Phylogeny and floral trait evolution in *Jaborosa* (Solanaceae)

Marcela Moré, Andrea A. Cocucci, Alicia N. Sérsic & Gloria E. Barboza

Instituto Multidisciplinario de Biología Vegetal (Universidad Nacional de Córdoba - CONICET), CC 495, CP 5000, Córdoba, Argentina
Author for correspondence: *Marcela Moré, mmore@efn.uncor.edu*
ORCID: MM, http://orcid.org/0000-0003-2982-8939; AAC. http://orcid.org/0000-0002-3464-3309;
GEB, http://orcid.org/0000-0003-1085-036X

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Abstract The genus *Jaborosa* (Solanaceae), which comprises 22 species endemic to southern South America, encompasses remarkable flower variation. To test if this interspecific variation is related to transitions in pollination mode and to major concomitant geological changes, phylogenetic relationships within the genus were reconstructed. To determine when such major transitions in flower traits occurred, divergence times were estimated and the evolution of relevant floral traits was studied. Sequences of four plastid spacer regions (*trnH-pbsA*, *trnD*^{GUC}*-trnT*^{GGU}, *rpl32-trnL*^{UAG}, *ndhF-rpl32*) and one nuclear region (granule-bound starch synthase) were used to resolve relationships among 18 *Jaborosa* species, using species of the "Atropina clade" as outgroup. Phylogenetic reconstruction strongly supports the monophyly of *Jaborosa*, with species resolved in two major clades: (1) a "Lowland clade" (L) comprised of three noticeably sphingophylous species distributed below 1000 m and north of 36° S latitude, and (2) an "Andean clade" (A) composed of the remaining species, which strongly differ in floral morphology and mainly occur at high altitudes (more than 3000 m in the Puna desert) or high latitudes (up to 53° S latitude in the Patagonia steppe of Tierra del Fuego). Species in the Andean clade have flowers that range from black, rotate, deceptive, saprophilous fly-pollinated flowers to green, tubular, nectar-producing flowers with a mixed pollination system. Estimation of divergence times suggests a split of *Jaborosa* from its sister genus *Atropa* L. at ca. 16.7 Ma. The split between the L and A clades possibly occurred ca. 10 Ma, with recent species diversification co-occurring with the uplift of the Andes during the Pleistocene (ca. 1–3 Ma). Reconstruction of ancestral states of pollination mode, altitudinal distribution, and floral traits (corolla colour, flower morphology, presence of nectar) suggests that pollination by moths probably evolved once within the L clade, whereas brood-site deceptive pollination probably evolved once within the A clade. These contrasting pollination modes are associated with changes in corolla colour, flower morphology and loss of a functional nectary.

Keywords hawkmoth pollination; *Jaborosa*; phylogeny; saprophilous fly pollination; Solanaceae; southern South America

Supplementary Material Electronic Supplement (Table S1; Fig. S1; Appendix S1) and alignment are available in the Supplementary Data section of the online version of this article at http://www.ingentaconnect.com/content/iapt/tax

■ INTRODUCTION

Several plant groups have originated and diversified in South America, such as Bignoniaceae, Calceolariaceae, Gesneriaceae and Solanaceae (Gentry, 1982; Cocucci, 1999; Hunziker, 2001; Smith & Baum, 2006; Cosacov & al., 2009; Olmstead, 2013; Perret & al., 2013). It has been suggested that the hyperdiverse Neotropical flora results from the combination of mature as well as recent and rapid radiations (e.g., in the Andes; Linder, 2008). In the latter case, the topographical and environmental changes that occurred during the Andean orogeny markedly influenced the diversification of the local biota (Gentry, 1982; Brumfield & Edwards, 2007). Although the Andean uplift was initiated ca. 20 Ma in the Early Miocene, high-elevation areas such as Puna and Páramo, where several endemic plant clades have radiated, are much younger. These areas date from ca. 2 to 4 Ma in the Pliocene, coinciding with the development of arid environments in South America (Hughes & Eastwood, 2006; Luebert & al., 2011; Särkinen & al., 2012). The diversification of Andean clades through geographical isolation likely occurred in concert with another major promoter of diversification: namely, adaptation to changing pollinator environments (Cocucci, 1999; Perret & al., 2001; Pérez & al., 2006; Muchhala, 2006; Smith & al., 2008; Cosacov & al., 2009).

The nightshade genus *Jaborosa* Juss. includes 22 species distributed from southern Peru and Bolivia to Tierra del Fuego in Argentina and Chile (Barboza, 2013). *Jaborosa* species are mostly rhizomatous perennial herbs that occur, with the exception of three species, primarily in semiarid regions located either at high altitudes in the Andes or at high latitudes in the Monte desert and the temperate Patagonian steppe of southernmost Argentina (Barboza & Hunziker, 1987). The remaining three species mainly occur in lowland grasslands either in the Parana and Uruguay River basins (*J. integrifolia* Lam., *J. runcinata* Lam.) or in the western Chaco region (*J. odonelliana* Hunz.). *Jaborosa* species exhibit astonishing interspecific variation in floral traits ranging from nocturnal white flowers with very long corolla tubes that are fragrant

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and produce abundant nectar, to diurnal, dark maroon flowers with rotate corollas that emit unpleasant odours and either lack or have a reduced nectary (Cocucci, 1988, 1999). Previous studies found that *J. laciniata* (Miers) Hunz., *J. leucotricha* (Speg.) Hunz., *J. rotacea* (Lillo) Hunz. & Barboza and *J. sativa* (Miers) Hunz. & Barboza are pollinated by carrion-seeking flies (Cocucci, 1988; Moré & al., 2013) and *J. integrifolia* is pollinated by nocturnal hawkmoths (Vesprini & Galetto, 2000; Moré & al. 2014). Pollination by long-tongued hawkmoths has evolved several times within Solanaceae, with at least 16 genera known or suspected to be pollinated by nocturnal moths (Knapp, 2010). By contrast, pollination by saprophilous flies has been confirmed for only some *Jaborosa* species (Cocucci, 1988; Moré & al., 2013), although *Larnax glabra* (Standl.) Sawyer (Deanna & al., 2014) and some Australian species of *Anthotroche* Endl. (L.A.R. Haegi, pers. comm.) have rotate flowers with maroon to black corollas, suggesting pollination by saprophilous flies.

