as mentioned, using Mesquite v.2.0 (Maddison and Maddison, 2007) and are listed in Appendix S2 (see Supplemental Data with the online version of this article).

Biogeographical patterns—To determine biogeographical patterns, we scored each species for its presence in the southern, central, and northern Andean regions (Fig. 4A), as delimited by Simpson (1975). Distribution information was taken from Molau (1988), Correa (1999), Ehrhart (2000), and Sérsic (2004) and is available in Appendix S2 (see Supplemental Data with the online version of this article).

RESULTS

Phylogenetic analyses—Morphological data—Parsimony analysis of the morphological data retrieved 32 most parsimonious trees (MPT; L = 484 steps, CI = 0.30, RI = 0.6). The strict consensus tree had low resolution (one of the MPT is shown in Fig. 1) with only five clades with both jackknife (jk) and Bremer support (BS). In the strict consensus tree, the *Porodittia-Calceolaria* clade was well supported (jk = 100%, BS > 10). *Calceolaria* was retrieved as a monophyletic group (jk = 73%, BS = 5).

Almost all the representative taxa of subgen. *Cheiloncos* and subgen. *Rosula* were clustered in their respective moderately supported clades. Subgen. *Cheiloncos* corresponds to clade I, which includes all the species from sect. *Rugosae* in addition to *C. thyrsoflora* (sect. *Integerrimae*, from subgenus *Calceolaria*); this clade was retrieved as the sister group of the remaining *Calceolaria* species (jk = 79%, BS = 4). The remaining *Calceolaria* species formed a clade (jk = 85%, BS = 3), and within this clade two major groups can be distinguished: clade II (jk = 75%, BS = 3), which includes almost all species previously assigned to subgen. *Rosula* (except for subclade IIa representing sect. *Micranthera* previously assigned to subgenus *Cheiloncos*) and a subclade (clade III) without support, which includes species previously assigned to subgenus *Calceolaria* (except for the southern species *C. tenella* assigned to subgenus *Rosula*).

Molecular data—The ITS matrix included 721 base pairs (bp), of which 186 (25.8%) were parsimony informative. The *matK* matrix included 836 bp, of which 51 (3.52%) were parsimony

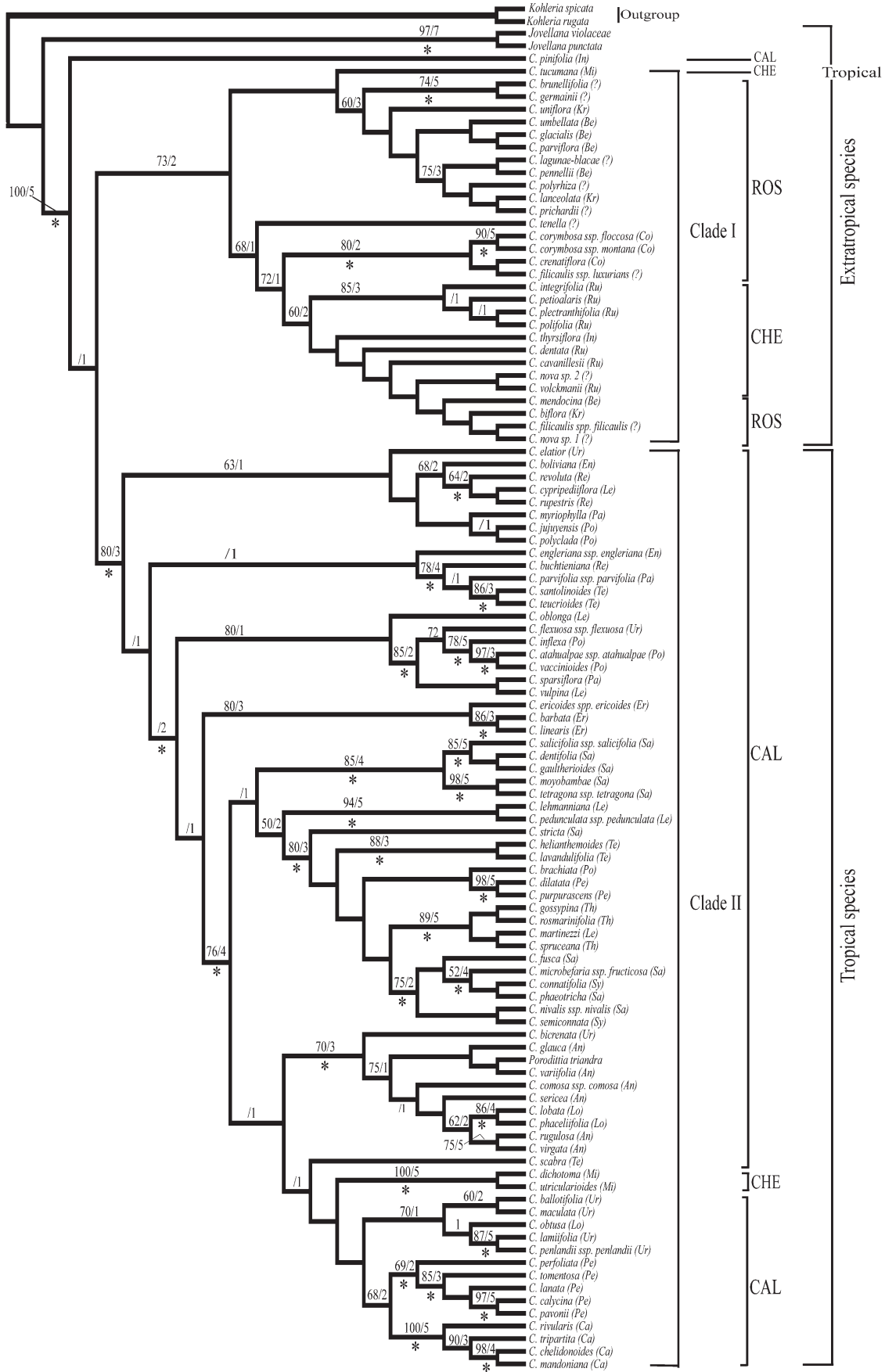
informative. The strict consensus tree of the *matK* analysis (not shown) resulted in a poorly resolved cladogram, with only a few moderately supported large clades, which were also found in the combined molecular analysis. The strict consensus tree of the ITS data set (not shown) retrieved many supported minor clades, which were also found in the MPT of the combined molecular analysis. The ILD results indicated that ITS and *matK* partitions were congruent ($P = 0.15$). The combined molecular matrix included 239 (11.01%) parsimony informative characters, and retrieved a single MPT (L = 647 steps, CI = 0.44, RI = 0.76) (Fig. 2). The Bayesian run reached a stable log likelihood value and standard deviation of split frequencies after about 5 000 000 generations. A burn-in fraction of 0.25 was used, and subsequent trees were used to estimate Bayesian posterior probabilities (BPP; only values >0.95 are reported).

This single MPT showed inconsistencies with the morphological data, such as the position of *Porodittia triandra*, which here appeared nested within *Calceolaria*, together with representative taxa from sect. *Anacyrta*, sect. *Lobatae* and *Urticopsis* (BPP = 1, jk = 100%, BS = 5; Fig. 2). Two major clades were retrieved (Fig. 2): clade I (jk = 73%, BS = 2) clustering the species of subgenus *Cheiloncos* and *Rosula*, except for *C. thyrsoflora* (sect. *Integerrimae*), previously assigned to subgenus *Calceolaria*, while the strongly supported clade II (BPP = 0.97, jk = 80%, BS = 3), exclusively clustered species of subgenus *Calceolaria*, except for the two species of section *Micranthera*, previously assigned to subgenus *Cheiloncos*. With the exception of *C. tucumana*, the species nested within clade I are extratropical, while exclusively tropical species clustered in clade II (Fig. 2).

Total evidence—The combined morphological and molecular matrix included 1593 characters, of which 275 (12.47%) were parsimony informative. Although the ILD results indicated that morphology and molecular partitions were significantly incongruent ($P = 0.02$), the combined evidence analysis retrieved more strongly supported clades. Five MPT (L = 1357 steps, CI = 0.36, RI = 0.67) were retrieved in the parsimony analysis (strict consensus tree in Fig. 3). The Bayesian run reached a near stable state for the log likelihood and standard deviation of split frequencies after about 6 000 000 generations, but values continued to fluctuate until 20 000 000 generations. For a representative sample, a burn-in fraction of 0.5 was used, and subsequent trees were used to calculate Bayesian posterior probabilities (BPP; only values >0.95 are reported).

