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NOTES ON NEGLECTED AND UNDERUTILIZED CROPS

Population genetics and ethnobotany of cultivated *Diospyros riojae* Gómez Pompa (Ebenaceae), an endangered fruit crop from Mexico

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Abstract The traditional cultivation of an endangered species, *Diospyros riojae*, in North-Central Mexico, and the sale of its fruit, is described for the first time. This is complemented by the first examination of genetic structure in New World Ebenaceae. Genetic comparisons are made among 27 accessions of *D. riojae* from across its known range of cultivation, and individuals of *Diospyros conzattii*, *Diospyros digyna*, *Diospyros californica*, *Diospyros rosei*, *Diospyros rekoi* and an unknown species of *Diospyros* from Oaxaca, Mexico. Morphological comparisons are made between *D. conzattii*, *D. riojae*, and the unknown species of *Diospyros* from Oaxaca. *D. riojae* is cultivated in two markedly different climates: arid and semi-arid localities west of the Sierra Gorda, especially near Tecozautla, Hidalgo, and humid areas of the Huasteca Region, east of the Sierra Gorda. Much lower levels of genetic diversity were detected in western populations, where populations are larger, and management intensity is the greatest. Neither the results of our genetic analysis, nor our morphological analysis of recent collections of *D. riojae*, *D. conzattii*, and an unknown species from Oaxaca, are consistent with the most recent revision of Neotropical *Diospyros*. Taxon-level divergence, rather than cultivar-level divergence, is suggested for *D. riojae* and *D. conzattii*.

Keywords *Diospyros riojae* · Ebenaceae · Genetic diversity · Mexico · Species circumscription · Tree domestication

Introduction

The genus *Diospyros* (Ebenaceae) is comprised of over 500 species of trees and shrubs best known for producing dense black wood (ebony) and tasty fruit (e.g. persimmons and *zapotes*). It has a pantropical-subtropical distribution, with several species also in temperate regions of Asia and North America (Duangjai et al. 2009). There are some 100–130 species in the New World (Wallnöfer 2009), with about 35 of these found in Mesoamerica. Recent phylogenetic work suggests that nearly all of the Mesoamerican species are in a clade that includes many of the species from the Indian Subcontinent, SE Asia, Oceania, and South America (Duangjai et al. 2009). Morphological studies suggest a suite of

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species complexes for the Mesoamerican taxa (Provance et al. 2008; Provance and Sanders 2006, 2009), but there have not been any population or phylogeographic studies of Mesoamerican *Diospyros*.

Here, we provide the first detailed account of the traditional cultivation of *Diospyros riojae* Gómez Pompa in Queretaro, Hidalgo, and San Luis Potosi, Mexico. We also conduct the first population study of a Mesoamerican *Diospyros*, based on 26 individuals of *D. riojae* from across its range of cultivation, and one putative wild individual from near the type locality in central Veracruz. Our immediate objective was to explore genetic structure within *D. riojae* using two geographically separated clusters: high elevation populations west of the Sierra Gorda, and low elevation populations east of the Sierra Gorda (Fig. 1). We sought to determine if populations on either side of the Sierra Gorda showed evidence of differentiation, and where unique genotypes and phenotypes occurred that could be important to future germplasm conservation efforts. Lastly, we investigated whether morphological and genetic evidence suggests cultivar or taxon-level divergences between *D. riojae* and *Diospyros conzattii* Standl.

Methods

Species examined

Diospyros riojae is a tree native to oak and mesophytic cloud forest between 100 and 1,350 m elevation in central and northern Veracruz (Gómez-Pompa 1964; Pacheco 1981; Provance and Sanders 2006). It is considered an endangered species in Mexico, and there is considerable interest in its conservation biology (Sosa et al. 1998; Luna Vega et al. 2006). A recent reassessment of herbarium material found that putative *D. riojae* collections often represented a similar species, *D. conzattii* (Provance and Sanders 2006). Despite female trees bearing edible fruits, *D. riojae* has seldom been reported as a cultivated tree (e.g. Alcorn 1984). The fruits (berries) are globose, and range from 3−5 cm in diameter. They have up to ten seeds, or are seedless. The leaves are elliptic to widely obovate, or sub-oval, and typically have an obtusely rounded apex. The lamina is shiny and relatively thick, with slightly revolute, thickened margins, and prominent 3° and 4° venation. The fruiting calyx is accrescent and firmly attached to the fruit, with 5(–6) spreading to reflexed sepals. Male flowers are relatively short and urceolate-campanulate (Provance and Sanders 2006).

Diospyros conzattii is primarily known from cloud and pine-oak forests of west-central Veracruz and nearby Puebla, although the type locality is in the Sierra Madre del Sur of southern Oaxaca (Provance and Sanders 2006; Wallnöfer 2007). *D. conzattii* is known for Oaxaca only from the type collection and trees in the Conzatti Garden in Oaxaca de Juárez, which have likely descended from seeds collected at the type locality. *D. conzattii* has leaves and flowers that are distinctly different from those of *D. riojae*, but the calyx and edible fruits are similar. Its leaves are lanceolate to elliptic, or ovate, with an apex that is distinctly acuminate, or sometimes tapered to an acute or obtuse rounded point. The lamina is dull, with thin, flat margins, and lacks prominent higher orders of venation (Provance and Sanders 2006). The male trees produce long-urceolate flowers.

