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Breakdown of species boundaries in *Mandevilla*: floral morphological intermediacy, novel fragrances and asymmetric pollen flow.

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Summary

- Phenotypic intermediacy is an indicator of putative hybrid origin, and has provided the main clues to discovering hybrid plants in nature. *Mandevilla pentlandiana* and *M. laxa* (Apocynaceae) are sister species with clear differences in floral phenotype and associated pollinator guilds: diurnal Hymenoptera and nocturnal hawkmoths, respectively. The presence of individuals with intermediate phenotypes in a wild population raises questions about the roles of visual and olfactory signals (*i.e.* corolla morphology and floral fragrances) as barriers to interbreeding, and how the breakdown of floral isolation occurs.

- We examined phenotypic variation in a mixed *Mandevilla* population analysing the chemical composition of floral fragrances, characterizing floral shape through geometric morphometrics and assessing individual grouping through taxonomically relevant traits and an unsupervised learning algorithm. We quantified the visitation frequencies of floral visitors and tracked their foraging movements using pollen analogues.

- The presence of morphologically intermediate individuals and pollen analogue movement suggested extensive hybridization between *M. laxa* and *M. pentlandiana*, along with asymmetrical rates of backcrossing between these putative hybrids and *M. laxa*. Floral volatiles from putative hybrid individuals showed a transgressive phenotype, with additional compounds not emitted by either parental species.

- Our results suggest the presence of a hybrid swarm between sympatric *M. pentlandiana* and *M. laxa* and indicate that initial hybridization events between these parental species are rare, but once they occur, visits between putative hybrids and *M. laxa* are common and facilitate continued introgression.

Introduction

The presence of hybrid individuals in nature, often characterized by intermediate phenotypes, raises questions about the breakdown of reproductive barriers between related species. In particular, pre-zygotic barriers to hybridization occur when floral morphology (*e.g.* posture or nectar spur length) reduces or prevents interspecific pollen transfer (*i.e.* mechanical isolation) or when specific floral traits (*e.g.* colour or scent) affect pollinator attraction and visitation behaviour (*i.e.* ethological isolation) (Grant 1994; Hodges & Arnold 1994). Pollinators are major drivers of plant hybridization because their foraging choices can determine the frequency and distribution of hybrid genotypes and the rate of gene flow between these hybrids and their parental species (Arnold 1997). Many studies have revealed that populations of related species often hybridize, despite some being pollinated by different functional groups (*e.g.* Hodges & Arnold 1994; Sérsic *et al.* 2001; Ippolito *et al.* 2004; Aldridge & Campbell 2007; Martin *et al.* 2008; Campbell *et al.* 2016).

A full understanding of the role of floral traits in reproductive isolation requires comparisons of the two parental species and natural hybrids with respect to floral traits (Campbell *et al.* 2015). Whether or not hybrids survive and produce fertile descendants by interbreeding with parental individuals depends to some extent on floral traits. Hybrid floral traits may prevent back-crossing to either parental species if they attract novel pollinator guilds (Vereecken *et al.* 2010; Ma *et al.* 2016; Marques *et al.* 2016), promote inappropriate pollen placement, or flower at different times than parental species (*i.e.* temporal or phenological isolation). Of all the traits known to affect reproductive isolation in plants, floral scent remains the least well studied (Waelti *et al.* 2008; Bischoff *et al.* 2014). Previous studies suggest that floral scent in hybrids can show at least three

different patterns in relation to parental species: 1) chemically additive, intermediate scent profiles (Cortis *et al.* 2009; Campbell *et al.* 2016), 2) the absence of certain compounds present in parental species (Bischoff *et al.* 2015) or 3) the presence of novel (transgressive) compounds not found in either parental species (Vereecken *et al.* 2010, Marquez *et al.* 2016). Manipulative experimental studies have shown that specific volatile compounds can mediate pollinator attraction in hybrid systems, and can elicit feeding behaviour in conjunction with flower colour and posture among hybrids of *Ipomopsis* in North America (Bischoff *et al.* 2015) and *Zaluzianskia* in South Africa (Campbell *et al.* 2016), respectively. One question emerging from these studies is whether pollen vectors (hawkmoths in both cases) were more likely to visit both parental species (promoting introgression) due to a plume of floral scent enveloping the hybrid swarm. This question mirrors the "hybrid bridge" hypothesis proposed by Floate & Whitham (1993), in that plant host shifts by herbivores can be facilitated by the presence of morphologically and/or chemically intermediate hybrid plants. If so, floral scent in a hybrid swarm might enhance gene flow between related parental species that are reproductively isolated under normal conditions, as a hybrid bridge for pollinators, as suggested by Aldridge & Campbell (2007).

A favourable system in which to study the role of floral scent in reproductive isolation and hybrid swarm dynamics is found in the genus *Mandevilla* (Apocynaceae) in southern South America. *Mandevilla pentlandiana* (DC.) Woodson and *M. laxa* (Ruiz & Pav.) Woodson are sister species (Simões *et al*. 2006) that occur in sympatry throughout Andean Bolivia and extreme north-western Argentina (Fig. S1), where they are pollinated by distinct pollinator groups, diurnal Hymenoptera and nocturnal hawkmoths, respectively (Torres & Galetto 1998; Moré *et al*. 2007). Across this large zone of sympatry, putative hybrids between *M. pentlandiana* and *M. laxa* have not been recorded, although the possibility that a third species, *M. grata*, may actually be of hybrid was suggested by Ezcurra (2005). According to Ezcurra, this rare endemic species is similar to *M. laxa* but it has smaller flowers and erect corolla lobes, features of *M. pentlandiana.* However, only two type specimens of *M. grata* are known from a single locality (now extinct), making it difficult to test whether it has a hybrid origin. Recently, we have discovered a new mixed population of *M. laxa* and *M. pentlandiana* at the

southern extreme of their distributions in north-central Argentina (Fig. S1), along with many individuals showing intermediate vegetative and floral traits, such as flower size, shape and leaf thickness. These individuals cannot be clearly assigned to any *Mandevilla* species, even to *M. grata*. There are at least three potential explanations for the phenotypic variation observed in this mixed population; 1) that it represents a rare breach of the species boundaries between *M. laxa* and *M. pentlandiana*, resulting in a recent hybrid swarm, 2) that intermediate individuals actually belong to one of the parental species, but having extreme phenotypic traits, or 3) the presence of a third species, without a hybrid origin. For the remainder of this paper, we refer to this morphologically intermediate population of *Mandevilla* plants as putative hybrids, with the understanding that we do not yet have genetic evidence that would allow us to determine their origin.

