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ORIGINAL ARTICLE

Interspecific and intergeneric hybridization in South American Rhamnaceae-Colletieae

Diego Medan · Marina Arbetman · Eugenia E. Chaia · Andrea C. Premoli

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Abstract In Gondwanic *Discaria*, phenotypically intermediate individuals exist between *Discaria chacaye* (G. Don) Tortosa and *Discaria articulata* (Phil.) Miers, and between *D. chacaye* and related *Ochetophila trinervis* (Gillies ex Hook. & Arn.) Poepp. ex Miers. We studied phenology, pollinators, parental crossability, morphological features, and variation patterns on neutral markers. Intermediates occur wherever parental taxa are sympatric or in close proximity. Flowering was synchronous in *D. chacaye* and *D. articulata* for 2 weeks. They also shared three to six pollinator species (depending on site). *O. trinervis*, which flowered later, occasionally overlapped with *D. articulata*, with which it shared two pollinators. Germinable seeds were obtained from hand pollinations only from crosses with *D. articulata* as paternal parent and *D. chacaye* as maternal parent. Sequencing of chloroplast DNA revealed only two haplotypes. One occurred in *D. articulata*, *D. chacaye*, and their intermediates, and the other in *O. trinervis* and its intermediate with *D. chacaye*. Only pure parentals had diagnostic alleles by isozymes, whereas intermediates mainly showed those of *D. chacaye*. Our results confirm intrageneric hybridization between *D. chacaye* and *D. articulata*, and intergeneric hybridization between *D. chacaye* and related *O. trinervis*.

Keywords Chloroplast DNA · *Discaria* · Hybridization · Isozyme electrophoresis · Leaf morphology · *Ochetophila* · Pollination experiments

Introduction

Natural hybridization is common in flowering plants (Stebbins 1959; Rieseberg and Ellstrand 1993; Arnold 1997) between species of both the same and different genera (Knobloch 1972). Hybridization is particularly common within genera and families with perennial habit, such as trees and shrubs with outcrossing breeding system (Ellstrand et al. 1996). For annuals and short-lived perennial herbs with much faster turnover, the presence of barriers to hybridization is a sine qua non for sympatric occurrence (Stebbins 1950). In contrast, recruitment of individuals into populations of long-lived plants may occur much more slowly, and barriers to hybridization may actually be disadvantageous in the long run; for example, some perennial species with distinct ecological, geographical, and phenological traits may retain the ability to hybridize and produce novel recombinants. These may be advantageous in changing environments (Raven 1976) such as during postdisturbance colonization or population expansion (Acosta and Premoli 2010).

Hybridization is particularly relevant in self-incompatible species naturally occurring at low densities and/or with relatively limited dispersal abilities, such as species of

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D. Medan (✉)
Cátedra de Botánica Agrícola, Facultad de Agronomía, Universidad de Buenos Aires, Avda. San Martín 4453, C1417DSQ Ciudad Autónoma de Buenos Aires, Argentina
e-mail: diemedan@agro.uba.ar

M. Arbetman
Universidad Nacional de Río Negro, Sede Andina, 8400 Bariloche, Argentina

M. Arbetman · E. E. Chaia · A. C. Premoli
Laboratorio Ecotono, Universidad Nacional del Comahue e INIBIOMA-CONICET, Quintral 1250, 8400 Bariloche, Argentina

Fig. 1 Representative branches of parentals (*upper row*) and intermediates (*lower row*). **a** *Discaria articulata*; **b** *D. chacaye*; **c** *Ochetophila trinervis*; **d** *D. chacaye* × *D. articulata*; **e** *D. chacaye* × *O. trinervis*. All from herbarium vouchers deposited at BAA. *Bar* 5 cm

Rhamnaceae inhabiting temperate latitudes of southern South America. Nevertheless, the maintenance of species identity in the face of potential interspecific gene flow is an evolutionary puzzle. Numerous studies have discussed the possibility that divergence may occur even in sympatry, i.e., in the absence of geographical barriers. Whereas adaptive divergence is possible under a model of ecologically driven disruptive selection, known examples are still very few (Barulenga et al. 2006). A major challenge is to demonstrate that species identity can be maintained after secondary contact between related species (Petit et al. 2003).

The family Rhamnaceae includes a single genus of trees and shrubs with Gondwanic distribution (*Discaria* Hook., Medan and Schirarend 2004). Two *Discaria* species, *Discaria chacaye* (G. Don) Tortosa and *Discaria articulata* (Phil.) Miers, together with two taxa recently transferred from *Discaria* to the genus *Ochetophila* Poepp. ex Endl., namely *O. nana* (Clos) Kellermann, Medan and Aagesen and *O. trinervis* (Gillies ex Hook. & Arn.) Poepp. ex Miers (Kellermann et al. 2005; Medan et al. 2009), inhabit southernmost South America (Tortosa 1983a). *D. chacaye* has the widest distribution area, which partially overlaps with those of all other species. It grows in diverse environments, ranging from temperate humid *Nothofagus* forest of Chile and Argentina to xeric *Austrocedrus chilensis* forest and Patagonian shrubby steppe, while *O. trinervis* and *D. articulata* are restricted to more xeric environments. In the drier habitats all three species may coexist (Tortosa 1983a, b, 1999).