We included several other taxa in the study for comparative purposes: *Diospyros digyna* Jacq., which is widely distributed in Mesoamerica; *Diospyros californica* (Brandegee) I. M. Johnst., which is endemic to the Cape Region of Baja California Sur; *Diospyros rekoi* Standl. and *Diospyros rosei* Standl., which are mainly from western Mexico; and an indefinitely determined species, *Diospyros* cf. *riojae*, from the Chimalapas region of Oaxaca that is morphologically most similar to *D. riojae*. Thus, a total of 27 collections of *D. riojae*; one accession each of *D.* cf. *riojae*, *D. conzattii*, *D. rosei*, *D. rekoi*, and *D. californica*; and two accessions of *D. digyna*, were included in the genetic analysis. Including 11 collections of *D. riojae*, and seven collections of *D. conzattii* cited in Provance and Sanders (2006), a total of 64 collections of *D. riojae*, eight collections of *D. conzattii*, and one collection of *D.* cf. *riojae*, were evaluated morphologically.

Study area and sampling method

The study was conducted in four areas of Mexico, including: (1) arid and semi-arid zones west of the Sierra Gorda, in Queretaro and northwestern Hidalgo at 1,300–2,100 m a.s.l. with mean annual precipitation (MAP) of 400–800 mm, mean annual temperature (MAT) of 16–20 °C, long dry periods, and

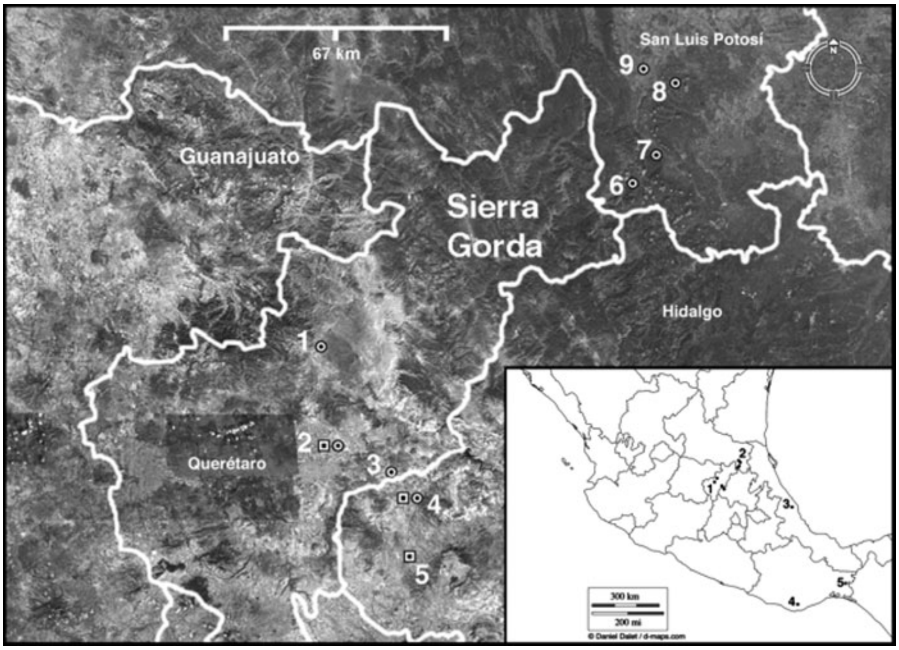

Fig. 1 Map of north-central Mexico, showing origin of genetically analyzed *Diospyros riojae* (circles: 1 near Toliman, 3 Pathé, 4 Tecozautla region, 6 San Antonio Xalcuayo Dos, 7 La Herradura, 8 Tanjasnec, 9 Aldzulup Poytzén), *D. riojae* fruit vendor locations (squares: 2 Ezequiel Montes, 5 Huichapan), and (inset: 1 west of the Sierra Gorda, 2 east of the Sierra Gorda, 3 Cruz Blanca, VE, 4 *D. conzattii*, Cafetal San Rafael, OA (native origin of tree in Conzatti Garden), 5 *Diospyros* cf. *riojae*, Chimalapas Region, OA

occasionally frosts; (2) the Sierra Gorda of eastern Queretaro and northern Hidalgo at 500–1,800 m a.s.l. with MAP of 800–1,800 mm, MAT of 20–24 °C, and a semi-humid atmosphere; (3) escarpment of the eastern Sierra Gorda, and the North Gulf Coastal Plain in San Luis Potosi and northern Hidalgo, at 60–900 m a.s.l. with MAP of 1,500–3,000 mm, MAT of 20–26 °C, and high humidity, with rain abundant in the summer, or most of the year; (4) Cafetal San Rafael and its vicinity in the Sierra Madre del Sur of the State of Oaxaca, at 800–1,200 m a.s.l. with MAP of 1,000–1,200 mm, MAT of 22–24 °C, and a sub-humid atmosphere (Fig. 1, inset) (García E - Comisión Nacional para el Conocimiento y Uso de la Biodiversidad 2001).