Our main goal is to find out whether these individuals showing intermediate floral morphology between *M. laxa* and *M. pentlandiana* actually represent hybrid individuals of these two species. Thus, We characterized the distribution of standing variation in flower shape and the chemical composition of floral scent of all flowering plants in our study population. We assessed individual grouping through taxonomically relevant traits and through an unsupervised learning algorithm. While the first is an *a priori* classification, the second is an unbiased attempt to identify morphological groupings without any prior information by combining geometric morphometric data with statistical procedures from the field of pattern recognition (Baylac *et al*. 2003). Moreover, to address the role of pollinators in the origin of these hybrid individuals we assessed the possibility of pollen flow among parental species and putative hybrids, using pollen analogues to experimentally evaluate the movement of floral visitors as pollen vectors.

Materials and methods

Study system, species description and distribution.

Mandevilla laxa and *M. pentlandiana* (Apocynaceae) are perennial vines that grow in woodland environments, and may occur in sympatry in central and northern Argentina and Bolivia (Fig. S1). Field observations were made during the flowering season, from November 2013 to

February 2014 in the "Reserva Hídrica Natural Parque La Quebrada", Córdoba Province, Argentina, representing the southernmost distribution of both species (Fig. S1). *Mandevilla pentlandiana* produces small and dense racemes, whereas *M. laxa* exhibits large and sparse racemes. *Mandevilla laxa* has salverform flowers with white corollas markedly larger than the tubular greenish flowers of *M. pentlandiana* (Fig. 1, upper panel). The flowers of *M. pentlandiana* emit a weak fruity fragrance resembling that of ripe melon and produce concentrated sucrose-rich nectar (44.7% w/w; Torres & Galetto 1998). In contrast, *M. laxa* flowers emit an intense jasmine-like fragrance and produce nectar with more variable sugar concentration than that of *M. pentlandiana* (27.7% w/w to 44.2% w/w; Torres & Galetto 1998). Flowers of both species remain open for 3-4 days. Elsewhere in Argentina, *Mandevilla laxa* is pollinated in the evening by nocturnal long-tongued hawkmoths (Lepidoptera: Sphingidae), and is also visited during daytime by hummingbirds and honeybees (Moré *et al.* 2007). In contrast, *M. pentlandiana* is pollinated primarily by honeybees and bumblebees during daytime and occasionally receives visits by hummingbirds (Torres & Galetto 1998).

Although both species are self-compatible, autogamy does not occur in unvisited flowers due to strict herkogamy, *i.e.* flowers depend upon the visit of pollinators to set fruits (Torres & Galetto 1999; Moré *et al.* 2007). *Mandevilla* species show an extremely precise and complex pollination mechanism. The anthers are united with the style-head, forming a complex organ, the gynostegium. The boundaries between adjacent anthers are represented by five longitudinal slits. Thickened stigmatic regions, called "style-heads", consist of an upper secretory part and a lower receptive part. The style-head is completely enclosed within the lower part of the anther-cone, with five grooves alternating with the slits between the anthers, forming five longitudinal pollination chambers. Pollination is achieved when pollinators' proboscis are inserted into the corolla tube through any of the five inter-staminal spaces between the fused anthers. When the proboscis is withdrawn, it is caught by the base of the corresponding anther-cone slit. During withdrawal pollen is attached onto the proboscis when it passes along each cone slit. If the proboscis was already carrying pollen, this is first deposited on the stigmatic concavity beneath the style-head (Moré *et al.* 2007).

Flower morphology

We used geometric morphometrics to characterize the flower shape variation among *M. laxa, M. pentlandiana* and their putative hybrids. Geometric morphometrics of floral shape is a growing field of study because it enables the interpretation and visualization of variation patterns at different levels: within populations (*e.g.* Gómez *et al.* 2006; Benitez-Vieyra *et al.* 2009), among populations (Gómez *et al.* 2008) or among species (van der Niet *et al.* 2010; Kaczorowski *et al.* 2012). In landmark-based geometric morphometrics, the spatial information missing from traditional morphometrics is contained in the data, because the data are coordinates of *landmarks*: discrete anatomical loci that are arguably homologous in all individuals in the analysis (Bookstein 1997; Zelditch *et al.* 2012).

One to five flowers (mean = 2.08) from all flowering individuals (n=63) in the community were sampled and preserved in 70% ethanol. Only first-day flowers were sampled to avoid differences due to flower senescence. Data were averaged per individual. Digital photos were taken of sagittal sections of flowers along with a 50 mm reference scale, such that three of the five petals were visible (Fig. 1, lower panel). A total of 13 "landmarks" were selected. Five landmarks were anatomical: insertion of the calyx into the petiole (1, 2), insertion of the style into the ovary (3), constriction of the entrance to the nectar chamber (4, 5) and the inflection point between the corolla tube and the corolla lobes (11, 12). The remaining eight landmarks were points of minimum or maximum curvature: tip of the sepals (6, 7), base of the anther-cone (8, 9), tip of the anther-cone (10) and tip of the central petal (13). Landmark configurations of each individual were obtained using the program TpsDig ver. 2.16 (Rohlf 2010). A generalized Procrustes analysis on the landmark coordinates considering the bilateral symmetry of flowers was performed using the program MorphoJ (Klingenberg 2011). This analysis involves three steps: (1) translation of the landmark configuration of all objects so that they share the same centroid; (2) scaling of the landmark configuration so that they have the same centroid size; and (3) rotation of the landmark configurations to minimize squared Euclidean distances between homologous landmarks (Zelditch *et al.* 2012). In this way, only shape information is retained in the data matrix of Procrustes coordinates. Landmark configurations from flowers of the same individual

were averaged after superimposition. A Principal Component Analysis (PCA) was then performed on the Procrustes coordinates. Morphological changes were visualized using the transformation grids from MorphoJ program (Klingenberg 2011).