Plants morphologically intermediate between *D. chacaye* and *D. articulata*, and between *D. chacaye* and *O. trinervis*, are found in sympatry with pure individuals (Fig. 1). Based on morphological evidence, Tortosa (1983b) suggested that intermediate forms are hybrids between the presumed parental species, but this hypothesis has so far remained untested. It is important to note that morphological intermediacy is not always associated with hybrids (Morrell and Rieseberg 1998; Park et al. 2003).

D. articulata, *D. chacaye*, and *O. trinervis* belong to the monophyletic tribe Colletieae (Aagesen 1999). They share characteristics such as proliferating synflorescences, presence of spines, opposite and decussate leaves with deciduous blades, general preference for xeric habitats with sandy or rocky soils, and considerable inter- and in some cases intraspecific variation in habit (e.g., from prostrate shrubs to 12-m-tall trees in *D. chacaye*). Structural

variations may be ascribed to different strategies for adaptation to environmental conditions, especially xeromorphy (Tortosa et al. 1996). Another common feature of these plants is that their roots may be colonized by the same strains of the endophytic nitrogen-fixing actinomycete *Frankia* (Chaia et al. 2006).

Previous reports indicate that pollen transport in *Discaria* and *Ochetophila* is mediated by nectar- and pollen-seeking insects, sometimes aided by wind, and that movement of pollen between plants is essential for reproduction due to prevailing self-incompatibility in both genera (Medan 1991, 1993, 2003; Medan and Devoto 2005). Thus, hybridization events could be the outcome of interspecific pollen transfer caused by flower pollinators, provided that (1) their reproductive phenologies are temporally coincident, and (2) the species share some flower pollinators.

We used hand pollinations, morphological assessments, and genetic analyses to characterize patterns of hybridization and reproductive isolating barriers between species of *Discaria* and *Ochetophila*. Specifically we asked: (1) Do the species share pollinators? (2) Are there phenological differences among these species? (3) Are intermediates present in sympatric populations? (4) Are sympatric species reproductively isolated? (5) Are pure entities morphologically distinct? (6) Do they hybridize at present? (7) Have pure taxa maintained species identity in the face of interspecific and intergeneric gene flow?

Materials and methods

Plant material

All study species are self-incompatible shrubs or small trees which reproduce only sexually (Medan et al. 1999; Medan and Devoto 2005; D. Medan, unpublished results). Flowers are perfect, small (4–5 mm diameter), and insect pollinated (Medan and Schirarend 2004). The widespread and southernmost *D. chacaye*, commonly known as *chacay* and *chacay de la cordillera*, grows from 33°S (Chile) and from NW of Neuquén (Argentina) to 55°S in Tierra del Fuego, while the relatively range-restricted *D. articulata*, called *mata negra*, occurs from 36°S to 38°S in Chile, and from the north of Neuquén to the west and central part of Chubut in Argentina, and the northernmost *O. trinervis*, known as *chacay*, occurs from 31°S to 48°S (Tortosa 1983a). The wide-ranging *D. chacaye* grows in a great variety of environments, where it attains different lifeforms, as a tree reaching 15 m of height in wet *Nothofagus dombeyi* to open, relatively dry *Austrocedrus chilensis* forest and as a shrub about 5 m tall forming dense matorrals at the forest–steppe ecotone. Also it may be found along streams and nearby permanent or temporarily inundated areas (SIB 2012). The geographically narrow *D. articulata* occupies open areas usually as understory vegetation, mostly as small shrub of dryland forest dominated by *Austrocedrus* (Cupressaceae) and mixed matorrals with *ñire Nothofagus antarctica*, *laura Schinus patagonicus*, and *retamo Diostea juncea* (SIB 2012). The small tree or shrub *O. trinervis* grows along a rainfall gradient in northwest Patagonia, from the ecotone between mesic forest and steppe to the shrubby steppe, either as a riparian plant along rivers and streams or distant from watercourses (Reyes et al. 2011). Under field conditions, individuals intermediate between pure taxa occur generally at low density, *D. chacaye* × *D. articulata* being far more frequent than *D. chacaye* × *O. trinervis* (Tortosa 1983b). These five forms (hereafter referred to as "parentals" and "intermediates") were considered in this work, with the addition of the hypothetically possible intermediate *D. articulata* × *O. trinervis*.