During 2009 and 2010, the first and third authors searched for populations of *D. riojae* at previously recorded localities, and new localities outside of the presumed range for the species. These localities included homegardens, managed forests, managed groves, and putative natural vegetation communities. Gardens adjacent to living quarters or work residences, whether in towns, rural areas, or intermediate settings, were considered homegardens. Community-managed forests were defined as natural settings with high concentrations of indigenous or nonindigenous species of useful trees, and were managed by people living in the area. Community-managed groves were characterized by the predominance of one species of useful tree. Otherwise, they were similar to community-managed forests. Tree occurrences were located by direct observation of trees while walking, or driving, and from leads provided by people. Thus, a mixture of intuitively directed searches and local knowledge was used to locate trees and informants. *D. riojae* fruits were sought at open-air markets in Xilitla (SL), Huichapan (HD), and Ezequiel Montes (QT). When adjacent trees appeared to be clonal, only one individual was sampled. Leaf samples were dried in silica gel for later extraction. Herbarium vouchers (CIMI), were made for all genetically tested individuals, except those from the market at Ezequiel Montés, Queretaro, for which photos of the fruits were obtained, and only dried calyces were preserved. Species determination was made following Provance and Sanders (2006) and by comparison with type specimens. Prior informed consent was obtained verbally from growers before informal conversation interviews, which were conducted in the immediate vicinity of *D. riojae*.

Genetic analysis

Primers were designed from sequences of *Diospyros kaki* Thunb. for the following loci (NCBI Genbank numbers in parentheses): phytoene synthase (PSY, FJ713744.1), lycopene beta-cyclase (LCYB, FJ940723.1), beta-carotene hydroxylase (BCH, GQ265553.1) (Zhao et al. 2011), and squalene synthase (SQS, FJ687954.1) (Zhou et al. 2012). The trnL intron was amplified from primers 'c' and 'd' (Taberlet et al. 1991). The trnL intron sequence for *D. digyna* was obtained from a sequence submitted to Genbank (Duangjai et al. 2009). Genomic DNA was extracted from lyophilized leaf tissue using a modified CTAB protocol (Saghai-Maroof et al. 1984) that included polyvinylpyrrolidone (PVP). DNA was quantified using nanodrop spectrometry. PCR amplification was done in 25 μl reactions containing 6 μl of 10 ng/μl DNA solution, 0.25 μl of TAQ polymerase, 12 μl of sterile water, 1 μl of dNTPs, 1 μl for each primer at 10 μM, 2.5 μl for 10 × TAQ buffer solution mix, and 1.25 μl of DMSO. An initial 1 min pre-melt was followed by PCR for 30 cycles, with denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, and extension at 72 °C for 5 min. Purified PCR products were obtained by mixing 12 μl with 3 μl of the exo-SAP purification solution (2 U of exonuclease 1, 2.5 U of Antarctic phosphatase) and placing them in a thermal cycler at 37 °C for 30 min, followed by 80 °C for 15 min, and finally 4 °C for 5 min. Purified PCR products were sequenced at the UC Davis College of Agriculture and Environmental Sciences Genomics Facility on an ABI 3730 XL capillary sequencer. The resulting sequences were aligned using Geneious Pro 5.0.4 (Biomatters) and sequence analysis used the analysis package of libsequence (Thornton 2003).

Results

Populations

Diospyros riojae is cultivated in two remarkably different climatic regimes, corresponding to areas east and west of the Sierra Gorda. The MAP at locations where *D. riojae* was found was 400–500 mm in arid (near Toliman, QT) and semi-arid (Tecozautla and its vicinity, HG, and Pathé, QT) places west of the Sierra Gorda, and 2,500–3,000 mm in humid areas on the eastern Sierra Gorda escarpment and the North Gulf Coastal Plain (García E—Comisión Nacional para el Conocimiento y Uso de la Biodiversidad). We located cultivated *D. riojae* at ten localities in western regions between 1,530 and 1,780 m elevation, and four localities east of the Sierra Gorda, including one on the escarpment at about 820 m elevation, and three on the coastal plain at 60–100 m (Table 1). Western populations represented cultivated trees in homegardens, community-managed forests, community-managed groves, along roadsides, and trees likely naturalized from cultivated settings. Trees east of the Sierra Gorda were only observed in homegardens. We did not observe *D. riojae*, or the closely related species *Diospyros gomeziorum* Provance et A.C. Sanders, in the Sierra Gorda proper. We were unable to locate *D. conzattii* at the type locality, but we found one female tree at the Conzatti Garden in Oaxaca City, which is presumed to have descended from material collected at the type locality.