In agreement with molecular data, the consensus tree of the total evidence approach showed *Porodittia triandra* grouped together with all *Calceolaria* species in a strongly supported clade (BPP = 1, jk = 100%, BS = 10; Fig. 3). Two morphological synapomorphic character states supported this clade: the presence of an infolded lower corolla lip (36) and fatty oils as a floral reward (33) (Fig. 3). These traits are lost in *P. triandra*, which appears nested with representative taxa from sect. *Anacyrta*, sect. *Lobatae*, and *Urticopsis* (BPP = 1, jk = 88%, BS = 6) within clade IIIC.

← Fig. 1. One of the 32 most parsimonious trees retrieved by the morphological analysis (L = 484 steps, CI = 0.30, RI = 0.6). Branches supported in the strict consensus are marked with bold lines. Numbers below branches indicate jackknife/Bremer support. Numbers above branches indicate number of changes. Subgenus abbreviations: CAL = *Calceolaria*; CHE = *Cheiloncos*; ROS = *Rosula*. Abbreviations within parenthesis indicate genus section according to Molau (1988): (An) *Anacyrta*, (Be) *Bellidifolia*, (Ca) *Calceolaria*, (Co) *Corymbosae*, (En) *Englerina*, (Er) *Ericoides*, (In) *Integerrimae*, (Kr) *Kremastochelios*, (Le) *Lehmannina*, (Lo) *Lobatae*, (Mi) *Micranthera*, (Pa) *Parvifoliae*, (Pe) *Perfoliatae*, (Po) *Polyclada*, (Re) *Revolutae*, (Ru) *Rugosae*, (Sa) *Salicifoliae*, (Sy) *Symphlocophylla*, (Te) *Teucriifoliae*, (Th) *Thamnobia*, (Ur) *Urticopsis*. Question marks indicate species not treated by Molau (1988).



The consensus tree displays three major clades, which strongly correspond to the three previously recognized subgenera (Fig. 3). Clade I (jk = 81, BS = 5) grouped the same taxa as the morphological analysis did, i.e., the majority of species in subgenus *Cheiloncos*. Moderately supported clade II (jk = 77, BS = 2) groups all the species previously assigned to subgenus *Rosula* with the synapomorphic character state: rosulate habit (1). Well-supported clade III (BPP = 0.99, jk = 86%, BS = 4) only included species of subgenus *Calceolaria* except for the two species of section *Micranthera*, previously assigned to subgenus *Cheiloncos* (Fig. 3).

In the strict consensus, all extratropical species formed a paraphyletic grade of two moderately supported clades at the base of the tree (clade I and II), and two species, *C. pinifolia* and *C. tenella* had an uncertain position (Fig. 3). Tropical species were retrieved as a well-supported monophyletic group (clade III, Fig. 3). Within tropical species, a moderately supported clade showed *Calceolaria tucumana* as the sister taxon of the remaining tropical species (jk = 75, BS = 5). Within this clade, three sets of species with the same geographic distribution and that mostly corresponded to the previous infrageneric classification were retrieved (Fig. 3). Group IIIA, included only species from southern Bolivia to central Peru, with the exception of *C. ericoides* from northern Peru and Ecuador; group IIIB, was retrieved in a more inclusive well-supported clade together with group IIIC (BBP = 0.95, jk = 79, BS = 3). The majority of species included in group IIIB are restricted to Peru; the exceptions are *C. dichotoma* from southern Peru to central Colombia, and *C. lamiiifolia* and *C. penlandii* from southern Colombia to northern Ecuador. Group IIIC, is a well-supported clade (BPP = 0.99, jk = 79, BS = 3) formed mainly by species from central Peru to the northern limit of *Calceolaria*. Only three sections in the tropical clade were retrieved as monophyletic: *Ericoides* (BBP = 1, jk = 85%, BS = 3), *Calceolaria* (BPP = 1, jk = 100%, BS > 7), and *Perfoliatae* (BPP = 0.96, jk = 79%, BS = 6).

Character evolution—A randomly chosen MPT from the “total evidence” analysis was used to visualize character evolution. A shrubby habit is a generalized condition within *Calceolaria*. The rosulate life-form evolved once and is a synapomorphic character of clade II, including almost all Patagonian species assigned to sect. *Rosula* (Figs. 3 and 4). The life form of perennial herb evolved independently at least nine times in different lineages, mainly in species located in the northern part of the distribution, while the annual herb life form is found in all species of section *Calceolaria*.

Several to many flowers per cyme is a general character in *Calceolaria*. A reduction in number to two flowers per cyme evolved nine times in different extratropical lineages; however, the extreme reduction to one flower per cyme was only observed in rosulate species (Fig. 4).

The loss of the elaiophore appears to be a secondary condition in *Calceolaria*, and its absence evolved independently in 14 lineages. The majority of elaiophore losses occurred in species located in the northern part of the distribution (Figs. 4 and 5).

Sternotribic pollination is exclusive to species in sect. *Rugosae* and to the tropical species *C. utricularioides*, while nototribic pollination is the most common mechanism within *Calceolaria*. This change in pollination mechanism appears to be associated with a shortening of filaments and styles and with a shift from a saccate to a nonsaccate upper lip (Figs. 4 and 5). The lever mechanism appeared associated with an unequal development of thecae and evolved in three evolutionary lineages within subgenus *Calceolaria*. This character is shared by all species of sect. *Calceolaria*.