Classification

Even though *M. pentlandiana* and *M. laxa* differ in many vegetative (leaf shape, thickness and hairiness, pers. obs.) and floral traits (corolla size and corolla lobe shape; Ezcurra 2005), the presence of intermediate individuals makes it difficult to test whether these individuals were extreme phenotypes of the putative parental species, or whether they were the result of hybridization. We followed two approaches to classify flowering individuals (n = 63) into groups, the first one following the taxonomic descriptions of the putative parental species, and the second using an unsupervised learning algorithm, allowing us to detect morphological discontinuities in the studied population. An *a priori* classification was needed for purely operative reasons, in order to examine pollen flow between different groups and taking samples for floral fragrances.

First, we divided the individuals into three groups using two taxonomically relevant floral traits (flower length and corolla lobe length) following Ezcurra (2005). An individual was considered to be an "intermediate" or putative hybrid when its flowers were too large to be assigned to *M. pentlandiana* (flower length > 15 mm or corolla lobes > 3mm) or too small to be assigned to *M. laxa* (flower length < 23 mm or corolla lobes < 13 mm). Second, we applied a Gaussian finite mixture analysis to the geometric morphometric data, a method used to identify morphological groupings without any prior information on the identity of the individuals (Baylac *et al*. 2003; Cordeiro-Estrela *et al*. 2008). This method assumes that observations are the result of mixed components, each one following a (multivariate) Gaussian distribution. Data generated by mixtures of multivariate normal densities are characterized by groups or clusters centred at the means, with increased density for points nearer the mean. Geometric features (shape, volume, orientation) of the clusters are determined by the covariance matrix. A 'best' model can be estimated by fitting models with differing

parametrizations (cluster mean, shape, volume and orientation) and/or numbers of components to the data by maximum likelihood, and then applying a statistical criterion for model selection. We performed this analysis using the *mclust* package (Scrucca *et al*. 2016) of R software (R Core Team 2018). We tested models with increasing number of clusters (from 1 to 9) and different parametrizations of the within-group covariance matrix (for details see Fraley & Raftery 2007), and choose the best model based on its Bayesian Information Criterion (BIC). The Gaussian finite mixture analysis was performed in the reduced space of the first two principal components of the Procrustes coordinates.

Characterization of the chemical composition of flower fragrance

Chemical composition of flower fragrance was studied by means of two complementary methods, solid phase microextraction (SPME) fibers (Goodrich *et al*. 2006) and dynamic headspace (Raguso & Pellmyr 1998) combined with gas chromatography and mass spectrometry (GC-MS; see Supplementary Material). Thirteen SPME samples were collected: three from *M. laxa*, six from *M. pentlandiana* and four from putative hybrids, according to the *a priori* taxonomic classification (see above). For dynamic headspace, 24 samples were obtained, 11 of them at night and 13 during daytime.

Fragrance emission rates were obtained from dynamic headspace samples. Differences in emission rates among parental species and the putative hybrids and between periods (diurnal vs. nocturnal) were compared using Kruskal-Wallis or Wilcoxon rank sum test, respectively. The chemical composition of floral fragrances was compared using Non-Metric Multidimensional Scaling (NMDS), an ordination method based on a dissimilarity matrix, that depicts sample coordinates in a low-dimensional geometric space. The Bray-Curtis index was used to build a dissimilarity matrix (Dötterl *et al.* 2009). This index generates values between 0 (all compounds are shared) and 100 (they are completely dissimilar). Stress, which illustrates how the obtained 2-D configuration departs from the observed distance matrix, was determined, with lower values of stress indicating a better fit. We

also performed an analysis of similarities (ANOSIM) to evaluate qualitative and quantitative differences in scent composition among samples. ANOSIM is a commonly used multivariate procedure that directly operates on a similarity matrix. It yields a test statistic *R* that is a relative measure of separation between *a priori* defined groups, based on differences of mean ranks between and within groups. An *R* value of "0" indicates a completely random grouping, whereas a value of "1" indicates that samples within groups are more similar to each other than to any sample from a different group (Dötterl *et al.* 2009). Statistical significance of *R* was assessed by 10,000 random permutations of the grouping vector to obtain an empirical distribution of *R* under the null model. All statistical analyses were performed using R software (R Core Team 2018).

Flower visitors

A total of fifty observation periods, each one of 30 min, were done throughout the flowering period in *M. laxa*, *M. pentlandiana* and putative hybrids between November 2013 and February 2014. In each observation period, a different focal plant was chosen. Flower visits were recorded during morning (09:00 to 11:30 hrs.), afternoon (14:00 to 18:30) and evening (19:00 to 21:00) in a total of 866 flowers (215 in *M. laxa,* 304 in *M. pentlandiana* and 347 in the putative hybrids). Observations were omitted between 11:30 and 14:00 hours because preliminary studies indicated no floral visitation during this time interval, presumably due to high temperatures. Animal visitors were identified to species whenever possible, and to order if not. Visitation frequency was calculated as visits $\cdot$ flower^{-1} $\cdot$ hour^{-1}. Differences in visitation rates among parental species and putative hybrids (following the taxonomic classification, see above) and among observation periods were examined using Kruskal-Wallis tests.