The two largest populations of *D. riojae* we observed were found in the arid region west of the Sierra Gorda, and both were located in the municipality of Tecozautla, Hidalgo. The largest, comprised of roughly 500 trees, occurs mostly in homegardens in residential, but also in rural areas of Tecozautla, and in some adjacent communities. Occasional trees of ambiguous ownership occur along roadsides and in other public areas. The species seems to have naturalized in gallery forest along the Rio Tecozautla. The second largest population we observed was comprised of about 100 trees, in a community-managed grove at El Paso, about 5 km north of Tecozautla. The largest population we observed east of the Sierra Gorda occurs on the coastal plain at Aldzulup (or Al d'hulup) Poytzén in San Luis Potosi, where there are about 30 trees in Teenek homegardens. Wild populations apparently do not occur in the adjacent environment, but occasional trees come up spontaneously in homegardens. On the eastern escarpment, tree owners reported putatively natural populations southeast of San Antonio Xalcuayo Dos, but this was not confirmed.

Genetics

High genetic differentiation, calculated as Fst (Hudson et al. 1992), was observed between *D. riojae* and *D. conzattii* for three of the nuclear loci, while

Table 1 Species, location, voucher (collector, collection no., herbarium), UTM, altitude, region, management setting, and tree origin of *Diospyros* analyzed

Species	Location	Voucher	UTM	Altitude	Region	Setting	Origin
riojae	La Herradura, SL	GR 8314 CIMI; P "19" UCR*	14 Q 505240 2371150	88 m	East	H	A
riojae	San Antonio Xalcuayo Dos, SL	GR 8310–8318 CIMI; P "20–24" UCR*	14 Q 498360 2362940	834 m	East	H	S, A
riojae	Tanjasnec, SL	GR 8316 CIMI; P "25–26" UCR*	14 Q 510910 2392350	271 m	East	H	A
riojae	Aldzulup Poytzén, SL	GR 8317–8319 CIMI; P "11–18" UCR*	14 Q 501490 2396490	60 m	East	H	S, A
riojae	Cruz Blanca, VE	T Vázquez 98-045 UCR*	14 Q 748030 2185800	1,072 m	East	N	S
riojae	La Cañada, QT	GR 8301 CIMI; P "9" UCR*	14 Q 406290 2314950	1,535 m	West	H	O
riojae	Ezequiel Montes, QT	P "6–7" UCR*	–	–	West	–	–
riojae	Arroyo Pequeño Salitre, QT	GR 8300 CIMI; P "10" UCR*	14 Q 405960 2314220	1,535 m	West	F	A
riojae	Pathé, QT	GR 8322 CIMI; P "8" UCR*	14 Q 426850 2278090	1,774 m	West	H	O
riojae	West Tecozautla, HG	GR 8323 CIMI; P "1–5" UCR*	14 Q 432240 2270820	1,710 m	West	H	S, A
riojae	Rancho Viejo, HG	P 17334 UCR	14 Q 428110 2273270	1,676 m	West	R	–
riojae	La Esquina, HG	P 17336 UCR	14 Q 427050 2272000	1,687 m	West	H	O
riojae	San Antonio, HG	P 17335 UCR	14 Q 426250 2271210	1,712 m	West	G	–
riojae	Rio Tecozautla, HG	P 17308–17310B UCR	14 Q 434440 2270460	1,693 m	West	H	S
riojae	Tecozautla, HG	P 17303–17307, 17311–17322 UCR	14 Q 433420 2271160	1,705 m	West	H, R	O, S, A
riojae	El Paso, HG	P 17323–17332 UCR	14 Q 433750 2275360	1,616 m	West	C, R	S, A
conzattii	Conzatti Garden, Oaxaca, OA	GR 8343 UCR*	14 Q 742490 1888670	1,566 m	South	B	S, A
cf. *riojae*	El Peñasco, OA	S Maya 3885 UCR*	15 Q 363150 1843120	1500 m	South	N	S
digyna	Marie Selby B. G., FL	M Chase 212 K*	17 R 347656 3023648	3 m	–	B	–
digyna	UCRBG, Riverside, CA	P "s.n. 1" UCR*	11 S 469918 3758759	400 m	–	B	O
californica	Cult. in private garden, Riverside, CA	P "s.n. 2" UCR*	11 S 463042 3758428	300 m	West	H	O
rekoi	San Jeronimo, JA	P 8152 UCR*	13 Q 605740 2256490	1,430 m	West	H	S, A
rosei	Chamela, JA	M Quigley 621 UCR*	13 Q 491480 2159390	90 m	West	N	S

GR = I García-Ruiz, P = MC Provance, * = sequence analyzed; H = Homegarden, N = natural vegetation, F = community-managed forest, R = street, old field, pasture, or vacant lot, G = abandoned homegarden, C = community-managed grove, B = botanical garden; O = germinated by owner, S = spontaneous, A = ambiguous comparisons of *D. riojae* and *D.* cf. *riojae* from Oaxaca did not show significant differentiation at any of the four loci (Table 2). TrnL intron sequences were identical for *D. riojae*, *D. californica* and *D. digyna*, while *D. conzattii* had a 2 bp insertion in trnL intron differentiating it from these species. Two 1 bp substitutions in trnL intron differentiated *D.* cf. *riojae* from *D. riojae*, *D. californica* and *D. digyna*. Diversity statistics are shown in Table 3.