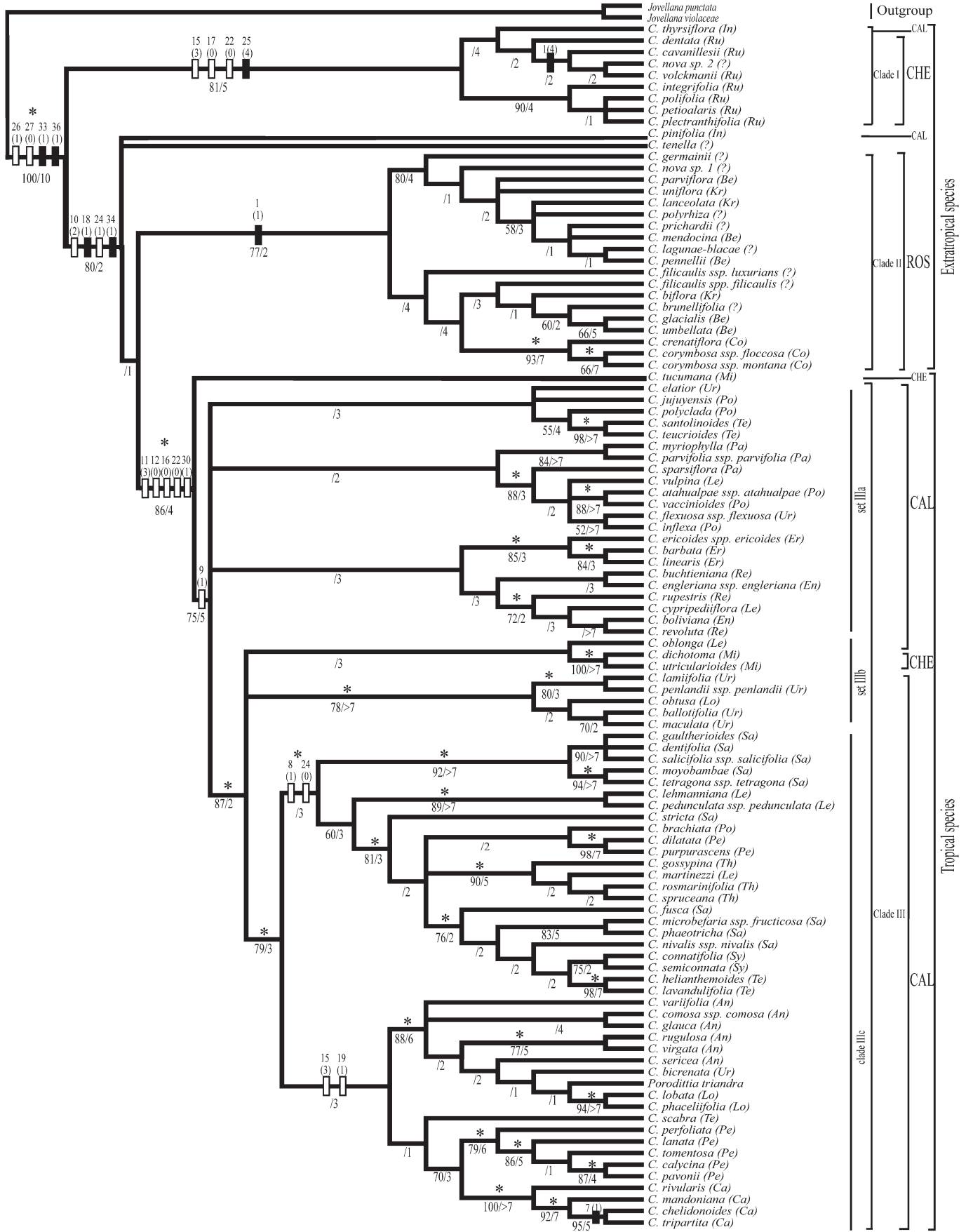
Data on chromosome number were available for 66 of the species studied (Molau, 1988; Ehrhart, 2000). The base chromosome number of Calceolariaceae is $x = 9$, except for species of sect. *Calceolaria* with a base number $x = 8$. All species nested in clade I are diploids ($2n = 18$), while in clade II almost all are diploids, but there are also tetraploids such as the two species of sect. *Kremastocheilos* (*C. uniflora* and *C. biflora*) and the species *C. filicaulis* ssp. *filicaulis* for which both diploids and tetraploids were recorded. On the other hand, almost all tropical species are tetraploid with $2n = 36$, the exception being *C. tucumana* (sect. *Micranthera*), which is a diploid species, two species of sect. *Calceolaria* (*C. chelidonoides* and *C. rivularis*), which are octoploids ($2n = 64$), *C. utricularioides* (sect. *Micranthera*), which is aneuploid ($2n = 30$), and the species *C. engleriana* (sect. *Engleriana*) and *C. tripartita* (sect. *Calceolaria*) for which both tetraploids and octoploids are known. Patterns of chromosome number are shown in Fig. 4.

Individuals of *Centris* are the most common oil-collecting bees visiting *Calceolaria* (Figs. 4 and 5), although shifts to *Chalepogenus* are common and usually associated with *Calceolaria* species occurring in moist habitats.

Biogeographical patterns—Distribution patterns that optimized onto one of the total evidence MPTs showed an important association between the phylogeny and the geographic distribution of *Calceolaria* species, suggesting an austral origin for *Calceolaria* and northward migration. The cladogram shows two bottom clades formed by species exclusively distributed in the southern region (Fig. 4). Three major evolutionary radiations are displayed, two in the southern Andes and extra-Andean Patagonia (i.e., the southern part of its distribution), and the other in the central and northern Andes (i.e., the middle and northern parts of its distribution). Only three species from the southern clade appear in the central Andes (Fig. 4). A single historical event explains the origin of the expansion from the south to the central area of distribution and the most important diversification of *Calceolaria*, while different parallel evolutionary lines show a northward expansion from the central to northern area of distribution across the Huancabamba Deflection.

Most of the species in the northern region are exclusively located north of the Huancabamba Deflection and are restricted to the southern and central areas of Ecuador, suggesting a diversification associated with this barrier. They were nested together in a clade (BPP = 0.87, jk = 60, BS = 3). Moreover, this clade appeared as the sister group of a clade (BBP = 1, jk = 92%, BS > 7) clustering species restricted to northern Peru, south of the

Fig. 2. The single most parsimonious tree retrieved by the molecular analysis (ITS + *matK*) (L = 647 steps, CI = 0.44, RI = 0.76). Numbers above branches indicate jackknife/Bremer support. Asterisks below branches indicate Bayesian posterior probabilities > 0.95 from 30 000 000 generations. Abbreviations in parentheses indicate genus section according to Molau (1988). Section and subgenus abbreviations as in Fig. 1.



Deflection (Fig. 4). This pattern of divergent areas of distribution for sister clades (i.e., north and south of the geographical barrier) suggests that allopatric speciation could have taken place.

DISCUSSION

In this study, a total evidence approach was used to reconstruct the evolutionary history of *Calceolaria*, one of the most characteristic genera of the Andean and Patagonian flora. Molecular and morphological data sets were combined for two reasons even though they were incongruent. Secondary phylogenetic signals emerge from both molecular and morphological characters sets when they are combined (Nixon and Carpenter, 1996). Moreover, combined analysis can improve the estimate by increasing the number of informative characters, revealing groups not seen in the trees generated with the separate data sets (Chippindale and Wiens, 1994).

Infrageneric groups—Traditionally, three subgenera with 24 sections were recognized within *Calceolaria* (Molau, 1988). The majority of the sections (19 of them) were placed in subgen. *Calceolaria*, which is mainly distributed in tropical regions (i.e., the central and northern part of its distribution), while in temperate South America two subgenera were recognized: *Cheiloncos* and *Rosula*. According to Molau (1988), in a few cases, species occur in other geographical zones outside the main distribution range of each subgenus. Such is the case for sect. *Micranthera* (subgenus *Cheiloncos*) with most of its species in the tropical zone and for sect. *Integerrimae* (subgenus *Calceolaria*) with most of its species in the southern temperate zone.

Our results suggest the need for a taxonomic revision at the level of sections because, for the most part, they were not retrieved as natural groups. By contrast, the classification of *Calceolaria* proposed by Molau (1988) into three subgenera is supported by our results, with some exceptions. *Calceolaria dichotoma* and *C. utricularioides* (sect. *Micranthera*, subgenus *Cheiloncos*) should be transferred to subgenus *Calceolaria*, and *C. thyrsoflora* (sect. *Integerrimae*, subgenus *Calceolaria*) to subgenus *Cheiloncos*. Moreover, morphological characters in the total evidence consensus retrieved the redefined subgen. *Cheiloncos* and subgen. *Rosula* supported by synapomorphic character states (i.e., long filaments and rosulate habit, respectively), while character states supporting subgenus *Calceolaria* clade were homoplastic (i.e., simple hairs on the adaxial and abaxial side of leaf, absence of glandular hairs on the leaf and calyx, closed flowers, and short styles). The redefined subgenus *Cheiloncos* was retrieved as the sister group of the remaining species, suggesting that this temperate group was an early lineage. These species are all diploids (Molau, 1988; Ehrhart, 1997, 2000) and possess the basic synflorescence type and habit (Sérsic and Cocucci, 1996b).

Despite differing from the rest of *Calceolaria* by having three stamens and a three-lobed corolla, *P. triandra* was retrieved nested within *Calceolaria*. Because the three-staminate