To analyse if *M. laxa, M. pentlandiana* and putative hybrids share pollinators and, thus, if pollen flow was possible among them, we calculated the proportional similarity indices (PS) of flower visitor assemblages (Smith *et al.* 2008). This measurement takes into account both the number of visitors shared and their visitation frequency. PS is calculated as $1 - \frac{1}{2} [(\Sigma(P_{ai} - P_{bi})]$, where P_{ai} and P_{bi}

are the proportions of the total visitation rate made up by the ith flower visitor to a^{th} and b^{th} taxa, respectively. PS values range from 0 to 1, with higher values indicating greater overlap in flower visitors.

Because moth pollination is difficult to detect by direct observation at night, and previous records had indicated that hawkmoths are important pollinators of at least *M. laxa* (Moré *et al.* 2007), flowers of *M. laxa* (n=75), *M. pentlandiana* (n=75) and putative hybrids (n=75) were inspected for the presence of moth scales using a binocular microscope (as described by Nilsson & Rabakonandrianina, 1988). In addition, we inspected the proboscides of hawkmoth individuals belonging to four species that were captured by our colleagues (Beccacece *et al.* 2011) at the study site during the flowering season of *M. laxa*, *M. pentlandiana* and putative hybrids, using a binocular microscope to confirm whether they were carrying pollen from any of them. Hawkmoth individuals are deposited at the GICLA (Group for Research and Conservation of Lepidoptera from Argentina) collection of the Museum of Zoology at the Universidad Nacional de Córdoba (MZUNC). Pollen loads attached to the hawkmoths' proboscises were compared with reference pollen samples taken from flowers of *M. laxa, M. pentlandiana* and their putative hybrids and mounted in glycerine jelly semi-permanent preparations (see Kislev *et al.* 1972).

Movement of flower visitors

This complex pollination mechanism of *Mandevilla* species (see above) makes it difficult to assess pollen transfer between flowers of different individuals in a straightforward manner. Thus, we used fluorescent powders as pollen analogues to estimate the movement of flower visitors in the population (Kearns & Inouye 1993). Individuals received a specific fluorescent powder of a given colour as follows: blue for *M. laxa*, yellow for *M. pentlandiana* and red for the putative hybrids, according to the *a priori* taxonomic classification. Fluorescent powders were placed within the corolla, surrounding the anther-cone using a fine bristle brush in about 1/3 of the open flowers at the study site. After three days, the remaining 2/3 of the open flowers were collected (n=335 for *M.*

pentlandiana, 190 for putative hybrid individuals and 44 for *M. laxa*). Flowers were inspected under an epifluorescence microscope (Leica DMLB equipped with a digital camera Leica DC 250) using filters of different colours to maximize visualization (blue filter for the blue powder and yellow filter for the remaining yellow and red powders). Thus, we determined the percentage of flowers that received analogues of pollen from the same or a different colour. We considered the presence of analogues of pollen in a flower as an indicator of a floral visit but not necessarily of a successful pollination. We tested whether the number of flowers that delivered or received pollen analogues departed from null expectations based on the abundance of *M. laxa, M. pentlandiana* and putative hybrids in the population using a χ^2 independence test. Expected numbers of flowers were calculated from marginal totals.

Results

Flower morphology

More than 95% of total variance in the configuration of floral landmarks was explained by the first two PCA axes (85.72% by PC1 and 9.62% by PC2). Individuals of the studied community were arranged in the morphospace determined by the first two axes, where the two putative parental species represented the minimum and maximum extremes of morphological variability (Fig. 2). Many putative hybrids appeared intermingled with *M. laxa* individuals. Variation in PC1 loadings was mainly represented by changes in the elongation of the corolla tube (landmarks 1, 2, 4 and 5) and in the corolla lobes shape (landmarks 11, 12 and 13; Figs. 1, 2). Individuals from *M. laxa* showed the maximum values in PC1 loading scores, showing both an elongated corolla tube and a well-developed, star-shaped limb. Meanwhile, *M. pentlandiana* individuals showed the minimum values in PC1 loading scores, showing a short corolla tube and reduced corolla lobes. Variation in the PC2 loadings was mainly represented by the position of the anther-cone (landmarks 8, 9 and 10) and the constriction to the nectar chamber entrance (landmarks 4 and 5; Figs. 1, 2), which is retracted in the minimum values but located towards the apex of the tube in the maximum ones. Moreover, in both

PC1 and PC2 the relative position of the sepals (landmarks 6 and 7) varied in relation to the corolla tube entrance (landmarks 11 and 12; Fig. 1).

Classification

Taxonomic classification based on two relevant floral traits assigned 17 individuals to *M. laxa* and 21 to *M. pentlandiana*. The 24 remaining individuals were not assignable to either of these taxa (Fig. S4). The Gaussian mixture analysis indicated that the best model included four groups. Alternative solutions had BIC values lower by at least two points, a difference which may be considered as significant following the empirical criteria of Fraley & Raftery (1998). The first cluster included all the individuals belonging to *M. pentlandiana* following the previous classification, and one putative hybrid or intermediate individual. The second and third clusters involved nine and eigth intermediate individuals, respectively. The fourth one included all the individuals belonging to *M. laxa* and six intermediate individuals (Fig. 2).

Characterization of the chemical composition of flower fragrance

Qualitative analysis

Nine volatile organic compounds (VOCs) were identified from floral headspace using SPME-GC-MS (Table S2). Two VOCs found in low amounts were exclusive to *M. pentlandiana* and were not detected using dynamic headspace analysis: an unidentified monoterpene and an unidentified sesquiterpene (classes suggested by mass spectral ion fragments). Sesquiterpenes were the most representative compounds in the floral scent of *M. pentlandiana*, dominated by (*E,E*)-α-farnesene, whereas monoterpenes dominated the scent of *M. laxa*, with linalool being the most abundant compound. Benzenoid esters also were present in *M. laxa*, with methyl benzoate present in greater amounts than methyl salicylate. The scent of intermediate individuals was dominated by two monoterpenoids, linalool and 1,8-cineole, and shared the presence of linalool and methyl salicylate with *M. laxa*, whereas no volatiles were shared with *M. pentlandiana* (Table S2).