We found no difference in diversity between males and females (data not shown). Both heterozygosity (He) and Simpson's index of diversity (SID) were greater in eastern populations than western populations, as was nucleotide diversity (pi) at the PSY locus. One tree from a community-managed forest near Toliman had a PSY genotype otherwise detected only in the putative wild tree from Central Veracruz. Samples from Tanjasnec had unique LCYB haplotypes. There was no variation of SQS in *D. riojae*. The BCH sequences of *D. conzattii*, *D.* cf. *riojae*, and three accessions of *D. riojae*, were each unique.

Morphology

Abaxial and adaxial leaf surfaces of typical mature leaves of *D. conzattii* and *D. riojae* are shown in Fig. 2. Leaf morphology of our *D. riojae* and *D. conzattii* vouchers, none of which were exposed to alcohol during processing, were consistent with their conspecifics, including the specimens of the Provance and Sanders (2006) data set. For example, *D. conzattii* leaves were dull on both sides, thin (pergamentaceous to chartaceous), had margins with flat edges, and inconspicuous higher orders of venation. *D. riojae* leaves were shiny, thick (subcoriaceous to coriaceous), had margins that were slightly thickened and slightly revolute, and prominent higher orders of venation. Leaf shape differed between the species, being consistent with the description of Provance and Sanders (2006).

Table 2 Observed Fst and permutation p-values for comparisons between *Diospyros riojae* and *D. conzattii*, for four nuclear genes

Comparison	Fst observed	P (1,000 replicates)
D. riojae versus *D. conzattii* (BCH)	0.6	0.033
D. riojae versus *D. conzattii* (LCYB)	0.14942	0
D. riojae versus *D. conzattii* (SQS)	1	0.001
D. riojae versus *D. conzattii* (PSY)	−0.0119219	0.514
D. riojae versus *D.* cf. *riojae* (BCH)	0.3	0.067
D. riojae versus *D.* cf. *riojae* (LCYB)	0.0108828	0.334
D. riojae versus *D.* cf. *riojae* (SQS)	0	–
D. riojae versus *D.* cf. *riojae* (PSY)	0.00843939	0.191

BCH beta-carotene hydroxylase, *LCYB* lycopene beta-cyclase, *SQS* squalene synthase, *PSY* phytoene synthase

Ethnobotany

The fruit of *D. riojae* are called *zapote negro* in predominately non-indigenous communities, and are sold in late fall and winter for 15–20 pesos/kg at *tianguis* (travelling outdoor markets), and by roadside vendors in western Hidalgo and western Queretaro. We observed fruit for sale in Ezequiel Montes (QT), and Huichapan, Tecozautla, and La Esquina (HG). Several growers stated that fruits were available in other towns as the outdoor markets changed venue.

In Tecozautla, *D. riojae* trees were found to be especially common along roadsides and in homegardens of residences near narrow stone and mortar canals that line the streets. According to one grower, water flows through the canals about every 40 days, feeding a subterranean system of *caños* that people in residential areas depend on for homegarden irrigation. In Tecozautla, trees are grown for supplementary food, and for a variety of personal reasons. Trees comprise an important component of the landscape, and sometimes people with trees on their property reported having little interest in the fruit. Fruit are usually eaten fresh, but can be prepared in any way that *D. digyna* fruits are prepared. A few people mentioned that an alcoholic drink could be made from the fruit, but they were without further details.

Commercial efforts exploited as few as one tree, and the largest private commercial grove of trees we visited had eleven female and three male trees. This grower reported typical annual fruit yields of 250 kg/tree, with rare yields of up to 500 kg/tree. The trees are reported to have a long juvenile stage, with fruit production starting at about 10 years of age. The fruit

Table 3 Summary statistics for LCYB, PSY, and SQS in populations of *Diospyros riojae* west of the Sierra Gorda, east of the Sierra Gorda, and for the populations combined

Locus	Region	n[a]	bp[b]	S[c]	S[d]	HN[e]	He[f]	pi[g]
LCYB	East	16	177	3	0	6	0.758065	0.00613951
PSY	East	18	408	15	1	11	0.852941	0.0128582
SQS	East	14	174	0	0	1	0	0
LCYB	West	10	177	1	1	2	0.1	0.000564972
PSY	West	6	408	10	10	3	0.318182	0.00410509
SQS	West	9	174	0	0	1	0	0

[a] Sample size

[b] Base pairs sequenced

[c] Segregating (polymorphic) sites

[d] Singleton polymorphisms

[e] Number of haplotypes

[f] Haplotype heterozygosity

[g] Nucleotide diversity

are usually harvested intermittently when olive-green, from late October until January. One grower demonstrated how green fruits were placed in direct sunlight until warm, then wrapped in newspaper, and then stored indoors in a wooden, blanket-wrapped bushel crate (Fig. 3). Using this treatment, fruit remained undamaged, and ripened in just a few days.

A nearly homogenous community-managed grove of *D. riojae* trees spans about seven acres of streambed and bottomland along the Río San Francisco near El Paso. The grove is adjacent to some cultivated plots, and is surrounded by terraces and low hills supporting cactus-rich xeric scrub. One fruit collector estimated that there were about 100 *zapote negro* in the grove. Following our exploration of the groves, we thought that that was a reasonable estimate. Large trees of both sexes were common, as was adventitious growth from roots and crowns. Seedlings and saplings were rarely observed.