Parentals and intermediates from all study sites (see below) were documented by depositing herbarium vouchers at the Herbario Gaspar Xuárez, Buenos Aires (BAA).

Study sites

We worked in the period 1995–2007 at nine sites located along 1.5° of latitude in the Provinces of Neuquén and Río Negro: Arroyo Pedregoso (hereafter AP), Bariloche (B), Catán Lil (CL), Confluencia Traful (CT), Lago Huechulafquen (LH), Lago Queñi (LQ), Lago Tromen (LT), La Lipela (LL), and Puerto Canoa (PC) (Table 1). Among the four sites that had populations of two or all three parentals, two sites (LH and B) included *D. chacaye* × *D. articulata* intermediates, and one site (B) also had *D. chacaye* × *O. trinervis* intermediates.

Community- and species-level pollination studies were simultaneously conducted at most sites (see Devoto et al. 2005, 2009 for additional details). Four to five observers collected data over a period of 7–8 days in mid-December over approximately 10 ha around a base camp. Sites CL and PC were visited for only 1 day for accessory sampling.

Pollinator assemblages

We observed flower visitors during walks along transects. We sampled on each plant species at different times of the day and on different days in order to record visitor profiles as completely as possible (cumulated observation time: ca. 270 h). An insect was recorded as a flower visitor when it foraged on a flower in such a way that pollen removal from anthers or deposition on stigma was possible. Following a common practice in community-scale studies, we assumed that all visitors defined in this way contribute to pollination, hereafter being referred to as pollinators. We collected 631

Table 1 Location of study sites, arranged by increasing latitude, and estimated size of populations of parentals and intermediates

Site (abbreviation) [date(s) of work at site]	Geographic coordinates	Number of individuals				
		D. articulata	*D. chacaye* × *D. articulata*	*D. chacaye*	*D. chacaye* × *O. trinervis*	*O. trinervis*
Lago Tromen (LT) [1998]	39°34′S 71°26′W			$n > 1{,}000$		100–1,000
Catán Lil (CL) [1995]	39°37′S 70°34′W					$n < 100$
Puerto Canoa (PC) [1997]	39°45′S 71°30′W			100–1,000		
Lago Huechulafquen (LH) [1997, 1999, 2003]	39°48′S 71°12′W	100–1,000	100–1,000	100–1,000		$n > 1{,}000$
Lago Queñi (LQ) [1999]	40°09′S 71°43′W			100–1,000		
Arroyo Pedregoso (AP) [2001]	40°37′S 71°35′W			100–1,000		
Confluencia Traful (CT) [2001]	40°43′S 71°05′W					100–1,000
La Lipela (LL) [2000]	40°48′S 71°06′W	$n > 1{,}000$		$n < 100$		$n > 1{,}000$
Bariloche area (B) [2001, 2007]	41°04′S 70°06′W	$n > 1{,}000$	$n < 10$	$n > 1{,}000$	$n < 10$	$n > 1{,}000$

insect individuals that were permanently mounted according to standard entomological methods, and identified to the lowest possible level, in many cases with the assistance of specialized taxonomists (see "Acknowledgments").

Using data gathered at all sites and years, we first defined pollinator assemblages for all parentals, and identified shared pollinator taxa. Because different pollinators are likely to contribute differently to interspecific pollen transfer, we restricted lists of shared pollinators to taxa expected to carry abundant pollen loads (bees and large-bodied syrphid flies). This subsets were further restricted to pollinator species that actually visited pairs of parentals at the same site and year.

Flowering phenology

An individual was considered in bloom when at least one branch exhibited open flowers that received insect visits. At each study site we first recorded the flowering status of at least 10 % of the individuals of each parental species, then used this figure to calculate the proportion of flowering individuals in the population.

Interspecific crossings

In randomly selected plants, flowering branches with unopened buds were covered with bridal veil bags until stigmas swelled and became covered by a clear secretion, which was considered an indication of stigmatic receptivity. With the aid of a 10× hand lens, stigmas were visually controlled for lack of self-pollen, then pollinated by gently touching them with an anther of a freshly opened flower of a designated heterospecific individual. Pedicels of pollinated flowers were marked with fluorescent dye, then unpollinated flowers and unopened buds were removed, and bags were closed. Fruits were harvested 1 month later. Seeds were scarified by removal of a small piece of the testa and germinated on moist filter paper in 6 × 5 × 1 plastic boxes at room temperature.