Quantitative analysis and emission rates

The chemical complexity, species-specificity and temporal patterns of scent composition varied markedly between taxa. Eighteen different VOCs were detected using dynamic headspace methods. These VOCs included monoterpenes, sesquiterpenes and benzenoids as described above, as well as hydrocarbons (Table S3, Fig. 3). A total of 11 additional VOCs not detected using SPME fibers were identified here, including monoterpenoids (sabinene, *β*-myrcene, (*E*)-*β*-ocimene, *α*-terpinolene and *α*-terpineol), irregular terpenoids (*trans*-TMTT ((*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene) and *trans*-DMNT ((*E*)-4,8-dimethylnona-1,3,7-triene), benzenoids (benzaldehyde, benzyl alcohol, benzyl benzoate) and the hydrocarbon pentadecane (see Table S3). In *M. pentlandiana*, eight volatiles were recorded, both in daytime and night-time samples, whereas *trans*-DMNT and *trans*-TMTT were detected at night but were present only in traces during daytime. The *trans*-DMNT and pentadecane were exclusive to *M. pentlandiana*. In *M. laxa*, 11 compounds were detected, two of them only in trace amounts (1,8-cineole and *α*-terpineol), with *β*-myrcene emitted exclusively at night and a farnesol isomer and *trans*-TMTT only during daytime. Benzaldehyde, methyl benzoate and benzyl benzoate were exclusive to *M. laxa*, whereas all samples emitted methyl salicylate (Table S3). Finally, the headspace of putative hybrids contained 13 VOCs, with seven emitted only during daytime: linalool, *α*-terpineol, methyl salicylate, benzyl alcohol, (*E,E*)-*α*-farnesene, farnesol and heptadecene. Four VOCs were specific to the putative hybrids: sabinene and *α*-terpinolene (emitted during day and night) and benzyl alcohol and *α*-terpineol (emitted only during daytime). Compounds shared with *M. pentlandiana* were 1,8-cineole, (*E,E*)-*α*-farnesene and heptadecene, whereas those shared with *M. laxa* were *β*-myrcene, linalool and *α*-terpineol (Table S3).

Total scent emission rates, calculated per g dry floral mass per inflorescence per hour, did not differ significantly between parental species and the putative hybrids (Kruskal-Wallis test, $H = 3.375$, d.f. = 2; $P = 0.185$) or between diurnal and nocturnal samples (Wilcoxon test, *M. pentlandiana*, $W = 4$, $P = 0.343$; putative hybrids $W = 9$, $P = 0.786$; *M. laxa*, $W = 8$, $P = 1.0$). However, the dry mass

of one flower of *M. laxa* (0.1043 ± 0.0229 g; *n* = 53) is, on average, five-fold larger than that of *M. pentlandiana* (0.0253 ± 0.0082 g; *n* = 201). Thus, each *M. laxa* flower produces much larger amounts of fragrance than *M. pentlandiana* flowers, due to scaling of floral mass.

The NMDS analysis of headspace data suggested the presence of clusters in the fragrance phenotypic space, each corresponding to *M. laxa, M. pentlandiana* and their putative hybrids, with the single exception of a diurnal sample from a putative hybrid individual, which clustered among *M. laxa* samples. These clusters differed significantly in fragrance chemical composition (ANOSIM, *R* = 0.807, *P* = 0.0001; Fig. 3). Six VOCs, mainly monoterpenoids, clearly separated putative hybrids from *M. laxa* and *M. pentlandiana* (compounds 1 to 6; Fig 3).

Floral visitors and visitation rates

Flowers of *M. pentlandiana* were visited by a wide array of animals including honeybees (*Apis mellifera*) and other Hymenoptera (*Bombus opifex* and wasps), Diptera (hoverflies and flesh-flies), hummingbirds (*Chlorostilbon aureoventris*; Trochilidae) and noctuid moths (Lepidoptera: *Rachiplusia* sp.). Hawkmoths (Sphingidae) were not directly observed during the formal observation periods of this study although an *Agrius cingulata* individual (Sphingidae) was seen visiting flowers outside the observation periods. No lepidopteran scales were recorded in the flowers of *M. pentlandiana*. Putative hybrid flowers were visited by *C. aureoventris* hummingbirds, flies, *Apis mellifera* and wasps. A total of 2.7% (n = 75) of examined flowers of putative hybrids had lepidopteran scales, and an unidentified hawkmoth was observed visiting its flowers outside the observation periods. Finally, flowers of *M. laxa* were visited by hummingbirds (*C. aureoventris* and *Sappho sparganura*) and Diptera (hoverflies and flesh-flies, Table 1). No scales were recorded in the examined flowers of *M. laxa*. Four out of ten trapped hawkmoths carried pollen from *M. laxa* attached to their proboscis (*Manduca rustica*, *n* = 2; *Manduca diffissa, n* = 1; and *Manduca sexta*, *n* = 1).

Flowers of *M. pentlandiana* were visited more frequently (Kruskal-Wallis test, $H = 9.80$, d.f. = 2, $P = 0.007$, Table 1) than those of *M. laxa* and the putative hybrids, which did not differ significantly from each other. No significant differences were recorded at different observation times (morning, afternoon, evening) or within parental species and the putative hybrids (*M. pentlandiana*, $H = 2.68$, d.f. = 2, $P = 0.268$; putative hybrids, $H= 2.31$, d.f. = 2, $P = 0.32$; *M. laxa*, $H= 1.75$, df = 2, P = 0.42). *Mandevilla laxa* and the putative hybrids showed the highest overlap in their visitor assemblage, according to proportional similarity index (*M. laxa*-putative hybrids, PS = 0.74, Table 1). There was a high degree of differentiation between the visitor assemblage in the other two pairs: *M. pentlandiana*-putative hybrids, PS = 0.08; *M. pentlandiana*-*M. laxa*, PS = 0.10 (Table 1).