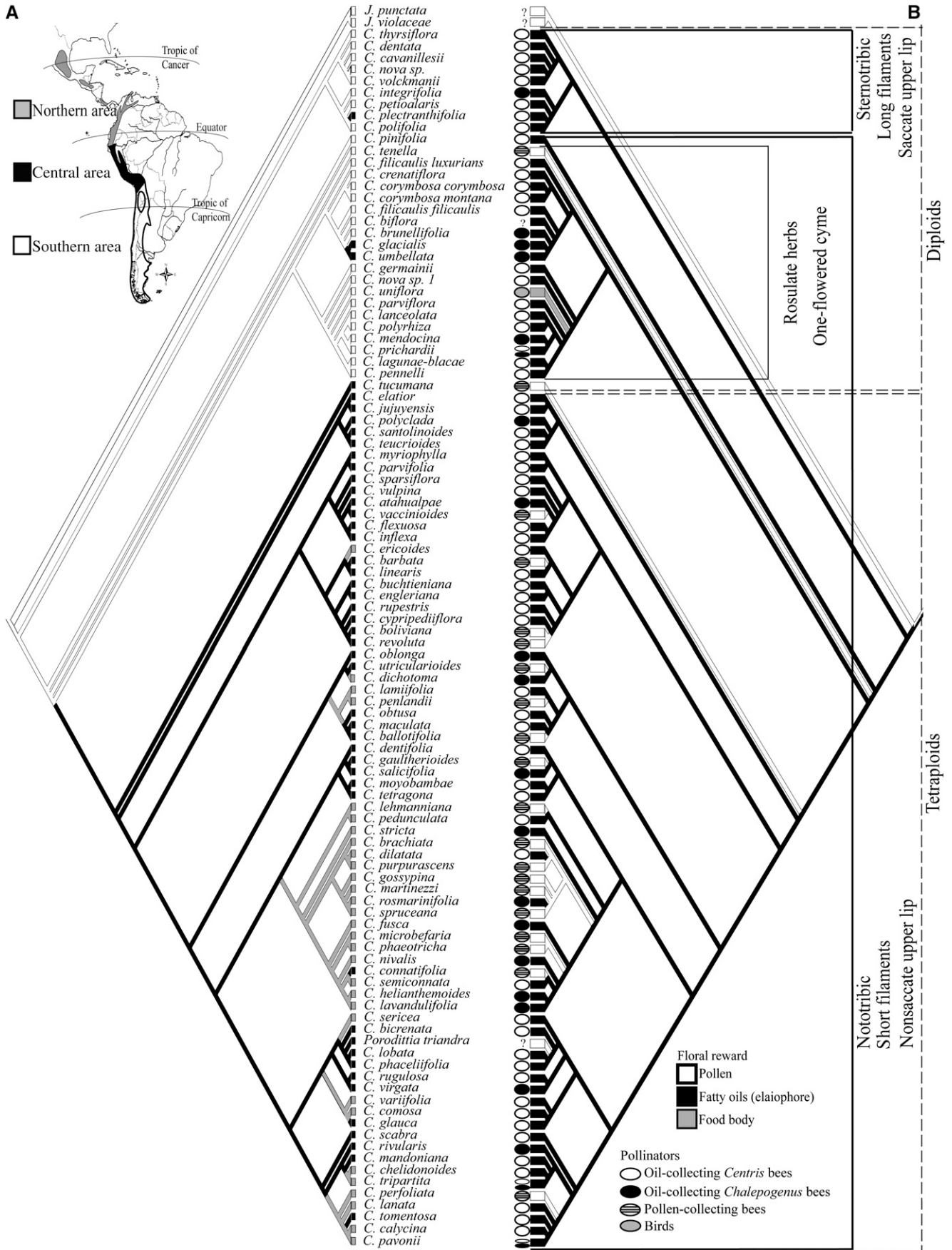
teratological flowers of *Calceolaria* are developmentally similar to those of *Porodittia* and considering that this monotypic genus has a narrow distribution sympatric with other *Calceolaria* species, the hypothesis of Mayr and Weber (2006) that *Porodittia* is a genetically fixed teratology seems probable. Therefore, we support Andersson's proposal (2006) that *Porodittia* be transferred as *C. triandra* (Cavanilles) Vahl., which has lost the two morphological synapomorphies of *Calceolaria*, i.e., the presence of an infolded lower corolla lip and fatty oils as floral reward.

Character evolution—It has been stressed that the origin of *Calceolaria* was concurrent with an association with oil-collecting bees (Molau, 1988; Sérsic, 2004), probably in the late Tertiary (Vogel, 1974), and that the absence of the elaiophore is a derived condition. Our phylogenetic hypothesis suggests that the association with oil-collecting bees was simultaneous with the evolutionary split between *Jovellana* and *Calceolaria*. The acquisition of the oil-producing trichomes is associated with strongly specialized pollination (e.g., Cocucci, 1991; Steiner and Whitehead, 1991; Johnson and Steiner, 2000; Sérsic, 2004). Interestingly, in *Calceolaria*, the repeated loss of the oil glands led to a less specialized pollination system resulting from the shift from oil- to pollen-rewarding flowers (Molau, 1988; Sérsic, 2004). The exception are the Patagonian species *C. uniflora* and *C. fothergillii* in which the loss of the oil glands led to a modified elaiophore in the form of a juicy food body associated with bird pollination (Sérsic and Cocucci, 1996a; Roitman et al., 2002; Sérsic, 2004).

Our results therefore suggest shifts of pollination systems in the groups of species distributed in areas where oil-collecting bees are scarce. At the southernmost part of the distributional range, there are two peculiar bird-pollinated species (Sérsic and Cocucci, 1996a), and at the northernmost part of the distributional range, most species lacking an elaiophore probably rely on self-pollination or on pollination by pollen-collecting bees (i.e., *Bombus*, *Xylocopa*). The frequent lack of elaiophore in the northern part of the distribution range is probably related to the scarcity of tropical oil-collecting bees at high elevations where northern *Calceolaria* species grow (up to 4700 m a.s.l.) (Molau, 1988).

Furthermore, different pollen deposition mechanisms (nototriby vs sternotriby) are related to distinctive morphologies because the long and slender stamen filament is associated with sternotribic pollination, while the short and thick filament is associated with nototribic pollination (Sérsic, 2004). Nototribic pollination is the most common mechanism in *Calceolaria* and appears to have evolved once, while sternotriby is mostly restricted to a temperate, early-branching clade. The shift from a sternotribic to nototribic mechanism could be seen as a trend to a more advantageous pollination because pollen is deposited in the dorsal part of pollinators, and it is therefore less susceptible to being groomed out (Thorpe, 2000). It seems that the nototribic pollination mechanism may represent a key innovation facilitating diversification within *Calceolaria* as suggested for other angiosperms (Givnish, 1997; Westerkamp and Claßen-Bockhoff, 2007).

← Fig. 3. Strict consensus tree for five most parsimonious trees retrieved by the combined analysis of molecular and morphological data (L = 1357 steps, CI = 0.36, RI = 0.67). Character numbers are above branches, together with character state numbers in parentheses. Numbers below branches indicate jackknife/Bremer support. Asterisks above branches indicate Bayesian posterior probabilities >0.95 from 30 000 000 generations. Black rectangles are synapomorphic character states, open rectangles are homoplastic character states. Section and subgenus abbreviations as in Fig. 1.



Growth habit is diverse and contrasting in *Calceolaria*. A shrubby habit is the most widespread in the genus. A loss of woody habit occurred at least in 10 lineages. The annual habit is restricted to the species of sect. *Calceolaria* and sect. *Micranthera* and evolved from perennial habit at least in three independent lineages, probably associated with a shift in habitat conditions since these annual herbs are common in moist sites in the high Andes. Molau (1988) suggested that the weedy habit (in part associated with reduction in flower size, self-fertilization, and high seed production) was a parallelism among these sections, a view that is supported by our results. This transition in growth form, which seems to be associated with some reproductive assurance mechanisms (e.g., self-fertilization), is probably related to a more general evolutionary pattern in plants, which suggests that environments with low survival of parents and high survival of seedlings favor annual against perennial habit (Fenner and Thompson, 2005).

Regarding chromosome number, it is interesting that all diploid species are clustered in the two southern clades, with the exception of *C. tucumana*, the sister taxon of all tropical species. All tetraploids and octoploids are nested in the cluster of tropical species, with the exception of three southern species, *C. filicaulis* ssp. *filicaulis*, for which both diploid and tetraploid morphs are known, and the tetraploids *C. uniflora* and *C. biflora* (Ehrhart, 2000). Thus, polyploidy seems to have evolved independently at least in three lineages. The transition to autogamy in the species of the tropical zone probably relates to the higher frequency of polyploidy, a pattern also observed in many groups of angiosperms (Barringer, 2007). A few aneuploids are known in *Calceolaria*, suggesting that aneuploidy has played a relatively small role in the evolution of persistent lineages. As a general pattern in the genus, aneuploidy appears to be restricted to tropical species with annual habit (Molau, 1988). A correlation between these character states (i.e., aneuploidy and annual habit) was also found in other groups of plants and would be related to general ecological characters of annual species that promote the fixation of chromosomal variants (Levin and Wilson, 1976; Abou-El-Enain, 2002; Ellison et al., 2006).