Crossings were made at LH and B. At LH, crossings involved *D. chacaye* and *D. articulata*. Here, mean distance between pollen donors and pollen receptors was ca. 60 m. At B, all three parentals were available. Plants of *D. chacaye* and *D. articulata* were ca. 150 m apart, but—although plants of *O. trinervis* were growing in their proximity—the nearest blooming population of this species was located 29.1 km away. For pollinations involving *O. trinervis*, branches with freshly opened flowers enclosed in plastic bags were transported by car. All pollinations were made within 2 h of removal of flowers from the donor plants.

The pollination experiment involved 261 flowers. Fruit- and seed-set data were analyzed using G^2 tests (Agresti 1996).

Morphological and molecular studies

We sampled material for molecular and morphological analyses at site B (*D. articulata*, $n = 14$ individuals; *D. chacaye*, $n = 35$; *O. trinervis*, $n = 18$; *D. chacaye* × *D. articulata*, $n = 8$; *D. chacaye* × *O. trinervis*, $n = 9$). All available intermediate individuals were sampled. Leaf material was kept in a portable cooler (0–5 °C) for later protein and DNA extraction in the laboratory, and additional material was herborized for morphological studies. All sampled individuals were used for both morphological and molecular studies.

For morphological analysis, five vegetative characters were recorded: internode length, presence of spines in young stems, leaf length, type of leaf venation (coded as 1 = retinerved, 2 = with two basal veins, 3 = trinerved), and type of leaf margin (1 = entire, 2 = dentate, and 3 = crenate). Measurements were made with a caliper, and leaf characters were recorded in a sample of five randomly

selected leaves per individual. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. The nonparametric Kruskal–Wallis test and multiple comparisons of mean ranks were used for variables not normally distributed, such as type of venation and type of leaf margin.

Genetic analyses were performed using nuclear markers (isozymes) and maternally inherited markers [sequences of noncoding regions of chloroplast DNA (cpDNA)]. We prepared homogenates for protein electrophoresis by grinding fresh leaves with liquid nitrogen. Then we added 2 mL extraction buffer as in Mitton et al. (1979) and performed horizontal electrophoresis on 12 % w/v starch gels (Starch Art Corporation, Smithville, Texas). Six enzyme systems coding for nine putative isozyme loci were resolved as follows: shikimate dehydrogenase (SKDH), phosphoglucoisomerase (PGI1, PGI2), and malate dehydrogenase (MDH1) in the morpholine-citrate buffer by Ranker et al. (1989), whereas malic enzyme (ME1, ME2), peroxidase (PERC1), and menadione reductase (MNR1, MNR2) were resolved in the system by King and Dancik (1983). Gels were run for approximately 5 h or until the dye front indicator had migrated about 10 cm, after which the anodic portion of gels (or cathodic in the case of PERC1) were sliced and stained following standard protocols. As no formal genetic analyses were performed for these species, the loci are considered putative. Nevertheless, the obtained banding patterns were homologous with those found in other species for which genetic control of the isozyme markers was determined (Murphy et al. 1996; Soltis and Soltis 1989).

For DNA extraction we used a modification of the Dumolin–Lapegue protocol (Dumolin et al. 1995) or followed a protocol which allows DNA extraction from isozyme extracts (Arbetman and Premoli 2011). Typically, three leaves were ground with liquid nitrogen, and 1 mL buffer [2 % alkyltrimethylammonium bromide (ATMAB), 0.5 M ethylenediamine tetraacetic acid (EDTA) pH 8, 1 M Tris-HCl pH 8, 5 M NaCl, 1 % dithiothreitol (DTT), 1 % polyvinyl pyrrolidone (PVP)] was added. After 1 h of incubation at 55 °C, samples were allowed to cool. Then, 400 µL dichloromethane was added, and centrifugation was performed for 10 min. The upper aqueous phase was isolated in a new tube, 750 µL isopropanol was added, and an overnight precipitation was permitted. A new centrifugation and discharge of the liquid phase, followed by air drying and ethanol precipitation, was performed. Samples were resuspended in 1× TE buffer with RNase (Qiagen). DNA concentration was estimated on 1 % agarose gels stained with SYBR Safe (Invitrogen). Polymerase chain reaction (PCR) amplification and sequencing was performed using the *trnL-F* intergenic spacer. Amplification consisted of 1–3 µL DNA in 25-µL reactions containing 1× PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 160 µM of each dNTP, 0.4 µM of each primer, and 1 U Taq DNA polymerase (Invitrogen). Primers used were E (5′-GG TTCAAGTCCCTCTATCCC-3′) and F (5′-ATTTGAACT GGTGACACGAG-3′) from Taberlet et al. (1991). Thermal cycling was performed on an Eppendorf Mastercycler gradient with one hold at 95 °C for 5 min proceeding to 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, followed by one last extension at 72 °C for 5 min. Amplification products were purified using ExoSAP-IT (USB). Purified DNA was used as a template for direct sequencing with BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems). Sequencing reactions were analyzed on an ABI 3100 Avant genetic analyzer (Applied Biosystems) at Laboratorio Ecotono facilities, Universidad Nacional del Comahue. DNA sequences were aligned using the program ClustalX 1.81 (Thompson et al. 1994) in the Mega 4.0 program (Tamura et al. 2007).

