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PHYLOGENETICS AND RETICULATE EVOLUTION IN *PISTACIA* (ANACARDIACEAE)[1]

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The systematic position and intrageneric relationships of the economically important *Pistacia* species (Anacardiaceae) are controversial. The phylogeny of *Pistacia* was assessed using five data sets: sequences of nuclear ribosomal ITS, the third intron of the nuclear nitrate reductase gene (*NIA-i3*), and the plastid *ndhF*, *trnL-F* and *trnC-trnD*. Significant discordance was detected among ITS, *NIA-i3*, and the combined plastid DNA data sets. ITS, *NIA-i3*, and the combined plastid data sets were analyzed separately using Bayesian and parsimony methods. Both the ITS and the *NIA-i3* data sets resolved the relationships among *Pistacia* species well; however, these two data sets had significant discordance. The ITS phylogeny best reflects the evolutionary relationships among *Pistacia* species. Lineage sorting of the *NIA-i3* alleles may explain the conflicts between the *NIA-i3* and the ITS data sets. The combined analysis of three plastid DNA data sets resolved *Pistacia* species into three major clades, within which only a few subclades were supported. *Pistacia* was shown to be monophyletic in all three analyses. The previous intrageneric classification was largely inconsistent with the molecular data. Some *Pistacia* species appear not to be genealogical species, and evidence for reticulate evolution is presented. *Pistacia saportae* was shown to be a hybrid with *P. lentiscus* (maternal) and *P. terebinthus* (paternal) as the parental taxa.

Key words: Anacardiaceae; ITS; *ndhF*; *NIA-i3*; phylogenetics; *Pistacia*; *trnC-trnD*; *trnL-F*.

Zohary (1952) recognized 11 species in the genus *Pistacia* L. (Anacardiaceae). *Pistacia* contains the economically important species, *P. vera*, the source of pistachio nuts and is an important floristic element in the vegetation of its distributional region. One of these species (*P. saportae*) was later suggested to be an interspecific hybrid (Zohary, 1972). *Pistacia aethiopica* J. O. Kokwaro was published as a new species in 1980 (Kokwaro and Gillett, 1980); however, its status has not been evaluated. *Pistacia integerrima* was proposed as a recently diverged subspecies of *P. chinensis* (Zohary, 1952). On the basis of results from plastid restriction site analyses and its flowering phenology, however, Parfitt and Badenes (1997) argued for the species status of *P. integerrima*. *Pistacia* sensu Parfitt and Badenes (1997) thus comprises 11 species disjunctly distributed in the northern hemisphere (Fig. 1), with seven species distributed from the Mediterranean basin to central Asia (*P. atlantica*, *P. integerrima, P. khinjuk*, *P. lentiscus*, *P. palaestina*, *P. terebinthus*, and *P. vera*), two species in eastern Asia (*P. chinensis* and *P. weinmannifolia*), and two species from the southwestern United States to Central America (*P. mexicana* and *P. texana*). *Pistacia chinensis* extends into tropical Asia as far as Myanmar and the Philippines (Zohary, 1952).

Pistacia is characterized by its dioecious reproductive system and homeochlamydic perianth (or naked flowers) (Mabberley, 1997). *Pistacia*, distinguished from other Anacardiaceae members by its reduced flower structure, plumose styles, and unusual pollen morphology (Pell, 2004), was described as a separate family, Pistaciaceae Adans. (Adanson, 1763). The synapomorphies of a single apotropous ovule per locule place *Pistacia* within the Anacardiaceae. This treatment is supported by morphological data (Wannan and Quinn, 1991) and recent molecular data (Pell, 2004; Yi et al., 2004, 2007). Many authors recognized the affinity of *Pistacia* with genera in the tribe Rhoeae, although *Pistacia* was treated as a distinct tribe or a subfamily (Marchand, 1869; Eichler, 1875–1878; Takhtajan, 1987, 1997; Mitchell et al., 2006). *Pistacia* resembles other Rhoeae members by having three syncarpous carpels, unilocular fruits, and a thin exocarp. Engler (1876) placed *Pistacia* in the tribe Rhoideae (= Rhoeae). This treatment was followed by Engler (1883, 1892) and Mitchell and Mori (1987). Wannan and Quin (1991) divided the Anacardiaceae into two groups, A and B, based on fruit and wood anatomy, flower morphology, and flavonoid chemistry. Wannan and Quin's groups A and B are similar to subfamilies Spondioideae and Anacardioideae within molecular studies of Terrazas (1994) and Pell (2004). *Pistacia* was included in subfamily Anacardioideae in both studies.