Some people, including vendors, mentioned Toliman, Queretaro, as a source of *zapote negro* fruit. We located two trees near Toliman, but neither of them were commercially harvested. One tree in a homegarden was grown from a seed obtained from a fruit

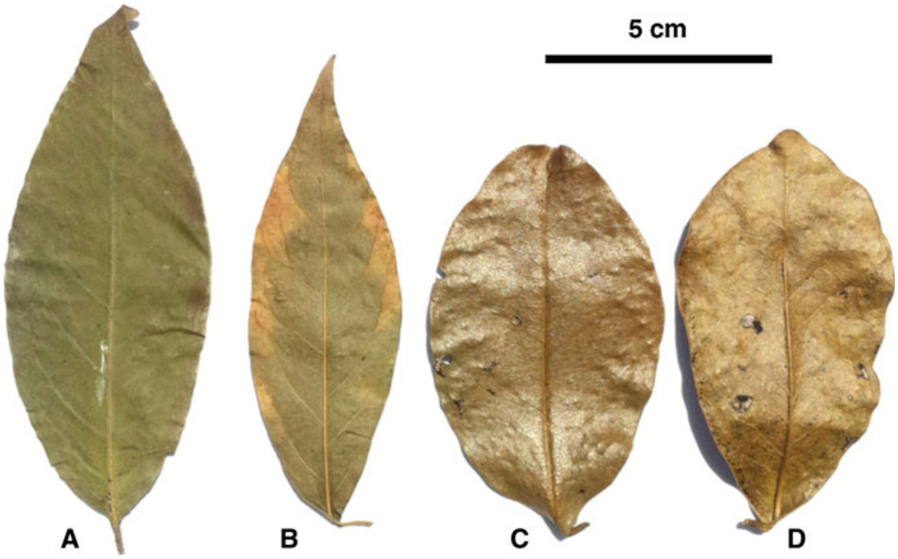

Fig. 2 Comparison of mature, air-dried leaves of *D. conzattii* (**A, B**) and *Diospyros riojae* (**C, D**). Both the adaxial **A, C** and abaxial **B, C** surfaces are shown

Fig. 3 Some recently ripened *Diospyros riojae* fruit in a crate in Tecozautla, which will likely be sold to a vendor in Huichapan, Hidalgo

bought at an outdoor market in Colón, Querétaro. We observed another tree, with an ambiguous origin, growing in a community-managed forest that was enriched with *Juglans*. Our elderly guide's familiarity with this individual tree went back 80 years.

Diospyros riojae and its fruits are called *monek*' by Teeneks in the Huasteca communities of Aldzulup Poytzén and Tanjasnec, San Luis Potosi. Our guide in Tanjasnec described two types of dark *zapotes* present locally, which were consistent with *D. digyna* and *D. riojae*. He stated that they both were called *monek'*. The largest eastern population we visited was in Aldzulup Poytzén, a small community with flat topography, and a relatively warm and humid climate. We observed at least 30 trees in at this location. Our guide stated that the species did not occur in the surrounding environs, and that trees were not intentionally planted in the gardens. In Aldzulup Poytzén, tree trunks were sometimes nearly 100 cm in diameter. These trees were presumed by locals to be over 100 years old. Although ambiguous in origin, trees often had adventitious shoots from roots near the ground surface, or coming from the base of the trunk. Some isolated saplings were observed that likely arose spontaneously from seeds. Management techniques reminiscent of coppicing and pollarding were noted, though these practices seemed to be related to space management within the homegarden.

We also found *D. riojae* trees east of the Sierra Gorda in the mestizo communities of San Antonio Xalcuayo Dos (Municipio Xilitla), and La Herradura (Municipio Alfred M. Terrazas). In San Antonio Xalcuayo Dos, trees of both sexes occurred in intensively managed homegardens. Growers stated the tree was rare in surrounding areas, but that scattered trees in a natural setting grew at lower elevations near Arroyo Seco. In La Herradura, we located one large tree with copious fruit in a homegarden. The origin of the tree was ambiguous, and the owner stated that his tree was locally unique. We did not observe *D. riojae* fruits nearby at a large outdoor market in Xilitla. Tree origin throughout the range of cultivated *D. riojae* was usually ambiguous. Growers indicated that new trees sometimes arose from adventitious root and crown shoots, as well as spontaneously from naturally dispersed seeds. Nevertheless, on the west side of the Sierra Gorda some people occasionally also spoke about particular trees that either they, or people that they knew, had intentionally grown from seeds. The seeds are said to be difficult to germinate, and young trees are reportedly slow to establish. On the west side of the Sierra Gorda, we observed one roughly 30 cm tall *D. riojae* sapling, that had reportedly been grown from seed, displayed for sale among several other species of fruit trees.