We also used two unpublished sequences of the *trnL* gene obtained from two *D. chacaye* × *D. articulata* intermediate individuals from site LH, kindly provided by L. Aagesen and collaborators (see Aagesen et al. 2005 for methods).

DNA sequences were compared with the ones available at the National Center for Biotechnology Information (NCBI) for the pure species (accession numbers for *D. articulata* are AY460414/AY642145, for *D. chacaye* AY460415/AY642146, and for *O. trinervis* AY460421/AY642151).

Isozyme genotypes and allelic frequencies were used to calculate parameters of genetic diversity and degree of identity of parentals and relationships with intermediates. We estimated genetic diversity parameters to test the hypothesis of higher heterozygosity in intermediates. We described allele profiles including the presence of diagnostic alleles, i.e., those characteristics of any given pure species, and gene frequencies across parentals and intermediates.

Results

Flowering phenology

Field observations in the period 1997–2007 showed that, at any given site and year, *D. articulata* started to flower somewhat earlier than *D. chacaye*, and *O. trinervis* flowered latest (Supplementary Table 1). The flowering periods of *D. chacaye* and *D. articulata* overlapped by at least 2–3 weeks at all sites where both species co-occurred, while the flowering of *O. trinervis* was so delayed that overlap occurred only for certain sites and years. For instance, at the LH site in mid-December 1999, all

Table 2 Size and degree of overlap of pollinator assemblages of parentals

Parental	*D. articulata*	*D. chacaye*	*O. trinervis*
Sampled sites	3	6	3
Pollinators			
Total species no.	41	126	33
Shared with *D. articulata*	–	21 (6/3)	10 (2)
Shared with *D. chacaye*	–	–	10 (0)

Number of site- and year-specific large-bodied shared pollinator species are given in *parentheses*. For *D. articulata* and *D. chacaye*, data on shared pollinator species are available for two site–year combinations

D. chacaye individuals were in bloom, while only half of the *D. articulata* population was still in flower (the other 50 % was already developing fruits) and no *O. trinervis* plants had started to flower yet.

Pollinator assemblages

Pollinators comprised 165 taxa distributed across five orders and 42 families, including fully identified species (47.8 %) and morphospecies identified to genus (19.4 %) or family (32.1 %) level (Medan et al. 1999; Medan and Devoto 2005; D. Medan, unpublished results; data consolidated in Table 2). While 84.2 % of pollinator species were parental-exclusive, all parentals shared pollinators with each other (Table 2). *D. articulata* and *D. chacaye* shared 21 spp., while *O. trinervis* shared ten spp. with *D. articulata* and 10 with *D. chacaye*. When only site- and year-specific bees and large-bodied syrphid flies were taken into account, the pollinators shared by *Discaria articulata* and *D. chacaye* dropped to six or three species (depending on site), and those shared by *D. articulata* and *Ochetophila trinervis* dropped to two species. *D. chacaye* and *O. trinervis* shared no pollinators of this type (Table 2).

Interspecific crossings

Interspecific crossings revealed significant differences in parental compatibility, including differences between direct and reciprocal crossings within a given species pair (for the whole experiment, $G^2 = 50.1$ for fruit-set values and $G^2 = 58.1$ for seed-set values, both $p \ll 0.01$). The two crossings including *Ochetophila trinervis* as pollen parent, the crossing *D. articulata* × *O. trinervis*, and the crossing at site LH having *Discaria chacaye* as pollen parent and *D. articulata* as ovule parent gave significantly low fruit- and seed-set values and yielded no germinable seeds. The crossing *Discaria chacaye* × *D. articulata* at site B had fruit- and seet-set values within statistical expectance, but also failed to yield germinable seeds (Table 3).

The crossing with *D. chacaye* as pollen donor and *O. trinervis* as ovule parent had unexpectedly high values of fruit and seed set, but none of the harvested seeds germinated. At both sites B and LH, the crossing of *D. articulata* as pollen parent and *D. chacaye* as ovule parent was particularly fecund: almost half of the flowers set fruit, 13.3–16.2 % of ovules became seeds, and two seeds germinated (Table 3).

Morphological and molecular evidence

Parental species formed homogeneous groups for leaf length (Table 4). While *O. trinervis* differed from the rest in leaf venation type, *D. chacaye* was significantly distinct from pure *D. articulata* and *O. trinervis* for leaf margin type (Table 4). No significant differences were found in internode length or spinescence (data not shown). Putative hybrids were overall intermediate to the presumed parentals. *D. articulata* × *D. chacaye* was intermediate in leaf length and had variable margin type. *D. chacaye* × *O. trinervis* was intermediate but not significantly different from either parental for leaf length and margin type (Table 4). As occurred with parental species, internode length and spinescence of putative hybrids varied indistinctly (data not shown).