Traditionally, the genus *Jaborosa* was grouped with *Salpichroa* Miers and *Nectouxia* Kunth in tribe Jaborosae Miers (Barboza & Hunziker, 1987; Hunziker, 2001). However, recent molecular studies place *Salpichroa* and *Nectouxia formosa* Knuth far from *Jaborosa*, in the small "Salpichroina clade" of unresolved position among the Capsiceae and Physaleae clades (Olmstead & al., 2008; Särkinen & al., 2013). By contrast, *Jaborosa* groups with tribes Hyoscyameae and Lycieae and the genera *Nolana* L.f., *Sclerophylax* Miers and *Latua* Phil. in the moderately well-supported "Atropina clade" (Olmstead & al., 2008; Tu & al., 2008, 2010; Levin & al., 2011; Särkinen & al., 2013).

To better understand the importance of historical biogeographic processes and floral adaptation in the evolutionary radiation of *Jaborosa*, we focused the present study on reconstructing the phylogenetic history against a backdrop of contrasting environments and pollination modes. Our specific goals are to: (1) assess the monophyly of *Jaborosa* and clarify relationships with respect to other representative members of the "Atropina clade", (2) estimate the age and geographic context of diversification of the genus, and (3) understand floral trait evolution and pollinator shifts among *Jaborosa* species.

■ MATERIALS AND METHODS

Taxon sampling and outgroup selection. — A total of 18 species of *Jaborosa* (including the two subspecies of *J. caulescens* Gillies & Hook.) were included in this study (Electr. Suppl.: Appendix S1). *Jaborosa chubutensis* Barboza & Hunz. is currently known only from the type locality, where we were unable to find any flowering individuals; thus, although included in this study, the species identification for this locality is tentative. *Jaborosa squarrosa* (Miers) Hunz. & Barboza sequences were downloaded from GenBank. We selected four plastid spacer regions (*trnH-pbsA*, $trnD^{GUC}$-$trnT^{GGU}$, *rpl32*-$trnL^{UAG}$, *ndhF-rpl32*) because they provided the highest number of parsimony-informative characters for the closely related *Lycium* L. (Miller & al., 2009). Moreover, the nuclear granule-bound starch synthase (GBSSI *waxy*) has been useful for inferring relationships among species in several other Solanaceae genera such as *Iochroma* Benth., *Lycium*, *Schizanthus* Ruiz & Pav. and *Solanum* L. (Peralta & Spooner, 2001; Pérez & al., 2006; Smith & Baum, 2006; Levin & al., 2007; Miller & al., 2011). We included representatives of *Salpichroa* (*S. origanifolia* (Lam.) Baill. and the two subspecies of *S. tristis* Miers), *Nectouxia formosa*, and several accessions of the "Atropina clade" sensu Olmstead & al. (2008) as outgroups: *Atropa belladonna* L. of tribe Hyoscyameae and *Lycium* spp., *Nolana* L.f. spp. and *Latua pubiflora* Baill. of Lycieae s.l. (Olmstead, 2013). *Datura stramonium* L. and *Solanum* spp. were used as distant outgroups within subfamily Solanoideae but outside the "Atropina clade" (Olmstead & al., 2008). Sequences of these outgroup species, except for *Salpichroa* spp., *Nectouxia formosa* and *Latua pubiflora*, were downloaded from GenBank (Electr. Suppl.: Appendix S1).

Sequence data. — Total genomic DNA was extracted from silica gel-dried leaf material using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The four plastid spacer regions (*trnH-pbsA*, $trnD^{GUC}$-$trnT^{GGU}$, *rpl32*-$trnL^{UAG}$, *ndhF-rpl32*) were amplified using the primers and protocols of Miller & al. (2009). For GBSSI *waxy*, we amplified exons 2 through 10 (ca. 1800 bp) using the forward primer 181F (5′-CGG GTA ATG ACA ATA TST CC-3′; Levin & al., 2006) and the reverse primer 2R (5′-GTT CCA TAT CGC ATA GCA TG-3′; Levin & al., 2006). Occasionally, internal primers CRmod (5′-GGC ATA GTA TGG GCT CAC AGT AA-3′, Levin & Miller, 2005) and IwaxyF (5′-ATT CCC TGC TAC CTG AAG TC-3′, Levin & Miller, 2005) were used to amplify the gene in two separate pieces following the protocol described by Levin & al. (2005). In two cases (*Jaborosa rotacea*, *Latua pubiflora*) only the second part of the GBSSI *waxy* gene (approximately 1000 bp) could be amplified. PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced in both directions by Macrogen (Seoul, Korea) with the same primers used for the amplifications. For GBSSI *waxy*, internal primers CRmod and IwaxyF were also used for sequencing (Levin & Miller, 2005; Levin & al., 2007).

Sequences from both strands of each PCR product were examined, compared, and corrected using the program BioEdit v.7.1.3.0 (Hall, 1999), from which a consensus sequence was generated. Sequence data were aligned using default settings in CLUSTAL W v.1.81 (Thompson & al., 1994) as implemented in BioEdit software (Hall, 1999) and then manually adjusted. Insertion/deletions were coded manually following the simple coding method proposed by Simmons & Ochoterena (2000).

Phylogenetic analyses. — Parsimony and Bayesian analyses were conducted for the following datasets: (1) the combined plastid data (*trnH-pbsA*, $trnD^{GUC}$-$trnT^{GGU}$, *rpl32*-$trnL^{UAG}$, *ndhF-rpl32*) and (2) the nuclear GBSSI *waxy* data. *Solanum* was defined as the outgroup (Olmstead & al., 2008) in all analyses. Indels were included as additional characters in all analyses.

Parsimony analyses were performed using only potentially informative characters with the program TNT v.1.1 (Goloboff & al., 2008). Heuristic searches were carried out with a total of 1000 random addition sequence replicates and

tree-bisection-reconnection (TBR) branch swapping. All most parsimonious trees were combined in a strict consensus tree using the Nelsen option in TNT (Goloboff & al., 2008). Support was estimated by jackknife as implemented in TNT, resampling 1000 times with TBR.