There is uncertainty about which oil-collecting bee genus first established a relationship with *Calceolaria*. Vogel (1974) indicated that it was *Chalepogenus*, while Sérsic (2004) postulated that the association with the two genera was nearly simultaneous. Our results suggest that the interaction was first established with *Centris* bees. A preponderance of *Centris*-pollinated species is evident along the entire tree, with consistently unidirectional shifts from *Centris* to *Chalepogenus* flower types. This leap-frog pattern is concurrent with migration to humid habitats, where *Chalepogenus* bees are apparently more abundant (Roig-Alsina 1999, 2000). Except for *Calceolaria integrifolia*, the small *Chalepogenus* bees only interact with nototribic flowers, while the larger *Centris* bees are able to manipulate nototribic as well as sternotribic flowers. Sternotribic flowers are characterized by their long stamens and styles that are enclosed completely by both inflated lips, which tighten together such that a certain degree of strength and ability is

required to open them and gain access to flower rewards (Sérsic, 1991, 2004; Rasmussen and Olesen, 2000).

The most remarkable changes in reproductive characters in *Calceolaria* such as the shift from sternotribic to nototribic mechanism, from bee to bird pollination, from long to short stamens, from many flowered to single-flowered cymes are observed in the southernmost clades. Moreover, species from Patagonia and southern Andes have the four pollination systems reported for the genus (i.e., bird pollination with a food body as reward, bee pollination with pollen as reward, and oil-producing flowers with nototribic and sternotribic pollination mechanisms). This pattern reinforces the idea that Patagonia and southern Andes are highly diverse areas, in terms of species diversity and interaction complexity, where particular floral and vegetative traits have evolved.

Geographical and historical context—Calceolariaceae is an Austral-Antarctic phylogeographical element (Cleef, 1979; Molau, 1988). Our results retrieved three main lineages, a northern lineage along the Central and Northern Andes and two southern lineages. The hypothesis of the origin of *Calceolaria* in southern Andes was proposed according to centers of diversity and cytological studies (Molau, 1988; Ehrhart, 1997, 2000; Sérsic, 2004). Our results support this hypothesis, suggesting an origin particularly in southern Chile, which seems to be consistent with the distribution of the sister genus *Jovellana*. The progressive elevation of the central Andes, together with climate changes that occurred after the mid-Miocene, may have produced an extension of the cooler climate of northern Patagonia to lower latitudes, allowing the dispersal of *Calceolaria* species toward the central and northern Andes, as also suggested for some groups of plants and animals (e.g., Simpson, 1975; Ezcurra, 2002; Doan, 2003). For many of these organisms, the Huancabamba Deflection in northern Peru represents the northern limit of their distribution, while in *Calceolaria* a few lineages were able to disperse over this barrier (Molau, 1988). During glaciation periods, the Huancabamba Deflection would have been covered by high-Andean vegetation, allowing migration across the depression, while during warmer interglacial periods, the aridization of this region became a strong barrier to migrations of high-Andean organisms (Simpson, 1975; Molau, 1988; Weigend, 2002). Because there are no adaptations to long-distance dispersal in Calceolariaceae, this corridor may have been an important factor in the evolutionary history of *Calceolaria*. Based on our results, a series of dispersal events occurred in both directions from the Huancabamba Deflection and one of them (or at most two) was particularly important because it explains the origin of almost all of the species located exclusively north of the deflection. Moreover, these species appeared as the sister group of a clade clustering species restricted to northern Peru, south of the deflection. This pattern of divergent distribution areas of sister clades (i.e., north and south of the geographical barrier) suggests a diversification process associated to this major depression in the high Andes.

← Fig. 4. One of the most parsimonious trees retrieved by the total evidence analysis with unambiguous optimization of (A) geographic distribution and (B) floral reward. Shading indicates in (A) white for southern area, black for the central area, gray for northern area; in (B) black is for presence of elaiophore, white for its absence and gray is for food body. Known and inferred pollinators for each species are also plotted using circles next to the taxon: black and white for *Chalepogenus* sp. and *Centris* sp. oil-collecting bees, respectively; hatched for pollen-collecting bees (e.g., *Xylocopa* sp., *Bombus* sp.); gray for bird-pollinated species; a question mark for pollinator status ambiguous. External boxes: ploidy levels (dashed lines), pollen deposition mechanism and feature of the upper lip (thick solid line) and rosulate species with reduced number of flowers per cyme (thin solid line).

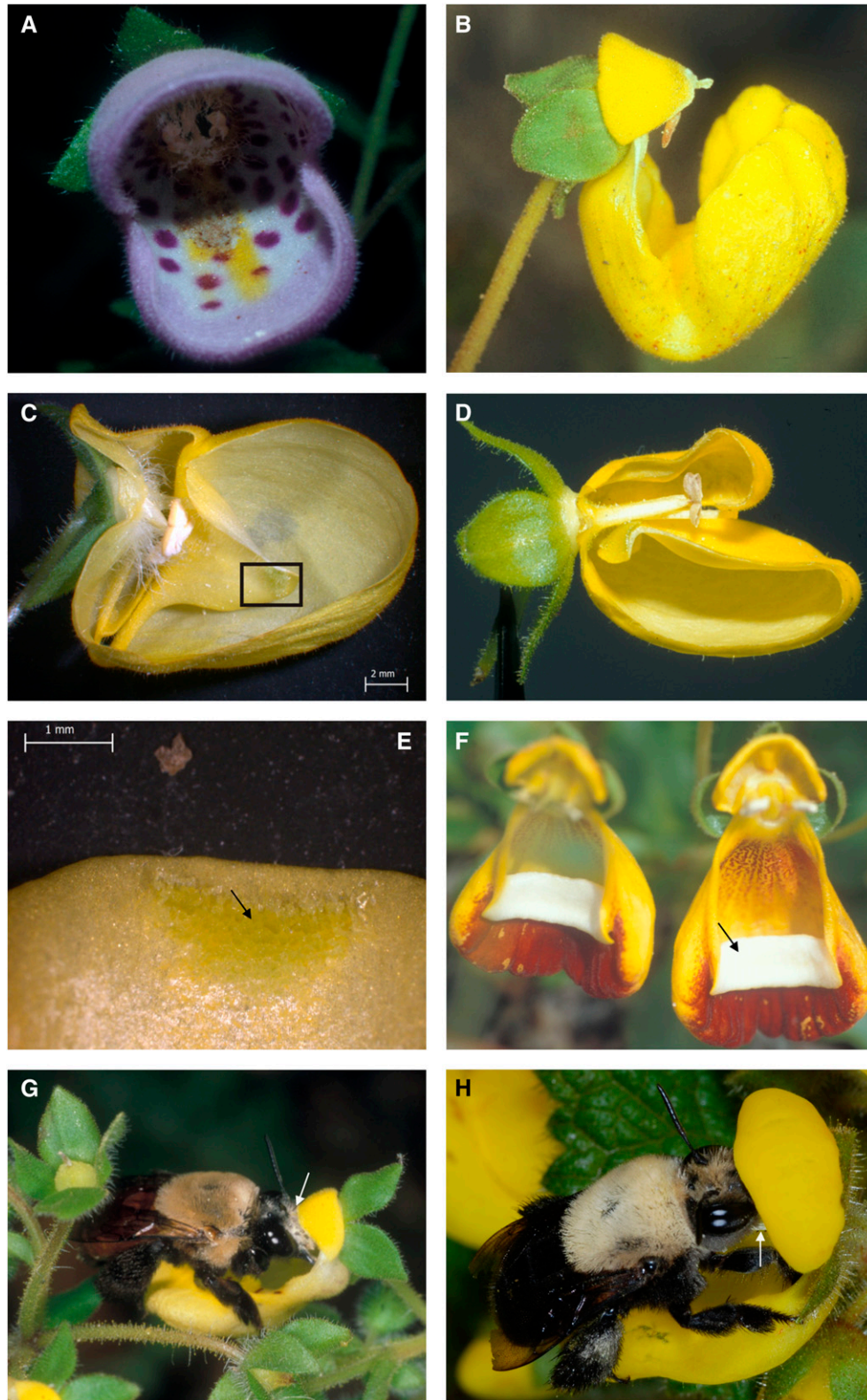


Fig. 5. Flowers of *Jovellana* (A) and *Calceolaria* (B–H). (A) *J. violaceae*. (B) Lateral view of *C. polyrhiza*, representative of the nototribic pollination mechanism. (C) Cross section of *C. elatior* showing features of its nototribic mechanism flower with nonsaccate upper lip and a saccate lower lip. The rectangle marks the area with the infolded lower corolla lip bearing the elaiophore. (D) Cross section of *C. dentata* showing a typical sternotribic flower with saccate upper and lower corolla lips. (E) Detail of infolded lower corolla lip bearing the elaiophore (arrow). (F) Flowers of the bird-pollinated *C. uniflora* showing the rewarding food body (arrow). (G) *Centris tricolor* pollinating *C. parviflora*. Note the stigma and stamens making contact with the dorsal part of the bee's head. (H) *Centris neffii* pollinating *C. plectranthifolia*. Note the fertile parts touching the ventral part of the bee's head.