Only the parentals had diagnostic alleles (Table 5). *D. articulata* had two unique alleles [MNR2 (1) and PERC1 (1)], *D. chacaye* had three [PGI2 (4), MNR1 (3), and MDH1 (5)], and *O. trinervis* had two [MDH1 (1 and 2)]. Intermediates mainly showed diagnostic alleles of *D. chacaye*. Intermediates *D. articulata* × *D. chacaye* had two of the three alleles of *D. chacaye* and none of *D. articulata*, and intermediates *D. chacaye* × *O. trinervis* had all three of *D. chacaye* and one out of two of *O. trinervis*. Mean expected heterozygosity (unbiased estimated by Nei 1978) was higher in intermediates (0.417 and 0.377) than in parentals (*D. articulata*, 0.294; *D. chacaye*, 0.322; *O. trinervis*, 0.321) (Table 6).

Sequences of chloroplast DNA of parentals and intermediates for two noncoding regions of the chloroplast revealed only two haplotypes. These haplotypes differed by six substitutions at the *trnL/trnF* region, while at the tRNA-Leu (*trnL*) region they differed by four substitutions and one indel. One haplotype was found in *D. articulata*, *D. chacaye*, and their intermediates (at both the LH and B sites), and the other haplotype was found in *O. trinervis* and its intermediate with *D. chacaye*. These sequences were identical to the ones already published in NCBI for the parental taxa (Aagesen et al. 2005).

Table 3 Results from hand crossings among parentals carried out at populations LH (December 1999) and B (December 2007)

Population	Pollen parent (no. of individuals)	Ovule parent (no. of individuals)	Percent fruit set (no. of pollinated flowers)	Percent seed set (no. of available ovules) [no. of germinated seeds]
LH	*D. chacaye* (4)	*D. articulata* (5)	*7.3* (41)	*1.6* (123) [0]
B	*D. chacaye* (3)	*D. articulata* (3)	21.2 (33)	8.0 (99) [0]
B	*D. chacaye* (3)	*O. trinervis* (3)	**34.6** (26)	**21.8** (78) [0]
LH	*D. articulata* (4)	*D. chacaye* (4)	**48.7** (39)	**16.2** (117) [1]
B	*D. articulata* (3)	*D. chacaye* (3)	**47.0** (35)	**13.3** (105) [1]
B	*D. articulata* (3)	*O. trinervis* (3)	*6.6* (30)	*1.1* (90) [0]
B	*O. trinervis* (3)	*D. chacaye* (3)	*3.7* (27)	*2.4* (81) [0]
B	*O. trinervis* (3)	*D. articulata* (3)	*10.0* (30)	*0.0* (90) [0]

Fruit- and seed-set values in *boldface* and *italics* are significantly above and below expected values, respectively (G^2 tests)

Table 4 Leaf morphological analysis of parental species and their intermediates

	No. of individuals	Leaf length	Type of venation	Type of margin
D. articulata	14	1.07 (0.27) a	1.00 (0.00) a	1.35 (0.75) a
D. chacaye × *D. articulata*	8	2.11 (1.05) c	1.00 (0.00) a	1.78 (0.83) ab
D. chacaye	35	3.94 (0.76) b	1.06 (0.23) a	2.68 (0.68) b
D. chacaye × *O. trinervis*	9	3.00 (0.82) bc	1.43 (0.79) a	1.86 (1.07) ab
O. trinervis	18	2.94 (0.55) c	3.00 (0.00) b	1.00 (0.00) a

Data are based on samples of five leaves per individual. For character coding see text. Values of leaf length in cm. Means and standard deviation (SD) are given. Within a column, different letters indicate significant differences at $p < 0.05$ (post hoc Tukey tests for leaf length, and multiple comparisons for mean ranks after Kruskal–Wallis nonparametric tests for type of venation and type of margin)

Discussion

This paper addresses instances of presumed hybridization within *Discaria* and between *Discaria* and the related genus *Ochetophila*, including (1) examination of pollination scenarios, (2) assessments of results of artificial interspecific crosses, and (c) comparative morphological and molecular characterization of parental species and intermediates.