Discussion

Genetic studies

Strong evidence of genetic differentiation exists for *D. conzattii* and *D. riojae*, but there is little evidence for genetic differentiation between *D.* cf. *riojae* in Oaxaca and *D. riojae*. The sequence of trnL-intron for *D. riojae* is identical to that of *D. californica* and *D. digyna*, but the latter two species differ considerably in morphology from *D. riojae*, and from each other. Conversely, *D. riojae*, *D. conzattii*, and *D.* cf. *riojae*, each have different trnL-intron sequences, even though they were considered the same species by Wallnöfer (2007). *D. conzattii* has a two base pair insertion that is currently not known to occur in any other species of *Diospyros*, based on Genbank submissions (data not shown). *D.* cf. *riojae* has two one base pair indels also known to occur in *D. rekoi* and *D. rosei* accessions from Jalisco. Explanations for the co-occurrence of the indels among these taxa include a close phylogenetic relationship and chloroplast capture involving a close relative. *D.* cf. *riojae* from the Chimalapas of Oaxaca may ultimately be shown to represent an undescribed species.

A genetic analysis of 27 *D. riojae* collections detected higher levels of diversity and heterozygosity (He) in eastern populations than in western populations. This is consistent with the hypothesis that populations of cultivated *D. riojae* are structured on either side of the Sierra Gorda, and may be the result of restricted gene movement or a bottleneck during the colonization of western populations. The minimum distance between eastern and western populations is about 106 km. In the intervening mountain terrain, there are occurrences of *D. gomeziorum*, a rare species that is most similar to *D. riojae*. This species seems to be separated geographically and ecologically from cultivated populations of *D. riojae*. We were unable to locate fresh material of this species for inclusion in our study. Whether or not this species can be genetically differentiated from *D. riojae* warrants further investigation.

Morphological studies and taxonomic status

Provance and Sanders (2006) examined the taxonomy of *D. riojae* and *D. conzattii*, and discovered several differences in leaf and flower morphology that could be used to differentiate the species. Based on herbarium specimens, they recognized three additional geographically coherent taxa. Subsequently, Wallnöfer (2007) treated all of these species as *D. conzattii*. He considered their morphological differences to be cultivar-level, ecologically induced, or artifacts of short-term alcohol preservation techniques. He also suggested that morphological differences among these species had resulted from gene flow between ancient cultivars and wild populations. Differences in flower size led Wallnöfer to suspect that changes in ploidy had also occurred. He considered the type of *D. conzattii* to be an ancient cultivar, and the distribution of the species to be distorted by relict cultivars surviving the decline of indigenous tribes following the colonization of Mexico by Europeans.

Previously, Provance and Sanders demonstrated the taxonomic utility of male flower size: long, urceolate flowers occur in *D. conzattii* and *Diospyros costaricensis* Provance et A.C. Sanders, and short, urceolate-campanulate flowers occur in *D. riojae* and *D. gomeziorum* (2006). We contend that changes in ploidy level, which can result in barriers to reproduction, and concomitant changes in morphology, argue in favor of recognizing multiple taxa. Ploidy levels are currently unknown for most Mesoamerican *Diospyros*, however, as cytological studies have only included *D. digyna* and *Diospyros xolocotzii* Madrigal et Rzed. Both species produce large edible fruit and long male flowers, but have typical ploidy levels of $2n = 30$ (Calderón-Olascoaga 2011).

Wallnöfer stated that perceived differences in brilliance reported for leaves of *D. riojae* and *D. conzattii* were artifacts of alcohol preservation, and that differences in vein prominence were induced by the local ecology. We eschewed alcohol while collecting, and all of our herbarium vouchers were air-dried in the same oven. Higher orders of venation were not prominent in our collection of *D. conzattii*, and consistently prominent in our *D. riojae* collections, even though we sampled at various elevations from a large geographical area, and from across local landscapes in both arid and humid climates. Consistent with findings of Provance and Sanders (2006), leaf brilliance, leaf venation, leaf shape, and leaf margin structure is considerably different in each species, while rather constant among members of the same species. Genetic and morphological evidence strongly suggests that *D. conzattii* and *D. riojae* are not cultivars of the same species, but species unto themselves. Some of the morphological and genetic differences observed in *D. riojae* and *D. conzattii* may reflect a long history of human cultigen selection, but we doubt this model, or an ecological one, adequately explains the totality of the observed morphological and genetic variation.

Diospyros cf. *riojae* from Oaxaca resembles *D. riojae*, differing primarily in leaf morphology. Because of the differences in morphology, and between the trnL-intron sequences of these two species, we suspect *D.* cf. *riojae* is distinct from *D. riojae*. However, differentiation was not demonstrated quantitatively by our data. Wallnöfer (2007) included this specimen in his concept of *D. conzattii*. Our results suggest that the Oaxaca specimen of *D.* cf. *riojae* has little to do with Oaxaca populations of *D. conzattii*, and more to do with *D. riojae* populations from North-Central Mexico. At this time, *Diospyros* aff. *riojae* Gómez Pompa, is likely to be the best determination for the specimen collected in the Chimalapas of Oaxaca.