Partitioned Bayesian phylogenetic analyses were conducted using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). The best-fit model of sequence evolution was determined for each partition using the Bayesian information criterion as implemented in jModelTest v.2.0.2 (Guindon & Gascuel, 2003; Posada, 2008). Best-fit models corresponded to TPM1uf+G for *trnH-pbsA*, GTR+G for *rpl32-trnL*^{UAG}, TVM+G for *trnD*-^{GUC}*-trnT*^{GGU} and *ndhF-rpl32*, and HKY+G for GBSSI *waxy*. The Markov chain Monte Carlo (MCMC) algorithm was run in two simultaneous and independent analyses starting from different random trees. Four Markov chains were run for 10 million generations, sampling trees every 1000 generations. Chain convergence was assessed with Tracer 1.5.0 and the first 25% of the sampled trees were discarded as burn-in. The remaining trees were used to construct a 50% majority-rule consensus tree. Final trees were visualized and edited using FigTree v.1.4.0 (Rambaut, 2009).

Divergence time. — Estimation of the divergence time of *Jaborosa* species was conducted in BEAST v.1.7.5 (Drummond & al., 2012) using the combined plastid data with *Solanum* as outgroup. We used the Akaike information criterion (AIC) as implemented in jModelTest v.2.0.2 (Guindon & Gascuel, 2003; Posada, 2008) to determine which nucleotide substitution model best fit our data. The best molecular clock model (strict or relaxed) was evaluated by comparing Bayes factors as implemented in Tracer v.1.5.0 (Suchard & al., 2001; Rambaut & Drummond, 2009), with the relaxed molecular clock model best fitting the data (log10 Bayes factor = 6.2). Thus, we performed the Bayesian analysis using BEAST v.1.7.5 under a relaxed clock model with branch-specific rates following a lognormal distribution and a GTR+G model of nucleotide substitution (Drummond & al., 2012). The analyses were completed with a randomly generated starting-tree topology and employed a Yule prior on the birth rate of new lineages. Due to the lack of fossils, age values previously estimated for two other genera belonging to the Lycieae s.l. clade were used to approximate divergence times in our study. We considered a normally distributed node age of 5±1 Ma for the origin of *Lycium* (Levin & al., 2007; Miller & al., 2011), and a normally distributed age of 10.3±2 Ma for the split between *Nolana* and *Lycium* (Dillon & al., 2009; Tu & al., 2010). A substitution rate estimate of 0.0028±0.0015 substitutions per site per million years was used as calculated for *trnH-psbA* and *trnS-trnG* in *Petunia* Juss. (Lorenz-Lemke & al., 2010).

Posterior estimates were obtained by sampling every 1000 MCMC steps for a total of 70 million generations. Log-files were analyzed in Tracer v.1.5.0 (Rambaut & Drummond, 2009), and the effective sample sizes (>200) were used to evaluate MCMC convergence within chains. The resulting trees were combined using Tree Annotator v.1.7.5 (Rambaut & Drummond, 2009), with the first 25% of the iterations discarded as burn-in. Final trees were visualized in FigTree v.1.4.0 (Rambaut, 2009).

Evolution of floral traits and pollination systems. — Floral traits (corolla shape, corolla colour, nectary disc), altitudinal distribution and pollination mode were scored for *Jaborosa* species excluding outgroups. Information was retrieved from direct observations in natural populations and from the literature (Barboza & Hunziker, 1987; Cocucci, 1988, 1999; Vesprini & Galetto, 2000; Moré & al., unpub. results). We selected qualitative floral traits that were pertinent to the pollinator interaction and that could be obtained for every species. These include corolla shape, corolla colour and nectary; combinations of these traits are roughly associated with different pollinator types, for example, salverform, white corollas and nectary present with moth pollinators; rotate, maroon corollas and lack of nectar with saprophilous fly pollination (Willmer, 2011). Three types of corolla shape were recognized: (0) subcampanulate-rotate, (1) salverform, and (2) short-tubular/urn-shaped. Corolla colour was coded as: (0) maroon to black, (1) white (including the whitish-pink coloured *J. cabrerae* Barboza and the whitish-pale blue *J. squarrosa*) or (2) yellowish to greenish. *Jaborosa sativa*, which has green flowers that often have maroon spots, and *J. reflexa* Phil., which also has some individuals with maroon flowers, were coded as yellowish to greenish. The presence of a nectary disc was coded as: (0) absent, (1) fully developed or (2) reduced. Four states were assigned to pollinator type: (0) mixed pollination (nectar flies, halictid bees, saprophilous flies), (1) moths, (2) saprophilous flies or unknown pollinators (coded "?"). Finally, altitudinal distribution was coded as: (0) below 1000 m including the lowland areas of the Pampas grasslands, Chaco forest and Patagonia steppe, (1) between 1000 and 3000 m including the Monte region, Prepuna desert and Yungas rainforest or (2) above 3000 m with high Andean vegetation (Electr. Suppl.: Table S1; Särkinen & al. 2012).

Ancestral state reconstructions of floral traits, altitudinal distribution and pollination mode in *Jaborosa* were implemented in Mesquite v.2.75 using maximum unordered parsimony and maximum likelihood (Maddison & Maddison, 2009). To estimate the frequency of shifts between states coded for the ancestral reconstructions we followed a similar approach to that of Valente & al. (2012). To account for uncertainty in tree topology and branch length, analyses were conducted over a sample of 1000 highly probable trees chosen at random from the combined plastid Bayesian analysis after excluding the initial 2.5 million generations from the burn-in (Navarro-Pérez & al., 2013). Reconstructions were performed using both unordered parsimony and maximum likelihood methods as implemented in Mesquite 2.75 (Maddison & Maddison 2009). For ML reconstructions we used the criterion with the Markov k-state 1 parameter default model; thus, all character states were equally weighted and considered unordered in all analyses. The analysis calculated, for each state at each node, the number of trees on which a given state was reconstructed as uniquely best state. When the ancestral state at a given node was ambiguous, the trait was reconstructed as "equivocal" (Maddison & Maddison, 2009). The estimated number of shifts between character states for each trait was determined using the "summarize state changes over trees" application (Maddison & Maddison, 2009).