Diversification in Calceolaria—*Calceolaria* is among the largest oil-producing genera. The low proportion of variable sites in *matK* and in particularly in the ITS sequences compared to other groups (e.g., Plunkett et al., 1997; Alvarez and Wendel, 2003) along with the numerous taxa of the genus, seem to support the idea of recent diversification. The high degree of inter-specific crossability (Molau, 1981, 1988; Ehrhart 2000, 2005) also supports the hypothesis of morphologically highly distinct, but young, lineages within *Calceolaria*; natural interspecific hybrids have been reported (Molau, 1981, 1988; Brücher, 1989; Ehrhart, 2005), even between species with different pollination syndromes (Sérsic et al., 2001). Even though hybridization can cause incongruence between trees inferred from nuclear vs. chloroplast DNA sequences (Wendel and Doyle, 1998), in our data set, there were no conflicts among ITS and *matK* tree topologies.

Ecogeographic isolation seems to be the main barrier to gene flow and, consequently, is the primary speciation mechanism (Molau, 1988; Ehrhart, 2005). Most *Calceolaria* species have a narrow distribution or are endemic to single valleys of the Andean mountain range, so rapid allopatric differentiation may have taken place. Similarly, previous studies of two Andean genera (Hughes and Eastwood, 2006; Ackermann et al., 2008) also reported an extraordinary rate of speciation, resulting in a large number of geographically isolated but closely related species, a phenomenon referred to as an island radiation on a continental scale.

The presence of elaiophores seems to be a key innovation favoring the evolutionary success of the genus, relative to its sister genus *Jovellana*. Over its distribution range, *Calceolaria* is associated with many different species of oil-collecting bees, and locally the association is often with a single bee species (Sérsic, 2004). This pattern of high pollinator specificity at the local scale has been reported in three different systems of oil-producing flowers (Steiner and Whitehead, 1990, 1991; Rasmussen and Olesen, 2000; Cosacov et al., 2008). This phenomenon suggests that these locally specialized interactions promote speciation through local evolution, reinforcing the idea that the diversification of this Andean genus is probably the result of local differentiation (Molau, 1988; Thompson, 1994; Rasmussen and Olesen, 2000). In addition, shifts between strobilic and notrobilic pollination suggests that speciation would be further promoted by floral as well as geographic barriers (Grant, 1994).

Conclusions—In this study, molecular and morphological evidence was used to reconstruct the evolutionary history of *Calceolaria*. Results partially support the previous infrageneric classification. Subgenus *Rosula* was retrieved as a monophyletic group supported by the synapomorphic character state rosulate habit. Our study also indicates the need to transfer *C. dichotoma* and *C. utricularioides* from subgenus *Cheiloncos* to subgenus *Calceolaria*, and *C. thyrsoflora* and *C. pinifolia* from subgenus *Calceolaria* to subgenus *Cheiloncos*. In addition, the formerly proposed transference of *Porodittia triandra* to *Calceolaria* is supported. Further phylogenetic studies that incorporate additional molecular markers are needed to clarify the circumscription of the sections. Diversification in *Calceolaria* was well correlated with geographic and geological processes, as evidenced by the high structure of the major lineages, with almost no geographic overlap. The acquisition of the elaiophore and a notrobilic pollination mechanism are important aspects of the evolutionary history in *Calceolaria*. The rosulate habit in

Patagonian species, the repeated loss of the oil gland related to shifts in pollinators (pollen-collecting bees and birds) and self-pollination in the northern species, as well as polyploidization, were also remarkable events in the evolutionary history of this Andean genus.

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APPENDIX 1. Species, vouchers for molecular and morphological analyses, their locations, GenBank accession numbers, and literature citations for the previously published sequences of DNA used in this paper (ITS; *matK*).

Taxon: Collection location, *Voucher* (Herbarium), GenBank accessions: ITS, *matK*, Citation.