Because of morphological intermediacy, putative hybrids have been reported several times within the tribe Colletieae of Rhamnaceae. The presumed parentals belonged to a unique genus (*Discaria*: Tortosa 1983b; Wright and Briggs 2000; *Colletia*: Tortosa 1989; *Retanilla*: Tortosa 1992) or to different genera (*Discaria* and *Ochetophila* (as "*Discaria*"): Tortosa 1983b; *Retanilla* and *Trevoa*: Tortosa 1992). In the present study intermediate individuals were commonly found wherever parental taxa existed in sympatry. In particular, hybridization took place between the most widespread and ecologically less restricted species *D. chacaye* with the northernmost distributed *O. trinervis* and the relatively range-restricted *D. articulata*. Therefore, current hybridization and potential introgression of *D. chacaye* with *D. articulata* and into *O. trinervis*, respectively, is probably favored by overlapping ranges and partially coincident phenologies and ecological niches.

Despite the potential gene flow—even between distinct genera—in Rhamnaceae, species identity seems to be maintained by diversifying selection, resulting in clearly identifiable taxa with distinct ecological characteristics. Similar scenarios have been described for sympatric *Nothofagus* (Nothofagaceae) (Premoli et al. 2012). Where species come into contact, e.g., in disturbed environments and under relaxed selection pressures, intermediates may originate as described below.

D. chacaye × *D. articulata*

This intermediate form has a wide distribution in Argentina and Chile, but only occurs at sites where both parentals coexist (Tortosa 1983b). In our study it was found at two of the three sites where it was expected, and it is known to have existed at the third site in the past (Tortosa 1983b). Across their ranges, parentals co-flowered over a period of 2–3 weeks and their insect pollinator assemblages had 21 species in common, of which 3–6 (depending on site) simultaneously visited both plant species. Therefore, a prediction of interspecific pollen flow could be reasonably made.

The outcome of the crossing experiment was consistent with the above expectation. The artificial crossing of *D. articulata* as pollen parent and *D. chacaye* as ovule parent was successful in terms of fruit and seed set. The

Table 5 Allele frequencies for seven polymorphic isozyme loci in the three parental species and their intermediates

Allele	*D. articulata*	*D. chacaye*	*O. trinervis*	*D. chacaye* ×	
				D. articulata	*O. trinervis*
MDH1					
1	0.000	0.000	**0.028**	0.000	0.000
2	0.000	0.000	**0.083**	0.000	0.111
3	0.000	0.162	0.194	0.188	0.056
4	1	0.779	0.694	0.750	0.667
5	0.000	**0.059**	0.000	0.063	0.167
ME2					
1	0.056	0.029	0.000	0.188	0.000
2	0.278	0.529	0.125	0.688	0.250
3	0.667	0.426	0.406	0.125	0.438
4	0.000	0.015	0.469	0.000	0.313
MNR1					
1	0.964	0.206	0.833	0.571	0.000
2	0.036	0.206	0.167	0.143	0.375
3	0.000	**0.588**	0.000	0.286	0.625
MNR2					
1	**0.077**	0.000	0.000	0.000	0.000
2	0.154	0.029	0.000	0.125	0.125
3	0.731	0.971	0.917	0.813	0.875
4	0.038	0.000	0.083	0.063	0.000
PERC1					
1	**0.056**	0.000	0.000	0.000	0.000
2	0.278	0.029	0.000	0.250	0.056
3	0.667	0.926	0.265	0.688	0.889
4	0.000	0.044	0.735	0.063	0.056
PGI2					
1	0.143	0.029	0.000	0.250	0.000
2	0.321	0.429	0.417	0.375	0.556
3	0.500	0.400	0.583	0.375	0.333
4	0.000	**0.071**	0.000	0.000	0.056
5	0.036	0.071	0.000	0.000	0.056
SKDH					
1	0.038	0.600	0.147	0.286	0.333
2	0.269	0.286	0.735	0.500	0.556
3	0.692	0.114	0.118	0.214	0.111

Alleles which are diagnostic for parentals are shown in *bold* ($n = 84$ individuals)

Table 6 Parameters of within-population diversity in parentals and their intermediates

	Sample size per locus	No. of alleles per locus	Percentage of loci polymorphic	Heterozygosity	
				Direct count	Hardy–Weinberg expected
D. articulata	12.7 (0.7)	2.4 (0.4)	66.7	0.189 (0.077)	0.294 (0.089)
D. chacaye × *D. articulata*	7.8 (0.1)	2.6 (0.3)	77.8	0.306 (0.091)	0.417 (0.087)
D. chacaye	34.6 (0.2)	2.8 (0.4)	77.8	0.263 (0.105)	0.322 (0.091)
D. chacaye × *O. trinervis*	8.7 (0.2)	2.6 (0.4)	77.8	0.239 (0.099)	0.377 (0.089)
D. trinervis	17.6 (0.2)	2.2 (0.3)	77.8	0.247 (0.091)	0.321 (0.075)