■ RESULTS

Phylogenetic analyses. — The sequence dataset of the four plastid regions was 3274 bp long, of which 351 (10.72%) were parsimony informative (Table 1). The aligned length of the plastid sequences ranged from 603 bp for *trnH-pbsA* to 1164 bp for *trnD*^{GUC}-*trnT*^{GGU} (Table 1). All aligned plastid DNA regions contained gaps; there were 92 indel characters in the plastid DNA dataset, of which 50 were parsimony informative (Table 1). The proportion of parsimony-informative characters was close to 13% in three of the four plastid markers (*trnH-pbsA*, *rpl32-trnL*^{UAG}, *ndhF-rpl32*) and 6.70% in the *trnD*^{GUC}-*trnT*^{GGU}. However, consistency and retention index values were considerably lower in *ndhF-rpl32* in comparison to the other three markers (Table 1). The aligned GBSSI *waxy* dataset was 1822 bp, of which 335 (19.16%) were parsimony informative. There were 47 coded indel characters, of which 20 were parsimony informative. Consistency and retention index values were higher than for any of the plastid markers (Table 1).

Both analyses, combined plastid and nuclear GBSSI *waxy* data, recognized *Jaborosa* as a strongly supported monophyletic clade (BPP = 1, jk = 100%; Figs. 1–2; Electr. Suppl.: Fig. S1). Besides, these results support two main clades within *Jaborosa*: (1) a clade (L) grouping the three species (*J. integrifolia*, *J. odonelliana*, *J. runcinata*; Fig. 3C, G, J) distributed in lowland areas, which clearly have floral traits associated with pollination by nocturnal hawkmoths and (2) an Andean clade (A) grouping the rest of the species distributed in the northern, central, and southern South American Andes, the Monte region, and the Patagonia steppe (Fig. 1–2). Within clade L, the position of *J. integrifolia*—which has a disjunct geographical distribution and grows in the eastern Pampean province and in the western Chacoan province sensu Morrone (2014)—is either sister to the eastern *J. runcinata* in the combined plastid tree (Fig. 1) or sister to the western *J. odonelliana* in the nuclear tree (Fig. 2). Species of the A clade have diverse floral traits associated with pollination by saprophilous flies, noctuid moths and mixed pollination systems, including nectar flies, saprophilous flies and halictid bees (Fig. 3 A, B, D–F, H, I, K–N). Within clade A, two well-supported sub-clades were recovered only in the nuclear data, one containing *J. chubutensis*, *J. reflexa*, *J. bergii* Hieron. and *J. laciniata* (A_1: BPP = 1, jk = 95%; Figs. 2, 3D, H, I) and the other containing the remaining species (A_2: BPP = 1, jk = 100%; Fig. 2). A group of species mainly distributed in the Andes of northern Argentina and Bolivia and the Yungas rainforest of Argentina (*J. lanigera* (Phil.) Hunz. & Barboza, *J. parviflora* (Phil.) Hunz. & Barboza, *J. oxipetala* Speg., *J. rotacea*, *J. sativa*, *J. squarrosa*) was resolved with moderately support within the A_2 sub-clade (BPP = 0.99, jk = 57%; Figs. 2, 3B, F, K, L). Moreover, both analyses recovered *Jaborosa caulescens* var. *caulescens* sister to *J. cabrerae* (combined plastid tree: BPP = 1, jk = 93%, Figs. 1, 3M; GBSSI *waxy* nuclear tree: BPP = 1, jk = 54%, Fig. 2); *J. chubutensis* sister to *J. reflexa* (combined plastid tree: BPP = 1, jk = 99%, Fig. 1; GBSSI *waxy* nuclear tree: BPP = 1, jk = 100%, Fig. 2) and *J. lanigera* sister to *J. parviflora* (combined plastid tree: BPP = 1, jk = 93%, Fig. 1; GBSSI *waxy* nuclear tree: BPP = 0.90, Fig. 2).

Among the taxa sampled, *Jaborosa* was sister to the Eurasian genus *Atropa* (tribe Hyoscyameae). Closely related to *Jaborosa*+*Atropa* was a clade consisting of *Lycium* spp. (including the former *Grabowskia* Schltdl.) and *Nolana*. Finally, *Jaborosa*+*Atropa*+*Lycium*+*Nolana* were sister to the Chilean endemic *Latua pubiflora* (Fig. 1). These genera comprising the Lycieae s.l. clade were sister to *Datura*. *Salpichroa* species were grouped with *Nectouxia formosa* in a strongly supported clade at the base of the phylogeny (Figs. 1 and 2).

Divergence times. — Using a combination of four plastid DNA regions calibrated with two previously estimated node ages (origin of *Lycium* and the split between *Nolana* and *Lycium*), the median divergence time of *Jaborosa* from its sister genus *Atropa* is estimated to be 16.7 (95% HPD: 9.5–25.8) Ma, during the early Miocene. The crown age of *Jaborosa* is estimated at ca. 10 (95% HPD: 5.4–15.6) Ma, during the late Miocene. The time of divergence of the most recent common ancestor of clade L is estimated at ca. 5.4 (95% HPD: 2.6–8.9) Ma, during the early Pliocene, while diversification within clade A was estimated to have occurred more recently, during the late Pliocene at ca. 3.7 (95% HPD: 1.9–6.0) Ma.

Evolution of floral characters and pollination systems. — Reconstructions of floral traits, geographical distribution and pollination mode from the maximum parsimony (MP) analyses

Table 1. Summary statistics and analysis parameters for individual and combined datasets for phylogenetic analyses of *Jaborosa* Juss.