- Calceolaria atahualpae* Kränzl. **ssp. Atahualpae**; Bolivia, Beck & Seidel 14454 (GB), AJ579420, AJ579813, Andersson (2006). *Calceolaria ballotifolia* Kränzl.; Peru, Schmidt-Lebuhn 474 (GB), AJ579434, AJ579812, Andersson (2006). *Calceolaria barbata* Molau; Peru, Molau & Eriksen 3362 (GB), AJ579409, —, Andersson (2006). *Calceolaria bicrenata* Ruiz & Pav.; Peru, Becker & Terrones 995 (GB), AJ579422, AJ580491, Andersson (2006). *Calceolaria biflora* Lam.; Argentina, Cocucci & Sérsic 1225 (CORD), FJ527784, FJ527805. *Calceolaria boliviana* (Rusby) Pennell.; Bolivia, Schmidt-Lebuhn 593 (GB), AJ579404, AJ579815, Andersson (2006). *Calceolaria brachiata* Sodiro ex Kränzl.; Ecuador, Steiner 234 (GB), AJ579462, —, Andersson (2006). *Calceolaria brunellifolia* Phil.; Argentina, Cocucci & Sérsic 2249 (CORD), FJ527795, —. *Calceolaria buchtieniana* Kränzl.; Bolivia, Moraes 686 (GB), AJ579411, AJ579816, Andersson (2006). *Calceolaria calycina* Benth.; Peru, Schmidt-Lebuhn 481 (GB), AJ579444, AJ579802, Andersson (2006). *Calceolaria cavanillesii* Phil.; Chile, Cocucci & Sérsic 2314 (CORD), FJ527791, —. *Calceolaria chelidonioides* HBK. Peru, Schmidt-Lebuhn 527 (GB), AJ579441, AJ579796, Andersson (2006). *Calceolaria comosa* Pennell **ssp. comosa**; Peru, Schmidt-Lebuhn 482 (GB), AJ579427, AJ579803, Andersson (2006). *Calceolaria connatifolia* Pennell.; Peru, Schmidt-Lebuhn 473 (GB), AJ579469, AJ579823, Andersson (2006). *Calceolaria corymbosa* Ruiz & Pav. **ssp. montana**; Argentina, Böcher & al. 1322 (C), AJ579393, —, Andersson (2006). *Calceolaria corymbosa* Ruiz & Pav. **ssp. floccosa**; Argentina, Cocucci & Sérsic 4136 (CORD), FJ527790, J527806. *Calceolaria crenatiflora* Cav.; Argentina, Cocucci & Sérsic 3423 (CORD), FJ527786, —. *Calceolaria cypripediiflora* Kränzl.; Peru, Molau 752 (GB), AJ579405, —, Andersson (2006). *Calceolaria dentata* Ruiz & Pav.; Argentina, Cocucci & Sérsic 952 (CORD), FJ527801, —. *Calceolaria dentifolia* Edwin.; Peru, Schmidt-Lebuhn 477 (GB), AJ579450, AJ579794, Andersson (2006). *Calceolaria dichotoma* Lam.; Ecuador, Harling & Andersson 18585 (GB), AJ579437, —, Andersson (2006). *Calceolaria dilatata* Benth.; Ecuador, Løjtmant & Molau 13950 (GB), AJ579464, AJ580492, Andersson (2006). *Calceolaria elatior* Griseb.; Argentina, Cocucci & Sérsic 1173 (CORD), FJ527788, —. *Calceolaria engleriana* Kränzl. **ssp. engleriana**; Peru, Molau & Öhman 1602 (GB), AJ579401, AJ579817, Andersson (2006). *Calceolaria ericoides* Vahl **ssp. ericoides**; Ecuador, Molau & Öhman 1138 (GB), AJ579408, AJ579825, Andersson (2006). *Calceolaria filicaulis* Clos. **ssp. filicaulis**; Argentina, Böcher & al. 1811 (C), AJ579386, —, Andersson (2006), Cocucci & Sérsic 2340 (CORD), —, FJ527808. *Calceolaria filicaulis* Clos. **ssp. luxurians** (Witasek); Chile, Rahn & Ødum 4790 (C), AJ579394, —, Andersson (2006); Argentina, Cocucci & Sérsic 2229 (CORD), —, FJ527807. *Calceolaria flexuosa* Ruiz & Pav. **ssp. flexuosa**; cultivated, C, Molau s.n., AJ579415, AJ579793, Andersson (2006). *Calceolaria fusca* Pennell. Ecuador, Øllgaard & al. 74155 (GB), AJ579466, AJ579824, Andersson (2006). *Calceolaria gaultherioides* Molau. Peru, Molau & Eriksen 3410 (GB), AJ579451, AJ579798, Andersson (2006). *Calceolaria germanii* W.t.; Argentina, Böcher & al. 1579 (C), AJ579387, —, Andersson (2006), Cocucci & Sérsic 3424 (CORD), —, FJ527815. *Calceolaria glacialis* Wedd.; Argentina, Cocucci & Sérsic 2668 (CORD), FJ527803, —. *Calceolaria glauca* Ruiz & Pav.; Peru, Schmidt-Lebuhn 519 (GB), AJ579423, AJ579805, Andersson (2006). *Calceolaria gossypina* Benth.; Ecuador, Franquemont 180A (GB), AJ579459, —, Andersson (2006). *Calceolaria helianthemoides* HBK.; Ecuador, Elleman 91661 (GB), AJ579461, —, Andersson (2006). *Calceolaria inflexa* Ruiz & Pav. Peru, Molau 777 (GB), AJ579418, —, Andersson (2006). *Calceolaria integrifolia* L. Chile, Steward 34 (C), AJ579396, —, Andersson (2006); *Calceolaria jujuyensis* Botta.; Argentina, Cocucci & Sérsic 1170 (CORD), FJ527789, —. *Calceolaria lagunae-blancae* Kränzl.; Argentina, Cocucci & Sérsic 978 (CORD), FJ527793, FJ527810. *Calceolaria lamifolia* HBK.; Ecuador, Schmidt-Lebuhn 360 (GB), AJ579435, —, Andersson (2006). *Calceolaria lanata* HBK.; cultivated, GBG, S. Andersson 3 (GB), AJ579447, AJ579826, Andersson (2006). *Calceolaria lanceolata* Cav.; Argentina, Cocucci & Sérsic 3580 (CORD), FJ527797, FJ527809; *Calceolaria lavandulifolia* HBK.; Ecuador, Harling 27232 (GB), AJ579460, AJ579818, Andersson (2006). *Calceolaria lehmannina* Kränzl.; Ecuador, Løjtmant & al. 12589 (GB), AJ579454, —, Andersson (2006). *Calceolaria linearis* Ruiz & Pav.; Peru, Molau & Eriksen 3481 (GB), AJ579410, —, Andersson (2006). *Calceolaria lobata* Cav.; Bolivia, Luteyn & Dorr 13783 (GB), AJ579428, AJ579791, Andersson (2006). *Calceolaria maculata* Edwin; Peru, Molau 891 (GB), AJ579433, AJ579810, Andersson (2006). *Calceolaria mandoniana* Kränzl.; Bolivia, Schmidt-Lebuhn 596 (GB), AJ579442, AJ579808, Andersson (2006). *Calceolaria martinezii* Kränzl.; Ecuador, Schmidt-Lebuhn 392 (GB), AJ579456, AJ579800, Andersson (2006). *Calceolaria mendocina* Phil.; Argentina, Cocucci & Sérsic 127 (CORD), FJ527800, FJ527811. *Calceolaria microbefaria* Kränzl. **ssp. fruticosa** (Pennell) Molau.; Ecuador, Holm-Nielsen & Øllgaard 24344 (GB), AJ579468, —, Andersson (2006). *Calceolaria moyobambae* Kränzl.; Peru, Schmidt-Lebuhn 478 (GB), AJ579449, AJ579827, Andersson (2006). *Calceolaria myriophylla* Kränzl.; Peru, Schmidt-Lebuhn 588 (GB), AJ579403, AJ579828, Andersson (2006). *Calceolaria nivalis* HBK. **ssp. nivalis**.; Ecuador, Harling 25373 (GB), AJ579465, —, Andersson (2006). *Calceolaria oblonga* Ruiz & Pav. Peru, Schmidt-Lebuhn 552 (GB), AJ579414, AJ579821, Andersson (2006). *Calceolaria obtusa* Molau; Ecuador, Azuay, Harling & al. 15074 (GB), AJ579432, —, Andersson (2006). *Calceolaria parviflora* Wedd.; Argentina, Cocucci & Sérsic 206 (CORD), FJ527802, FJ527812. *Calceolaria parvifolia* Wedd.; Bolivia, Gutte 86 (GB), AJ579412, —, Andersson (2006). *Calceolaria pavonii* Benth.; Peru, Schmidt-Lebuhn 459 (GB), AJ579445, AJ579814, Andersson (2006). *Calceolaria pedunculata* Molau **ssp. pedunculata**; Ecuador, Schmidt-Lebuhn 355 (GB), AJ579453, AJ579809, Andersson (2006). *Calceolaria penlandii* Pennell **ssp. penlandii**; Ecuador, Molau & Eriksen 2173 (GB), AJ579436, AJ579807, Andersson (2006). *Calceolaria pennellii* Desc. & Bors.; cultivated, GBG, S. Andersson 1 (GB), AJ579389, AJ580488, Andersson (2006). *Calceolaria pefoliata* L.f.; Ecuador, Løjtmant & Molau 12857 (GB), AJ579443, AJ579797, Andersson (2006). *Calceolaria petiolaris* Cav.; Chile, Cocucci & Sérsic 352 (CORD), FJ527794, —. *Calceolaria phaceliifolia* Edwin; Peru, Schmidt-Lebuhn 501 (GB), AJ579429, AJ579831, Andersson (2006). *Calceolaria phaeotricha* Molau; Ecuador, Molau & al. 2929 (GB), AJ579470, AJ579830, Andersson (2006). *Calceolaria pinifolia* Cav.; Argentina, Pedersen 15255 (C), AJ579392, —, Andersson (2006). *Calceolaria plectranthifolia* Walp.;