Movement of flower visitors

Roughly one third of the 569 analysed flowers (189 of 335 for *M. pentlandiana*; 24 of 44 for *M. laxa* and 115 of 190 for hybrid flowers) received fluorescent powders delivered by flower visitors as pollen analogues. Observed visitor movements departed significantly from null expectations based on relative abundance ($\chi^2 = 221.98$, df = 4, P < 0.0001; Fig. 4 a, b). Intraspecific movement was higher than expected in *M. laxa, M. pentlandiana* and their putative hybrid, indicating that some degree of assortative flower visitation is occurring (Fig. 4 b). However, observed flow of pollen analogues between *M. laxa* and putative hybrids did not differ from expected values, indicating that flower visitors are freely moving between them. In contrast, despite the greater abundance of flowers of *M. pentlandiana* in the community, movement of flower visitors between *M. pentlandiana* and other individuals was lower than expected (Fig. 4 a, b).

Discussion

Our findings suggest incomplete reproductive isolation among the parental species and the putative hybrids. This suggestion is supported by the morphological intermediacy of most putative hybrids, and the presence of some individuals which cannot be morphologically distinguished from

M. laxa. Remarkably, we detected a transgressive segregation pattern in the chemical composition of floral fragrances, *i.e.* the presence of novel or extreme traits in hybrid organisms beyond the phenotypic range of parental species. Even though we did not observe hawkmoths visiting during night time, evidence from circumstantial observations, pollen loads carried on probosces, and the presence of scales, suggest that they could pollinate flowers from *M. laxa*, *M. pentlandiana* or putative hybrids. In addition, putative hybrids and *M. laxa* shared hummingbirds as main flower visitors and, accordingly, the movement of pollen analogues was stronger between them. However, whether they effectively act as pollinators could not be confirmed. Preliminary molecular data available for this system (Supplementary Methods and Results, Table S1, Fig S2-S3) are inconclusive: plastid *trn*L-*trn*F was invariable, and the nuclear markers only varied at very few positions. Thus, we are aware that we do not have the genetic data needed to confirm the hybrid nature of intermediate individuals in our study. Alternative hypotheses cannot be completely disregarded: First, intermediate individuals may belong to one of the parental species, but having extreme phenotypic traits. However, *Mandevilla laxa* and *M. pentlandiana* are common species, well represented in botanical collections, yet extreme phenotypic variants have not been recorded before across their combined geographic ranges. Second, a third species without a hybrid origin may be present in the study site. This scenario is unlikely because this hypothetical third species has not been collected, up to the present, in allopatric populations. Instead, intermediate individuals were only found in two contact areas between *M. laxa* and *M. pentlandiana* (Ezcurra, 2005; this study).

Putative hybrid individuals showed an intermediate shape in relation to *M. laxa* and *M. pentlandiana*, a corolla tube less developed than in *M. laxa* but more developed than in *M. pentlandiana*, and a corolla lobe shape which varies in a gradient between the two parental species, being closer to the shape of *M. laxa*. Phenotypic intermediacy has been traditionally used as an indicator to assess the putative hybrid origins in wild populations (Ellis & Johnson 1999; Campbell & Aldridge 2006). Thus, our results are consistent with a hybrid nature of intermediate individuals. The *a priori* taxonomic classification, based on two relevant floral traits, and a finite Gaussian mixture analysis which does not rely on any *a priori* information, revealed three and four groups, respectively.

However, these alternative classifications were not exactly the same: from 24 individuals which were not taxonomically assignable to either *M. laxa* or *M. pentlandiana*, the finite Gaussian mixture analysis assigned 17 to two "morphologically intermediate" groups, one to *M. pentlandiana* and the remaining six clustered together with *M. laxa* individuals (Fig. 2). Geometric morphometric analysis has been applied to discriminate among parental species and their hybrids in plants (Shipunov & Bateman 2005; Lexer *et al.* 2009; Peñaloza-Ramírez *et al.* 2010; McCarthy *et al.* 2016). To our knowledge, modern pattern-recognition approaches like finite Gaussian mixture analysis have not yet been applied in preliminary investigations of ill-defined plant species. However, this approach is growing as a useful tool in the identification of animal cryptic species after the proposal of Baylac *et al.* (2003).

In contrast with floral shape, NMDS ordination of floral fragrance revealed that the putative hybrid individuals do not produce a chemically additive, intermediate scent between the two parental species. Instead, the fragrance patterns among *M. laxa, M. pentlandiana* and their putative hybrids include transgressive segregation (Rieseberg *et al.* 1999; Seehausen 2004). Putative hybrid individuals emitted exclusive VOCs -benzyl alcohol and two structurally related monoterpenes (sabinene, α-terpinolene)- as well as increased amounts of the monoterpenes β-myrcene and α-terpineol (Tables S2, S3). The most parsimonious explanation for these patterns is that these plants emit larger amounts of 1,8-cineole (eucalyptol) than either parental species, and that the additional/unique monoterpenoid compounds actually represent pleiotropic artefacts of increased enzymatic activity or pathway flux leading to 1,8-cineole emission (Table S2). This hypothesis derives from chemical studies of *Nicotiana* (sect. *Alatae*; Solanaceae) pollinated by the same hawkmoth guild in the same region of South America (Raguso *et al.* 2003 & 2006). Enzymatic and genetic studies with these species confirm that the actions of a single multi-product enzyme are sufficient to account for 1,8-cineole as a major product and the suite of monoterpene hydrocarbons (*e.g.* β-pinene, sabinene, β-myrcene, α-terpineol) as minor products (Fähnrich *et al.* 2012), as was determined previously for *Salvia officinalis* (Lamiaceae; Wise *et al.* 1998). The role of novel/transgressive volatiles among putative hybrids is difficult to ascertain without additional behavioural experiments. Our data show that

asymmetrical patterns of pollen movement/gene flow among parental species and putative hybrids may be correlated with or influenced by novel volatile compounds that are not simply additive or intermediate versions of parental phenotypes.