Region	*trnH-psbA*	*trnD*^{GUC}-*trnT*^{GGU}	*rpl32-trnL*^{UAG}	*ndhF-rpl32*	Combined plastid DNA	GBSSI *waxy*
Aligned length (bp)	603	1164	1163	920	3850	1822
Included characters	456	1089	989	740	3274	1748
PI characters/proportion	56/12.28%	73/6.70%	129/13.00%	96/12.95%	351/10.72%	335/19.16%
MPT/tree length	94/112 steps	100/114 steps	70/209 steps	1/170 steps	100/589 steps	24/615 steps
Consistency/retention index	0.589/0.779	0.634/0.838	0.679/0.864	0.340/0.521	0.668/0.856	0.719/0,889
# coded gaps (informative gaps/autapomorphies)	16 (10/6)	28 (20/8)	26 (9/17)	22 (11/11)	92 (50/42)	47 (20/27)
Best-fitting Bayesian model	TPM1uf+G	TVM+G	GTR+G	TVM+G		HKY+G

PI, parsimony informative; MPT, number of most parsimonious trees

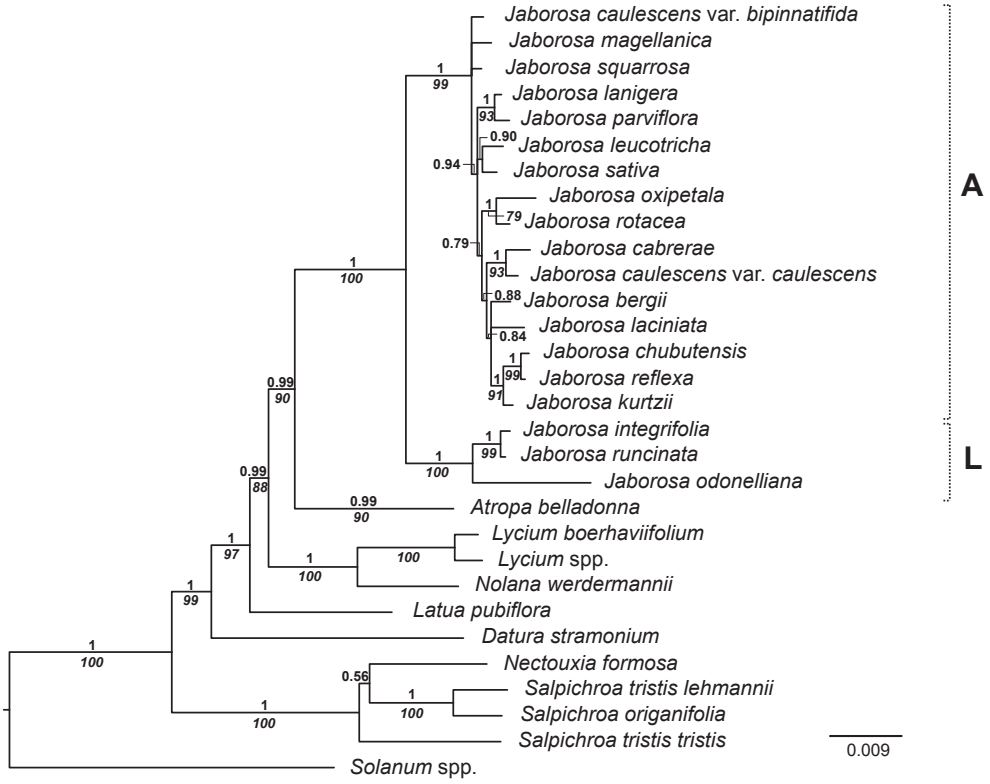

Fig. 1. Majority-rule consensus of trees sampled by the Bayesian analysis of *Jaborosa* and related taxa based on the combined plastid data (*trnH-pbsA*, *trnD*^{GUC}-*trnT*^{GGU}, *rpl32-trnL*^{UAG}, *ndhF-rpl32*). Numbers above branches are Bayesian posterior probabilities. Numbers below branches are jackknife values) for maximum parsimony. A and L stand for Andean and Lowland clades respectively.