On the basis of the morphology of leaves, leaflet, inflorescence, flowers, fruits, and the seedlings, Zohary (1952) divided *Pistacia* into four sections: *Lentiscella* Zoh., including *P. mexicana* HBK, and *P. texana* Swingle; *Eu Lentiscus* Zoh., including *P. lentiscus* L., *P. saportae* Burnat., and *P. weinmannifolia* Poisson; *Butmela* Zoh., including *P. atlantica* Desf.; and *Eu Terebinthus* Zoh., including *P. chinensis* Bge., *P. khinjuk*

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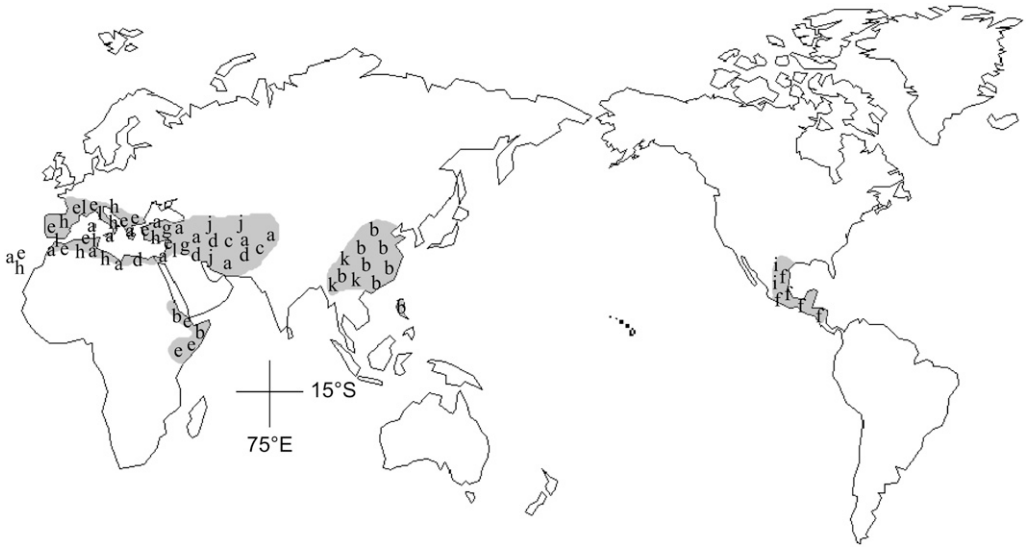

Fig. 1. The present distribution of *Pistacia* sampled in this study. The light gray shading indicates the general locations of 11 *Pistacia* species and one putative hybrid: a = *P. atlantica,* b = *P. chinensis,* c = *P. integerrima*, d = *P. khinjuk*, e = *P. lentiscus*, f = *P. mexicana*, g = *P. palaestina*, h = *P. terebinthus*, i = *P. texana*, j = *P. vera*, k = *P. weinmannifolia*, l = *P. saportae*.

Stocks, *P. palaestina* Boiss., *P. terebinthus* L., and *P. vera* L. On the basis of plastid restriction site analysis and morphological characters, Parfitt and Badenes (1997) suggested the division of the genus into two sections, *Lentiscus* and *Terebinthus*. Section *Lentiscus* includes Zohary's (1952) sects. *Letiscella* and *Eu Lentiscus* and consists of the evergreen species with paripinnate leaves and smaller seeds. They also suggested that Zohary's (1952) sects. *Butmela* and *Eu Terebinthus* be combined as sect. *Terebinthus*, which includes the deciduous species with imparipinnate leaves and large seeds. Section *Terebinthus* was supported by recent molecular studies on Mediterranean *Pistacia* species (Kafkas and Perl-Treves, 2001, 2002; Golan-Goldhirsh et al., 2004; Kafkas, 2006). However, the sampling scheme of the previous molecular studies was limited for sect. *Lentiscus*. The phylogenetic relationships among *Pistacia* species were also estimated by plastid DNA restriction site analysis and RFLP (Parfitt and Badenes, 1998), RAPD (Katsiotis et al., 2003), and RAPD and AFLP (Katsiotis et al., 2003). The current study extends this work by sampling 11 species and one putative hybrid using two nuclear (ITS and *NIA-i3*) and three plastid (*ndhF*, *trnC-trnD,* and *trnL-F*) markers.

Hybridization is presumed to be common among some *Pistacia* species (Zohary, 1952; Crane and Forde, 1976; Crane and Iwakiri, 1986; Morgan et al., 1992). Parfitt has hybridized a number of *Pistacia* species and has not seen evidence of genetic crossing barriers (Parfitt, 2003). *Pistacia saportae* shares similar morphology of leaves, winged rachis, inflorescence, and fruits with *P. lentiscus*, but the shape of its leaflets as well as occurrence of a terminal leaflet resemble *P. lentiscus* and *P. terebinthus*. *Pistacia saportae* was originally described as a hybrid between *P. lenticus* and *P. terebinthus* by Burnat (1896). However, some botanists have disputed the hybrid origin hypothesis of this species (Zohary, 1952). Zohary (1952) treated *P. saportae* as separate species. Later, Zohary (1972) treated *P. saportae* as a hybrid based on its intermediate morphology between the putative parents, *P. palaestina* and *P. lentiscus.* The hybrid status of *Pistacia saportae* was supported by wood anatomy (Grundwag and Weaker, 1976) and RAPD analysis (Werner et al., 2001).