cultivated, GBG, *Molau s.n.* (GB), AJ579398, —, Andersson (2006). *Calceolaria polifolia* Hook; Chile, *Steward 32* (C), AJ579397, —, Andersson (2006). *Calceolaria polyclada* Kränzl.; Bolivia, *Killeen 2712*, AJ579402, —, Andersson (2006). *Calceolaria prichardii* (Rendle) Kränzl.; Argentina, *Cocucci & Sérsic 969* (CORD), FJ527798, FJ527813. *Calceolaria purpurascens* (Kränzl.) Molau; Ecuador, *Molau & al. 2441* (GB), AJ579463, AJ579792, Andersson (2006). *Calceolaria revoluta* Pennell.; Peru, *Molau 760* (GB), AJ579407, —, Andersson (2006). *Calceolaria rivularis* Kränzl.; Bolivia, *Beck & Seidel 14535* (GB), AJ579440, —, Andersson (2006). *Calceolaria rosmarinifolia* Lam.; Ecuador, *Harling & Ståhl 27035a* (GB), AJ579457, AJ579804, Andersson (2006). *Calceolaria rugulosa* Edwin.; Peru, *Schmidt-Lebuhn 409* (GB), AJ579430, AJ579795, Andersson (2006). *Calceolaria rupestris* Molau; Peru, *Schmidt-Lebuhn 560* (GB), AJ579406, AJ579811, Andersson (2006). *Calceolaria sp. nova I*; Argentina, *Cocucci & Sérsic 3580* (CORD), FJ527785, —. *Calceolaria sp. nova II*; Argentina, *Cocucci & Sérsic 3408* (CORD), FJ527799, —. *Calceolaria salicifolia* Ruiz & Pav. subsp. *salicifolia*; Peru, *Molau 825* (GB), AJ579452, —, Andersson (2006). *Calceolaria santolinoides* Kränzl.; Argentina, *MNI97* (CORD), FJ527787, —. *Calceolaria scabra* Ruiz & Pav.; Peru, *Molau 802* (GB), AJ579421, —, Andersson (2006). *Calceolaria semiconnata* Pennell.; Ecuador, *Harling & Andersson 22050* (GB), AJ579467, —, Andersson (2006). *Calceolaria sericea* Pennell.; Ecuador, *Schmidt-Lebuhn 334* (GB), AJ579426, —, Andersson (2006). *Calceolaria sparsiflora* Kuntze.; Bolivia, *Beck 3019* (GB), AJ579417, AJ579829, Andersson (2006). *Calceolaria spruceana* Kränzl.; Ecuador, *Schmidt-Lebuhn 390* (GB), AJ579458, AJ579799, Andersson (2006). *Calceolaria stricta* HBK.; Ecuador, *Harling & Andersson 14086* (GB), AJ579455, —, Andersson (2006). *Calceolaria tenella* Poepp. & Endl.; Argentina, *Cocucci & Sérsic*

524 (CORD), FJ527796, —. *Calceolaria teucroides* Griseb. Bolivia, *Molau 710* (GBG), AJ579413, —, Andersson (2006). *Calceolaria tetragona* Benth. subsp. *tetragona*.; Peru, *Schmidt-Lebuhn 471* (GB), AJ579448, AJ579801, Andersson (2006). *Calceolaria thyriflora* Grah.; Chile, *Steward 23* (C), AJ579395, —, Andersson (2006). *Calceolaria tomentosa* Ruiz & Pav.; Peru, *Schmidt-Lebuhn 420* (GB), AJ579446, AJ579820, Andersson (2006). *Calceolaria tripartita* Ruiz & Pav.; Ecuador, *Jørgensen & al. 5* (GB), AJ579439, —, Andersson (2006). *Calceolaria tucumana* Des.; Bolivia, Mendoza, *Molau 708* (GB), AJ579399, AJ579806, Andersson (2006). *Calceolaria umbellata* Wedd.; Argentina, *Bothmer & Hjerting 6341* (C), AJ579384, —, Andersson (2006). *Calceolaria uniflora* Lam.; cultivated, GBG, *S. Andersson 2* (GB), AJ579388, AJ580489, Andersson (2006). *Calceolaria utricularioides* Benth.; Peru, *Molau & al. 1800* (I) (GB), AJ579438, —, Andersson (2006). *Calceolaria vaccinioides* Kränzl.; Bolivia, Luteyn & Dorr 13485 (GB), AJ579419, AJ580490, Andersson (2006). *Calceolaria variifolia* Edwin.; Ecuador, *Schmidt-Lebuhn 405* (GB), AJ579425, —, Andersson (2006). *Calceolaria virgata* Ruiz & Pav.; Peru, *Schmidt-Lebuhn 483* (GB), AJ579431, AJ579819 Andersson (2006). *Calceolaria volckmanii* Phil.; Argentina, *Cocucci & Sérsic 3418* (CORD), FJ527792, FJ527814. *Calceolaria vulpina* Kränzl.; Peru, *Lourteig 3138* (GB), AJ579416, —, Andersson (2006). *Jovellana punctata* Ruiz & Pav.; Chile, *Cocucci & Sérsic 959* (CORD), FJ527783, FJ527804. *Jovellana violacea* (Cav.) G. Don; cultivated, GBG, *S. Andersson 4* (GB), AJ579385, AJ580487, Andersson (2006). *Kohleria spicata* (Kunth) Oerst., cultivated, GBG, *S. Andersson 5* (GB), AJ579471, AJ580486, Andersson (2006). *Kohleria rugata* (Scheidw.) L.P. Kvist & L.E. Skog, *Zimmer & al.*, AY047075, —, Andersson (2006). *Porodittia triandra* (Cav.) G. Don. Peru, *Molau & Erikson 3486* (GB), AJ579424, AJ579832, Andersson (2006).

APPENDIX 2. Morphological characters used in the phylogenetic analyses.

1. Habit (CI = 26; RI = 75): (shrub 0, rosulate 1, annual herb 2, perennial herb 3, subrosulate 4)
2. Petiole (CI = 16; RI = 54): (absent 0, present 1)
3. Petiole pilosity (CI = 10; RI = 50): (absent 0, present 1)
4. Petiole wings (CI = 16; RI = 58): (absent 0, present 1)
5. Leaf margin (CI = 7; RI = 35): (entire 0, toothed/serrate 1, crenate 2)
6. Leaf venation (CI = 5; RI = 46): (absent 0, revolute 1)
7. Leaf divisions (CI = 33; RI = 50): (simple 0, pinnate 1, lobate 2)
8. Leaf texture (CI = 13; RI = 62): (membranaceous 0, coriaceous/subcoriaceous 1, succulent 2)
9. Leaf base (CI = 12; RI = 54): (truncate 0, acute 1, cordate 2, attenuate 3, blunt 4)
10. Leaf shape (CI = 11; RI = 43): (oblong-lanceolate 1, ovate-elliptic 2, triangular 3, intermediate (between 1 and 2) 4; linear-lanceolate 5)
11. Leaf hairs simple (CI = 11; RI = 34): (absent 0, adaxial 1, abaxial 2, both sides 3)
12. Leaf hairs glandular (CI = 11; RI = 45): (absent 0, adaxial 1, abaxial 2, both sides 3)
13. Calyx marginal hairs (CI = 5; RI = 56): (absent 0, present 1)
14. Calyx margin (CI = 25; RI = 50): (entire 0, toothed 1)
15. Calyx hairs simple (CI = 9; RI = 51): (absent 0, adaxial 1, abaxial 2, both sides 3)
16. Calyx hairs glandular (CI = 10; RI = 50): (absent 0, adaxial 1, abaxial 2, both sides 3)

17. Upper lip (CI = 33; RI = 80): (saccate 0, nonsaccate 1)
18. Upper lip length (CI = 14; RI = 62): ($\geq 1/3$ of lower lip 0, $< 1/3$ of lower lip 1)
19. Lower lip position (CI = 10; RI = 45): (horizontal 0, bent upward 1, vertical 2)
20. Lower lip outline (CI = 10; RI = 44): (inflated 0, flat 1)
21. Throat (CI = 7; RI = 50): ($<$ instep 0, \geq instep 1)
22. Opening (CI = 5; RI = 64): (closed 0, opened 1)
23. Color (CI = 23; RI = 16): (yellow 0, lemon-yellow 1, white 2, violet 3)
24. Color variegation (CI = 5; RI = 68): (absent 0, present 1)
25. Filament length (CI = 13; RI = 52): (0–4 categories)
26. Theca position (CI = 8; RI = 18): (deflexed 0, divaricate 1, ascendent 2)
27. Theca size (CI = 8; RI = 54): (equal 0, unequal 1)
28. Confluent thecae (CI = 5; RI = 37): (present 0, absent 1)
29. Lever mechanism (CI = 20; RI = 63): (absent 0, present 1)
30. Style length (CI = 16; RI = 48): (0–3 categories)
31. Fruit hairs simple (CI = 7; RI = 75): (absent 0, present 1)
32. Fruit hairs glandular (CI = 7; RI = 36): (absent 0, present 1)
33. Reward (CI = 11; RI = 27): (absent 0, elaiophore 1, food body 2)
34. Pollen deposition (CI = 50; RI = 90): (sternotribic 0, nototribic 1)
35. Number of flowers/cyme (CI = 16; RI = 33): (one 0, two 1, more than two 2)
36. Infolded lobe (CI = 50; RI = 50): (absent 0, present 1)