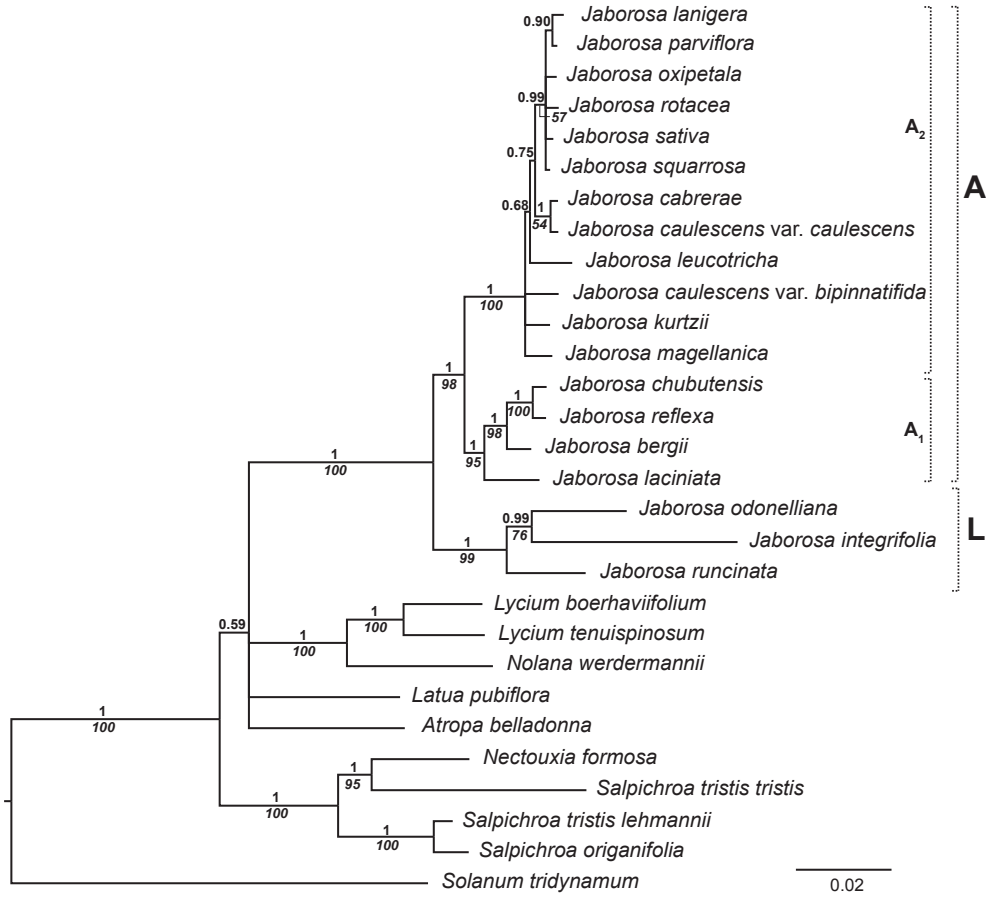

Fig. 2. Majority-rule consensus of trees sampled by the Bayesian analysis of *Jaborosa* and related taxa, based on the nuclear GBSSI *waxy* data. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are jackknife values for maximum parsimony. A and L stand for Andean and Lowland clades respectively.

Fig. 3. Flower diversity in *Jaborosa* (Solanaceae). **A,** *J. leucotricha* (Speg.) Hunz.; **B,** *J. sativa* (Miers) Hunz. & Barboza; **C,** *J. runcinata* Lam.; **D,** *J. bergii* Hieron.; **E,** *J. magellanica* (Griseb.) Dusén; **F,** *J. rotacea* (Lillo) Hunz. & Barboza; **G,** *J. integrifolia* Lam.; **H,** *J. laciniata* (Miers) Hunz. & Barboza; **I,** *J. reflexa* Phil.; **J,** *J. odonelliana* Hunz.; **K,** *J. parviflora* (Phil.) Hunz. & Barboza; **L,** *J. lanigera* (Phil.) Hunz. & Barboza; **M,** *J. caulescens* Gillies & Hook. var. *caulescens*; **N,** *J. kurtzii* Hunz. & Barboza. — Scale bar equals 0.5 cm.

was generally consistent with the maximum likelihood (ML) analyses, although the ML reconstructions were less resolved. Thus, only parsimony results are depicted here (Fig. 5). The ancestor of clade L was reconstructed as being moth-pollinated, distributed in lowland areas and having white flowers with a well-developed nectary disc in 100% of the 1000 trees of the Bayesian posterior distribution; the ancestral state for floral morphology was equivocal (Fig. 5). Further, the ancestor of clade A was reconstructed as having saprophilous fly pollination in 43% of the 1000 trees, having short-tubular/urn-shaped flowers in 76% of the trees, with a rudimentary nectar disc in the 82% of the trees, and with maroon to black corollas in 27% of the trees. Ancestral distribution was reconstructed from 1000 up to 3000 m only in 28% of the trees, being equivocal in 72% of the trees. Analyses failed to resolve the ancestral character states for the ancestor of the entire *Jaborosa* clade (Fig. 5).

Although with different resolution, transition estimates between different character states yielded congruent results using MP and ML analyses, but the latter were more conservative (Table 2). Eight of the possible 24 transitions occurred on average once or more times according to the MP analysis, while only one occurred according to the ML analysis (Table 2). Transitions common and most frequent in both analyses were from short-tubular/urn-shaped to rotate/subcampanulate (on average 4.26 or 0.65 times in MP and ML, respectively), from maroon/black to white corollas (2.58 or 0.07 times in MP and ML, respectively), from reduced nectary to either well-developed (2.94 or 0.83 times in MP and ML, respectively) or absent (1.68 or 1.15 times in MP and ML, respectively) and, finally, from mixed pollination to saprophilous flies pollination (2.33 or 0.01 times in MP and ML, respectively).

Only two transitions that, according to both analyses, never occurred were: change from moth to either saprophilous flies or mixed pollination (Table 2).

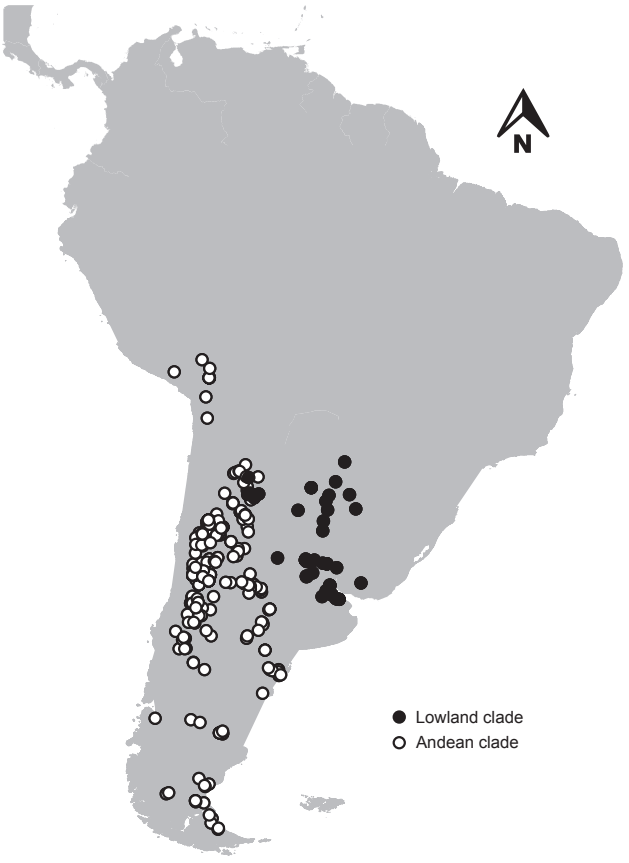

Fig. 4. Geographical distribution of Lowland and Andean clades of *Jaborosa*.