Molecular sequence analyses can be a powerful tool to identify hybrid taxa (Rieseberg and Wendel, 1993). Hybrids can be identified directly from sequence data as indels or SNPs (single nucleotide polymorphisms) in the ITS sequences (Rieseberg and Ellstrand, 1993; Baldwin et al., 1995; Wolfe et al., 1998). If both parental alleles are maintained at a nuclear locus in the hybrid genome, they can be cloned, then analyzed cladistically, together with the parental genotypes. Cladistic analysis of low-copy nuclear genes was successfully used to identify a few *Paeonia* hybrids (Sang and Zhong, 2000). When a hybrid fails to maintain sequence polymorphism at the nuclear loci, e.g., from allele loss, it may be identified from incongruence between the organelle- and the nuclear-based phylogenies (Rieseberg, 1991, 1997). Molecular phylogenies based on multiple, unlinked loci and multiple sample populations per species may successfully elucidate reticulate evolution (Rieseberg, 1997). The putative *Pistacia* hybrids were identified from SNPs in the ITS sequences, and cladistic analysis of the low-copy *NIA-i3* gene region. The putative paternal and maternal parents of proposed hybrids were identified by comparing the incongruent systematic positions between organelle- and nuclear-based phylogenies.

The objectives of this study were to (1) construct the phylogeny of *Pistacia* based on both nuclear and plastid sequences, (2) test the intrageneric classification of *Pistacia*, (3) elucidate the extent and nature of reticulate evolution among *Pistacia* species, and (4) discuss the taxonomic delimitation of *Pistacia* species.

MATERIALS AND METHODS

Species examined—All 11 *Pistacia* species recognized by Zohary (1952) and Parfitt and Badenes (1997) were included in this study (Table 1). We also included the putative hybrid *Pistacia saportae*. Because the sister group of *Pistacia* was not resolved (Pell, 2004), six genera from the tribe Rhoeae of Anacardiaceae were selected as outgroups (Table 1).

DNA extraction, PCR amplification, cloning, and sequencing—Total DNA was extracted from silica-gel dried or fresh leaf materials using the CTAB method (Doyle and Doyle, 1987). Amplifications were performed in 20-μL

TABLE 1. Accessions of *Pistacia* and outgroup taxa.