Means and SD are given

reciprocal crossing (with *D. chacaye* as pollen parent) was consistently infecund, which suggests that naturally occurring intermediates derive from insect pollination of *D. chacaye* plants with *D. articulata* pollen. Since there is no obvious reason for lack of natural pollen transport in the reverse direction, a postpollination barrier is hypothesized to operate in *D. articulata* when pollinated with *D. chacaye* pollen. Asymmetric interspecific crossability has been reported elsewhere in plants (e.g., Kameyama et al. 2005; Wood and Nakazato 2009). The disparity in style and pollen tube length could represent a structural prezygotic barrier that would result in unilateral success of interspecific hybridization when small-flowered species are used as seed parents (Williams and Rouse 1988; Gore et al. 1990). The style of *D. chacaye* is shorter than that of *D. articulata* (Medan and Aagesen 1995), and this structural difference may represent such a barrier. Additional research, however, is required to reveal whether this sole factor accounts for asymmetric crossability between *D. chacaye* and *D. articulata*. Presumed hybrids *D. chacaye* × *D. articulata* were morphologically intermediate between parentals (although somewhat closer to *D. articulata*). Molecular evidence (isozymes) indicated more closeness to *D. chacaye*, while the maternal-inherited marker was of little value here because both parentals shared the same haplotype.

D. chacaye × *O. trinervis*

This intermediate form has been found at three sites only (Tortosa 1983b), one of which (B) was included in our study. Here *D. chacaye* and *O. trinervis* were sympatric, as they also were at our sites LT and LL, where—in contrast—no intermediates were observed. Lack of opportunities for interspecific pollination (because of the delayed blooming of *O. trinervis* and the scarcity of shared pollinators) probably contributes to explain the rarity of this particular hybrid.

Intermediates were biased towards *D. chacaye* as regards morphology and isozymes, indicating possible backcrossing. Hand crossings suggested that natural interspecific pollinations have *D. chacaye* as pollen parent and *O. trinervis* as ovule parent, because the reciprocal combination was significantly less fecund. This is consistent with the intermediates sharing the haplotype of *O. trinervis*, and with a report by Tortosa (1983b), who raised plants with intermediate phenotypes from seeds collected from an *O. trinervis* individual.

Intergeneric hybridization is frequent among flowering plants, and it may be much more common than usually considered (Knobloch 1972). A recent study by Wu et al. (2010) discusses a case of intergeneric hybridization in the woody family Altingiaceae, which shows similarities to the *Discaria-Ochetophila* hybrid treated here: geographical ranges of parentals are partially overlapping, their reproductive phenologies and ecological traits are coincident to some degree, and hybrids occur intermixed with parentals but are uncommon. For Wu et al. (2010), this evidence is suggestive of the intermediate being an infrequently produced F_1 hybrid, rather than a stabilized species of hybrid origin.

D. articulata × *O. trinervis*

No such intermediates have been found in nature, although the flowering times of parentals show some overlap, and shared pollinators exist. When cross-pollinated, these parentals yielded the lowest fecundity values in the whole experiment, which is suggestive of the presence of a strong reproductive barrier.

Conclusions

Analysis of pollination scenarios (phenology and shared pollinators) predicted stronger pollen flow between *D. articulata* and *D. chacaye* than between *D. chacaye* and *O. trinervis*, and hand crossings suggested that the viability of *D. chacaye* × *D. articulata* intermediates is higher. Morphology and molecular analyses confirmed the intermediacy of putative hybrid individuals. Maternal-inherited markers and artificial crossings strongly suggest that in the intermediate *D. chacaye* × *O. trinervis* the former was the pollen parent and the second the ovule parent. In the case of the intermediate *D. chacaye* × *D. articulata*, artificial crossings point to *D. articulata* as pollen parent and to *D. chacaye* as ovule parent. Thus, our results confirm intra- and intergeneric hybridization in Rhamnaceae.

The fact that intermediates occur (1) only where parentals coexist, and (2) in abundances proportional to interparental pollen flow, suggest that the permanence of hybrid populations depends on long-term survival of intermediate individuals and/or continuous generation of F_1 individuals. The latter is in turn contingent upon the permanence of current pollination scenarios. Studies focused on hybrid populations are needed to assess their reproductive output, the long-term viability of hybrid individuals, and the stability of novel characters or character combinations observed, as a way to understand the possible role of hybridization in the evolution of this tribe.

The Andes are a hotspot of biodiversity, but hybridization in Andean taxa has so far not been investigated intensively (e.g., Premoli 1996; Marchelli and Gallo 2004; Ackermann et al. 2008; Acosta and Premoli 2010). For the Southern Andes, our study shows that, when morphologically pure entities are currently found in sympatry,

interspecific gene flow occasionally occurs due to incomplete reproductive barriers. However, species identities are ecogeographically maintained and deserve their current taxonomical recognition.

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