Ethnobotany

The gathering of useful trees into protected areas can be viewed as an early step in the process of tree domestication (e.g. Casas et al. 2007). Here, we present the first detailed description of *D. riojae* cultivation, occurring primarily in homegardens and community-managed forests and groves in North-Central Mexico. The fruits have an appearance, taste, and flesh texture similar to *D. digyna* fruits. In its stronghold, Tecozautla, Hidalgo, a number of growers reported that the species was in decline. They attributed this drop in the population to increasing disinterest in the fruit by younger generations. One grower remarked that nowadays spontaneous trees are less likely to be conserved, and that mature trees are more likely to be cut down, than in previous years. It was further stated that increasingly, growers are apt to cut down *zapote negro* trees to create space for more popular crops, such as wild pecans known locally as criollas (*Carya* sp.).

Narrow canals attributed to the Toltec were used to irrigate residential gardens 60 km southeast of Tecozautla at Tula, Hidalgo, as early as ca. A.D. 950–1,200 (Doolittle 1990). Early references to irrigation canals in Tecozautla suggest they existed before 1600 (Melville 1997), though who constructed the putative canals is unknown. Reports of an early canal system that is no longer apparent raises the suspicion that the current canal system was built over an earlier, and perhaps indigenous canal system, as occurred in other parts of Mexico (Doolittle 1990). If it is true, then the distribution of *D. riojae* in Tecozautla may at least in part be explained as an artifact of indigenous plantings along canals.

Alternatively, the current distribution may be a result of post-contact enrichment. However, pre-contact establishment seems more likely, since seeds and seedlings seem to require some human management in the Municipio of Tecozautla, even if minimal or indirect, to achieve adulthood. Remarkably, healthy mature trees clearly survive in this area without supplemental water, as they are occasionally encountered in the rural outskirts along the fringes of non-irrigated fields, at the sites of deserted homes, and other semi-natural settings where supplemental water is apparently not available. Further research into the local hydrology may be enlightening. The population in Tecozautla seems too extensive to have been cultivated into its present existence post-contact without some kind of historical account of enrichment. Thus, we doubt the *zapote negro* population in Tecozautla has come about entirely post-contact. Rather, the species seems to have been introduced by people relatively early to these arid lands west of the Sierra Gorda, especially around Tecozautla.

Although we suspect *D. riojae* is not indigenous to Tecozautla, we cannot be certain of this without further research. Areas along the Río Tecozautla may have served as refugia for a formerly more widespread indigenous *D. riojae* population in the Tecozautla region. These refugia may have been, or may still be, ecologically and climatologically similar to the cloud and pine-oak forests of steep terrain in Veracruz and Puebla, where *D. riojae* is typically considered indigenous. It is noteworthy that one grower reported that *D. riojae* used to occur in gallery forests at Cañón del Infiernillo, a location now underwater following the construction of the Presa de Zimapan. Interestingly, a hypothesis of drastic post-contact environmental change, largely induced by grazing livestock, has been proposed for Western Hidalgo (Melville 1997).

Opossums (*Didelphis* sp., locally *tlacuache*) are common in Tecozautla courtyards, and growers closely associate them with *D. riojae* fruits. The reputation of the Virginia opossum (*Didelphis virginiana* Kerr) as a disseminator of American persimmon (*Diospyros virginiana* L.) is controversial (e.g. Worth 1975), since it has also been reported that they do not swallow the seeds when they eat the fruit (Reynolds 1945). Nevertheless, it appears that opossums do eat large amounts of *D. riojae* fruit, and we suspect they occasionally disperse the seeds through endozoochory or may encourage seed germination by consuming flesh. Thus, distribution patterns may partly reflect this relationship.

Our guide in Tanjasnec provided the word *monek'* for *D. riojae* and *D. digyna* trees, and for their fruits. Our spelling of the word reflects his insistence on writing the correct spelling of the word down for us. Alcorn (1984) reported the Teenek name *munek'* for *D. digyna*. Alcorn did not mention the occurrence of two species of *Diospyros* in the Huasteca flora, but her collections included some specimens that would later be determined as *D. riojae* (Wallnöfer 2007), which suggests that her data for *D. digyna* is a composite. Our guide was familiar with two species, including *D. riojae* and *D. digyna*, which he explained are known by the same name. Clearly, more-in-depth studies related to the use and cultivation of *D. riojae* and *D. digyna* are warranted.

The climate east of the Sierra Gorda is warmer, and receives more rain than the cloud forests at and near the type locality in Veracruz (subhumid, with MAP = 1,000–1,800 mm and MAT = 16–22 °C) where *D. riojae* is putatively native (García E—Comisión Nacional para el Conocimiento y Uso de la Biodiversidad). The high levels of genetic diversity observed in eastern populations, raise the suspicion that *D. riojae* could be native to low elevations east of the Sierra Gorda, including the gulf coastal plain in San Luis Potosi. Further studies will be needed to address this question. Our sample was too small to strongly advocate conservation of individual genotypes. However, the higher genetic diversity and unique haplotypes found in Tanjasnec and Aldzulup Poytzén suggest germplasm conservation should emphasize eastern populations. Finally, the *D. riojae* orchard in El Paso might have a particularly high conservation value. It supports a large population of *D. riojae*, and similar groves of semi-domesticated trees have been shown to harbor diverse assemblages of wildlife (Reitsma et al. 2001; Perfecto 1997; Estrada et al. 1993).

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