■ DISCUSSION

Phylogenetic relationships. — The results of the combined plastid and the nuclear GBSSI *waxy* analyses confirm that *Jaborosa* is a well-supported monophyletic group within the "Atropina clade" sensu Olmstead & al. (2008). Our analysis recovered two strongly supported *Jaborosa* clades, a "Lowland clade", including the three species with sphingophilous flowers inhabiting lowland areas, and an "Andean clade", including the rest of the species distributed mainly across the Andes and in temperate South America. These findings differ slightly from the previous division of the genus into two sections (sect. *Jaborosa*, sect. *Lonchestigma* (Dunal) Wettst.) based on palynological (Barboza, 1986) and morphological traits (Barboza & Hunziker, 1987). Section *Jaborosa* was then characterized by entire leaves and smooth, porate pollen grains and included *J. integrifolia*, *J. odonelliana*, *J. oxipetala* and *J. runcinata*. The remaining species were placed in sect. *Lonchestigma*, characterized by pinnate leaves and reticulate colporate pollen grains. The only difference is that *J. oxipetala*, a small-flowered species restricted to the Yungas cloud forest of Argentina, is here recovered within the Andean clade and not within the Lowland clade. Future studies should confirm if the high mountain species *J. pinnata* Phil., *J. riojana* Hunz. & Barboza and *J. volkmanni* Reiche, which were not included in the present study, are also part of the Andean clade.

Previous studies have not resolved relationships between *Jaborosa* and their closest relatives (Olmstead & al. 2008; Tu & al., 2010; Särkinen & al., 2013). Our combined plastid dataset clearly supports *Atropa* (tribe Hyoscyameae) as sister genus to *Jaborosa* and the clade comprised of *Lycium* and *Nolana* as sister to *Jaborosa* plus *Atropa*. In addition, *Latua* is recovered as sister to the clade comprised of *Jaborosa*, *Atropa*, *Lycium* and *Nolana* (Fig. 1). However, elucidating which is actually the sister genus of *Jaborosa* and unraveling the basal relationships among the genera comprising the "Atropina clade" must await a more thorough sampling scheme.

Timing and geographic context of diversification. — Our results show that the *Jaborosa* ancestor diverged approximately 16.7 Ma in the early Miocene. Divergence in two main clades occurred approximately 10 Ma in the late Miocene when the Andes were still relatively low (400–2500 m) and climate was warm and humid at southern latitudes (Hughes & Eastwood, 2006). The split of these two major clades has a strong geographical structure, with one in lowlands and the other mainly

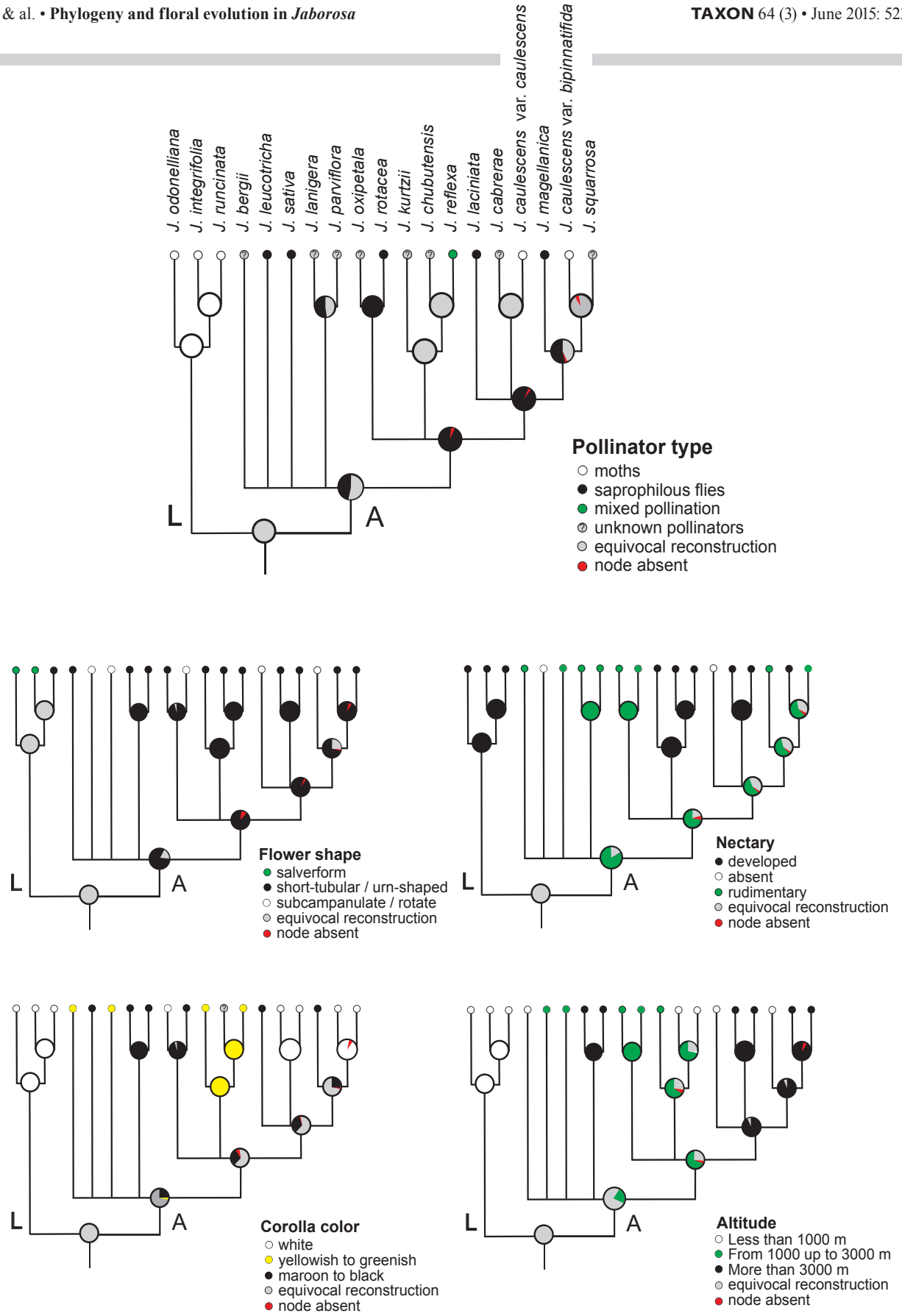

Fig. 5. Reconstruction of pollination mode, floral traits and geographical distribution with maximum parsimony character optimization in Mesquite. The majority-rule consensus tree from the Bayesian analysis with unsupported nodes collapsed is shown. Pie charts show the percentage of trees (from 1000 highly probable trees) for which a given state was reconstructed as ancestral for that node.