Relatively few field studies have been conducted on floral scent in hybrid plants. Campbell *et al.* (2016) reported both, intermediate flower morphology and volatile compound emission rates of the natural hybrids between *Zaluzianskya natalensis* and *Z. microsiphon* (Orobanchaceae) in South Africa. In constrast, Bischoff *et al.* (2014) described a similar system to our own, with *Ipomopsis aggregata* (Polemoniaceae; primarily hummingbird pollinated) and *I. tenuituba* (primarily hawkmoth pollinated) in the Rocky Mountains of western North America. In their study, hybrid *Ipomopsis* showed a transgressive phenotype; volatiles present in one or both of the parental *Ipomopsis* taxa either were absent in the hybrids (benzyl acetate) or were emitted in significantly greater or lesser amounts in the hybrids than in the parental taxa (pentan-1-ol, lavender lactone, α-pinene).

In our study system, olfactory signals may have contributed to the breakdown of ethological isolation, as floral bouquets combine attractants of different guilds of pollinators. For instance, *M. pentlandiana* flowers are characterized by high emission rates of the sesquiterpenoid (*E,E*)-α-farnesene, a compound common to many bee-pollinated plants (Dötterl & Vereecken 2010), but it also emits low amounts of farnesol, methyl-salicylate, *trans*-DMNT and *trans*-TMTT, all of which have been recorded in moth-pollinated plants (Kaiser 1993; Knudsen & Tollsten 1993; Raguso *et al.* 2006). In turn, *M. laxa* and putative hybrids emit compounds which are characteristic of hawkmoth-pollinated plants (Riffell *et al.* 2013; Raguso *et al.* 2006; Klahre *et al.* 2011), but they also produce terpenoids in lower proportions, which are widely present in bee-pollinated plants (Dobson 2006; Dötterl & Vereecken 2010). Along with morphological features, these mixed volatile bouquets may reduce visitor specificity across floral phenotypes, and thus may have facilitated the initial hybridization between *M. laxa* and *M. pentlandiana* and the subsequent unidirectional interbreeding between *M. laxa* and the putative hybrids, allowing the breakdown of ethological barriers. It remains unclear why similar populations have not been documented from the extensive zone of sympatry between *M. laxa* and *M. pentlandiana*, extending northwards from Jujuy, Argentina into much of

Bolivia. Extensive field and herbarium studies will be needed to determine whether additional introgressive populations have been overlooked, or simply do not occur.

Mandevilla laxa and putative hybrids shared hummingbirds as main flower visitors and, accordingly, the movement of pollen analogues was stronger between them (Fig. 4 b). In contrast, *M. pentlandiana* was mainly pollinated by bees and, despite its abundance, showed the lowest pollen analogue interchange, suggesting that in this system, floral traits are more responsible than the density of floral units in driving pollen transfer dynamics. Previous studies led us to expect higher levels of hawkmoth visitation to flowers of *M. laxa*, which they effectively pollinate elsewhere in Argentina (Moré *et al.* 2007). Spatiotemporal variation in abundance is a hallmark of hawkmoth pollination (Willmott & Búrquez 1996; Aldridge & Campbell 2007; Miller *et al.* 2014), which counterbalances their advantages as long distance pollen vectors and outcrossing agents (Brunet & Sweet 2006; Rhodes *et al.* 2017).

Even though olfactory signals and morphological intermediacy may have contributed to the breakdown of ethological isolation, from the point of view of mechanical isolation, the Apocynaceae family features a complex reproductive system that diminishes the potential for most floral visitors to fertilize these plants. A previous study revealed that the pollination mechanism of *M. laxa* requires a precise trait-matching with the proboscis width of hawkmoth pollinators. Despite being visited by a spectrum of hawkmoth species, it was found that only those with proboscises narrower than 0.7 mm effectively pollinate flowers (Moré *et al.* 2007). The same mechanism seems to operate in *M. pentlandiana* flowers (pers. obs.). We suspect that hummingbirds would be nectar thieves because of the mismatch between beak width and anther cone slit width. However, we do not completely discard that hummingbirds may eventually pollinate some flowers. Future studies using virgin flowers immediately bagged after a hummingbird visit and later checked for fruit set could shed light on this point. In relation to dipteran floral visitors, their proboscides are too short to reach either the nectar or pollen of *Mandevilla* flowers in our study.

Future studies on this system will evaluate the cytogenetic and genetic aspects, along with controlled crosses to produce F1 and F2 hybrids and backcrosses to the parents, to characterize baseline floral morphology and scent chemistry for each hybrid generation. This kind of balanced template for assessing how floral scent and morphology segregate is lacking from most relevant studies of floral introgression (*e.g.* Ippolito *et al.* 2004; Martin *et al.* 2008), and should allow a more meaningful interpretation of segregating traits, and their behavioural consequences, in natural hybrid zones.

Acknowledgements

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Table 1. Visitation rate of flower visitors to *M. laxa*, *M. pentlandiana* and their presumed hybrids expressed as visits per flower per hour. Rates are shown for all the floral visitors together and for each pollinator group in three periods (M = morning, A = afternoon and N = night) and adding up all the periods together. Visitation rate percentages of each pollinator group are shown between parentheses. Different upper case letters show significant differences. No significant differences among observation periods were detected.