Table 2. Summary of estimated pollination mode and floral trait transitions in *Jaborosa* Juss. based on ancestral state reconstructions over 1000 highly probable trees.

Transition	Parsimony	Maximum likelihood
Corolla shape (7 steps)		
rotate/subcampanulate → salverform	0.0410 (0–1)	0 (0)
rotate/subcampanulate → short-tubular/urn-shaped	0.3843 (0–3)	0.0100 (0–1)
salverform → rotate/subcampanulate	0.0717 (0–1)	0 (0)
salverform → short-tubular/urn-shaped	1.0309 (0–2)	0.0300 (0–1)
short-tubular/urn-shaped → rotate/subcampanulate	4.2647 (2–5)	0.6500 (0–4)
short-tubular/urn-shaped → salverform	0.8875 (0–2)	0.0700 (0–1)
Flower colour (8 steps)		
maroon/black → white	2.5763 (0–5)	0.0700 (0–1)
maroon/black → yellowish/greenish	2.0271 (0–3)	0 (0)
white → maroon/black	1.2154 (0–5)	0.0400 (0–1)
white → yellowish/greenish	0.3279 (0–2)	0 (0)
yellowish/greenish → maroon/black	0.6344 (0–3)	0 .0100 (0–1)
yellowish/greenish → white	0.1988 (0–2)	0 (0)
Nectary (5 steps)		
absent → reduced	0.0241 (0–1)	0 (0)
absent → well-developed	0.0635 (0–1)	0 (0)
reduced → absent	1.6755 (1–2)	1.1500 (0–2)
reduced → well-developed	2.9434 (0–4)	0.8300 (0–1)
well-developed → absent	0.3245 (0–1)	0 (0)
well-developed → reduced	0.9489 (0–4)	0 (0)
Pollination system (4 steps)		
moths → saprophilous flies	0 (0)	0 (0)
moths → mixed pollination	0 (0)	0 (0)
saprophilous flies → moths	6×10^{-4} (0–1)	0 (0)
saprophilous flies → mixed pollination	0.6742 (0–3)	0 (0)
mixed pollination → moths	0.9993 (0–1)	0 (0)
mixed pollination → saprophilous flies	2.3258 (0–3)	0.0100 (0–1)

Columns show the average number of unequivocal transitions between states using maximum parsimony and maximum likelihood methods (Maddison & Maddison, 2009). In parentheses are the minimum and maximum numbers of transitions inferred across all trees.

Andean, suggesting early isolation of these two ancestral lineages (Fig. 4). Interestingly, there is paleo-environmental evidence supporting the existence of a geographic barrier at the time of early divergence in *Jaborosa*. Studies have shown that in the middle and late Miocene (ca. 10 to 17 Ma) three successive Atlantic marine transgressions, informally known as the "Paranean Sea", resulted in a flooded area in southern South America possibly separating the ancestors of these lineages (Ortiz-Jaureguizar & al., 2006; Tambussi & Degrange, 2013).

Diversification of the Lowland clade, which consists of the three sphingophylous species (*J. integrifolia*, *J. odonelliana*, *J. runcinata*), is estimated at ca. 5 Ma. Recent paleontological evidence (Genise & al., 2014) indicates that, at that time, this lowland area was already inhabited by hawkmoths very similar to modern ones, such as species of *Manduca* and *Eumorpha*, known to pollinate *J. integrifolia* (Vesprini & Galetto, 2000; Moré & al., 2014). Diversification of the Andean clade was estimated to have occurred later than of the Lowland clade at ca. 4 Ma. This more recent diversification of the highland species correlates with the uplift of the Andes to its current altitudes and the establishment of the Patagonian steppe east of the Andes (Young & al., 2002; Hughes & Eastwood, 2006; Cosacov & al., 2010; Luebert & al., 2011). This suggests that these biomes were colonized by *Jaborosa* relatively recently, following Pliocene and Pleistocene uplift events.

Evolution of floral traits and pollination system in *Jaborosa*. — Our findings suggest that floral diversification in *Jaborosa* could have occurred in concert with a shift from ancient

subtropical lowland habitats that originated during Paleozoic and Mesozoic to the younger high Andes and Patagonian steppe that originated during the Pliocene. Evidence also suggests that early flower diversification involved a shift from moth pollination to saprophilous fly pollination. According to the phylogenetic reconstruction, pollination by moths was ancestral in the Lowland clade and arose again, at least twice, in the Andean clade. The reportedly or presumably moth-pollinated species (*J. integrifolia*, *J. odonelliana*, *J. runcinata*, *J. caulescens* var. *caulescens*, *J. caulescens* var. *bipinnatifida* (Dunal) Reiche) have flowers with salverform or short-tubular and white corollas, a conspicuous nectary disc and are fragrant at night. Hawkmoth-pollinated plant diversity is higher in subtropical lowland areas where ambient temperatures at dusk are moderate (Cruden & al., 1976), and where nocturnal hawkmoths are a substantial component of the pollinator fauna (Schreiber, 1978; Haber & Frankie, 1989; Raguso & al., 2003; Moré & al., 2005; Amorim & al., 2009).

Pollination by saprophilous flies was reconstructed as ancestral in the Andean clade, with reversals either to moth-pollination or mixed pollination. Moreover, within this clade, pollination by saprophilous flies is often associated with flowers that have dark-coloured (with the exception of *J. sativa*), rotate or sub-campanulate corollas, lack or have reduced nectaries, and emit foul odours (Moré & al., 2013). Fly-pollinated species are known to be more abundant in the high Andes than in the subandean zone (Arroyo & al. 1982).

In conclusion, phylogenetic analyses based on plastid and nuclear data support the monophyly of the genus *Jaborosa* with species retrieved in two major clades. Ancestral reconstructions suggest that a pollinator shift from hawkmoths to other insects, including saprophilous flies and settling moths among others, occurred in concert with changes in floral traits (morphology, colour, and nectar production) and dispersal from warm and humid lowland to cooler and drier regions during the initial radiation of *Jaborosa* across southern South America.

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