	Period	All floral visitors	Apis mellifera (honeybee)	Chlorostilbon aureoventris (hummingbird)	Rachiplusia sp. (settling moth)	Other Hymenoptera (vespids and bumblebees)	Diptera	Main floral visitors
M. laxa	M	0,033	-	-	-	-	0.033	Diptera
	A	0,032	-	0.032	-	-	-	C. aureoventris
	N	0,144	-	0.118	-	-	0.026	C. aureoventris
	All periods	0,07 [A]	-	0.050 (71.43 %)	-	-	0.020 (28.57 %)	C. aureoventris
Putative hybrids	M	0,033	0.008	-	-	0.004	0.021	Diptera
	A	0,089	-	0.089	-	-	-	C. aureoventris
	N	0,872	-	0.872	-	-	-	C. aureoventris
	All periods	0,331 [A]	0.003 (0.91%)	0.320 (96.97%)	-	-	0.007 (2.12%)	C. aureoventris
M. pentlandiana	M	0,49	0.156	0.026	-	0.185	0.123	Other Hymenoptera
	A	1,114	1.114	-	-	-	-	Apis mellifera
	N	0,711	0.344	-	0.033	-	-	Apis mellifera
	All periods	0,660 [B]	0.538 (75.78%)	0.009 (1.27%)	0.122 (17.18%)	-	0.041 (5.77%)	Apis mellifera

Figure legends

Fig. 1. Diversity of flower forms in the studied population. (a) *Mandevilla pentlandiana.* (b) Putative hybrid. (c) *Mandevilla laxa.* Lower panels show the position of the 13 landmarks used for the geometric morphometric analysis. Scale bars equal to 1 cm.

Fig. 2. Flower shape variation within and between *Mandevilla laxa, M. pentlandiana* and their putative hybrids. Shape variation explained by the first two axes of a PCA (n = 62). Symbols indicate the *a priori* classification, based on two relevant taxonomic floral traits: triangles, *M. pentlandiana*; squares, putative hybrid individuals and circles, *M. laxa*. Grey polygons delimits groups according to a finite Gaussian mixture analysis, and unsupervised learning algorithm. Maximum and minimum values from PC1 and PC2 are indicated in relation to the consensus shape.

Fig. 3. Floral scent variation within and between *Mandevilla laxa, M. pentlandiana* and their putative hybrids. Upper panel: Pie diagrams showing the relative contribution of different compound groups to the floral scent bouquets. VOCs were grouped into different chemical classes according to Knudsen *et al.* (2006). Medium panel: Non-metric multidimensional scaling (NMDS) of the floral scent profile based on Bray-Curtis dissimilarities, which were calculated from the total standardized peak areas of the scent compounds collected through dynamic headspace analysis. Symbols indicate: circles, *M. laxa*; triangles, *M. pentlandiana* and squares, putative hybrid individuals. Open symbols represent daytime samples, filled symbols represent night-time samples. Bottom panel: Identified floral volatile compounds and their associated chemical structures. The loci for these compounds in scent space (small black diamonds in NMDS) indicate the importance of these compounds in explaining differences between the samples nearest to these loci and those of other regions in MDS space, as determined by SIMPER analysis. Note that the position of the double bond in compound 9 has not been determined.

Fig. 4. Movement of flower visitors within and between *Mandevilla laxa, M. pentlandiana* and their putative hybrids, as visualized using pollen analogues. (a) Contingency table. Each cell shows the observed (above diagonal) and expected (below diagonal) number of flowers that received fluorescent powders. Their signed contribution to χ^2 (Pearson's residuals) is indicated. (b) Observed movement of flower visitors. The thickness of the arrows represents the observed number of visits. The size of the circles represents the sample size: white for *M. pentlandiana* (n= 335); grey for putative hybrids (n = 190) and black for *M. laxa* (n= 44).

Supporting Information

Supporting Material and Methods. Molecular analyses and characterization of the chemical composition of flower fragrance.

Table S1. GenBank accession numbers and variable sites for all *Mandevilla* sequences used in this study.

Table S2. Volatile organic compounds (VOCs) detected using SPME-GC-MS analyses of floral fragrances in the study system.

Table S3. Volatile organic compounds (VOCs) detected using dynamic headspace-GC-MS of floral fragrances in the study system.

Figure S1. Geographical distribution of *Mandevilla laxa* (blue dots) and *M. pentlandiana* (orange dots). The study population, where we found the morphological intermediate individuals, is shown with a white triangle.

Figure S2. Fragment of ITS alignment (between 50 and 210 bp) of *Mandevilla pentlandiana, Mandevilla laxa* and putative hybrid individuals. Genbank accession numbers and polymorphic sites are shown in Table S1.

Figure S3. Fragments of GBSSI waxy alignment (between 490 and 660 bp above, and 720 and 900 bp below) of *Mandevilla pentlandiana, Mandevilla laxa* and putative hybrid individuals. Genbank accession numbers and polymorphic sites are shown in Table S1.

Figure S4. Linear measurements of two key floral traits (flower length and corolla lobe length). Open dots show individuals not assignable to any taxa. Black dots show individuals that fit either in the typical *Mandevilla laxa* measures (blue box) or in *M. pentlandiana* measures (orange box). Grey box indicates the typical measures of *M. grata*, a third species supposed to be a hybrid according to Ezcurra (2005).

Monoterpenes
Alkenes
Alkanes
Benzenoids
Sesquiterpenes

M. pentlandiana   Hybrids   *M. laxa*

2-D stress = 0.151

Dimension 1

Dimension 2

1. 1,8 cineole
2. sabinene
3. α-terpinolene
4. β-myrcene
5. benzyl alcohol
6. α-terpineol
7. farnesol isomer
8. (E)-β-ocimene
9. heptadecene

10. pentadecene
11. (E)-4,8-dimethylnona-1,3,7-triene
12. (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene
13. linalool
14. benzyl benzoate
15. methyl salicylate
16. methyl benzoate
17. benzaldehyde
18. α-farnesene isomeres

Towards

(a)

	Putative hybrids	M. laxa	M. pentlandiana
Putative hybrids	109 / 63.2	10 / 17.6	38 / 76.2
M. laxa	29 / 23	27 / 6.3	1 / 27.7
M. pentlandiana	42 / 93.8	13 / 26.1	178 / 113.1

Pearson residuals:

8.2

4.0

2.0

0.0

-2.0

-4.0

-5.4

p-value = < 2.22e-16

(b)