Taxon	Voucher	Locality	Distribution	GenBank accessions				
				ITS	*NIA-i3*	*ndhF*	*trnC-trnD*	*trnL-F*
Pistacia atlantica Desf. #1	*Golan 1.114* (F)	Israel (cult.)	W Asia to Mediterranean	EF193076	EF190543	EF193106	EF193140	EF193123
P. atlantica Desf. #2	*Ickert-Bond 1300* (F)	USA, Arizona (cult.)		EF193077		EF193107	EF193141	EF193124
P. atlantica Desf. #3	*Wen 7133* (US)	USA, California (cult.)		EF193078		EF193108	EF193142	EF193125
P. chinensis Bge. #1	*Golan 1.412* (F)	Israel (cult.)	E Asia	EF193079	C1, EF190515 C2, EF190516 C3, EF190517 C4, EF190518	EF193109	EF193143	EF193126
P. chinensis Bge. #2	*Ji 0174* (KUN)	China, Kunming		EF193080	EF190519	EF193110	EF193144	EF193127
P. chinensis Bge. #3	*Wen 7090* (F)	USA, California (cult.)		DQ390466	DQ382323	DQ390462	DQ400560	DQ390470
P. integerrima Parfitt D. E. & Badenes M. L.	*Parfitt 54* (F)	USA, California (cult.)	EN Africa	EF193081	EF190520	EF193111	EF193145	EF193128
P. khinjuk Stocks	*Golan 1.149* (F)	Israel (cult.)	W Asia to Mediterranean	C1, EF193104 C2, EF193105	C1, EF190526 C2, EF190527 C3, EF190528 C4, EF190529	EF193112	EF193146	EF193129
P. lentiscus L. #1	*Golan 1.1009* (F)	Israel (cult.)	Mediterranean	C1, EF193082 C2, EF193083	EF190535	EF193113	EF193147	EF193130
P. lentiscus L. #2	*Ickert-Bond 1299* (F)	USA, Arizona (cult.)		DQ390467	DQ382324	DQ390463	DQ400561	DQ390471
P. mexicana H. B. K.	*Parfitt 27* (F)	USA, California (cult.)	Mexico, Texas	DQ390468	DQ382325	DQ390464	DQ400562	DQ390472
P. palaestina Boss. #1	*Golan 1.222* (F.)	Israel (cult.)	Mediterranean	C1, EF193084 C2, EF193085	C1, EF190521 C2, EF190522	EF193116	EF193150	EF193133
P. palaestina Bois. #2	*Golan 1.202* (F)	Israel (cult.)		EF193095	EF190523	EF193114	EF193148	EF193131
P. palaestina Bois. #3	*Golan 1.215* (F)	Israel (cult.)		C1, EF193097 C2, EF193096	EF190524	EF193115	EF193149	EF193132
P. saportae Burnat #1	*T. Yi 4* (US)	Israel (cult.)	Mediterranean	C1, EF193098 C2, EF193099	C1, EF190532 C2, EF190533 C3, EF190534	EF193117	EF193151	EF193134
P. saportae Burnat #2	*T. Yi 10* (US)	Israel (cult.)		C1, EF193100 C2, EF193101 C3, EF193102 C4, EF193103	C1, EF190530 C2, EF190531	EF193118	EF193152	EF193135
P. terebinthus L.	*Golan 1.350* (F.)	Israel (cult.)	Mediterranean	EF193086	EF190525	EF193119	EF193153	EF193136
Pistacia texana Swingle #1	*Ickert-Bond 1301* (F)	USA, Arizona (cult.)	Mexico, Texas	EF193087	C1, EF190536 C2, EF190537	EF193120	EF193154	EF193137
Pistacia texana Swingle #2	*Wen 7285* (F)	USA, Texas	S. Texas & NE Mexico	EF193088	C1, EF190538 C2, EF190539 C3, EF190540	EF193121	EF193155	EF193138
P. vera L. #1	*Golan 1.539* (F.)	Israel (cult.)	W Asia	AY677201	DQ382326	AY677209	DQ400563	AY677204
P. vera L. #2	*Wen 7099* (F)	USA, California (cult.)		C1, EF193089 C2, EF193090 C3, EF193091	C1, EF190541 C2, EF190542	EF193122	EF193156	EF193139
P. weinmannifolia Poisson	*Li 1630* (KUN)	China, Yunnan	E Asia	C1, EF193092 C2, EF193093 C3, EF193094	DQ382327	DQ390465	DQ400564	DQ390473
Actinocheita filicina (D. C.) Barkl.	*Panero s. n.* (CS)	Mexico	S Mexico	AY641509	DQ382321	AY640640	DQ400558	AY643120
Malosma laurina (Nutt.) Nutt. ex Engl.	*Miller 34* (CS)	Rancho Santa Ana Bot. Gard., CA (cult.)	S California and N Lower California	AY641510	DQ382322	AY640461	DQ400559	AY643121
Rhus aromatica Ait.	*Wen 7086* (F)	USA, Illinois	E North America	AY641493	C1, DQ382284 C2, DQ382285	AY640447	DQ400535	AY643107
R. chinensis Mill.	*Wen 6389* (F)	Morton Arb., IL (cult.)	E Asia to SE Asia	AY641480	DQ382286	AY640435	DQ400536	AY643095
R. glabra L.	*Wen 7171* (F)	USA, Alabama	North America	AY641486	DQ382292	AY640440	DQ400541	AY643100
R. virens Lindh. ex Gray	*Wen 7282* (F)	USA, Texas	SW America to N Mexico	AY641506	DQ382320	AY640458	DQ400557	AY643118
Schinus molle L.	*Wen 6686* (F)	USA, Los Angeles, CA (cult.)	California and Texas	AY641512	DQ382333	AY640463	DQ400565	AY643123
S. quartiniana (A. Rich.) A. J. Miller #1	*Miller 51* (CS)	Phoenix Desert Bot. Gard., AZ (acc. # 1980007001)	Africa	AY641517	DQ382331	AY640468	DQ400566	AY643128
S. undulata (A. Rich) T. S. Yi, A. J. Miller & J. Wen	*Miller s.n.* (CS)	Phoenix Desert Bot. Gard., AZ (acc. # 19800071)	Africa	AY541519	DQ382332	AY640640	DQ400567	AY643130
Toxicodendron diversilobum	*Wen 6693* (US)	USA, California	W North America	AY677202	DQ382328	AY677208	DQ400568	AY677205
T. radicans	*Wen 6236* (US)	USA, Illinois	North America	AY677203	DQ382329	AY677207	DQ400569	AY677206
T. vernix (L.) Kuntze	*Wen 7146* (F)	Morton Arb., IL (cult.)	E North America	AY541520	DQ382330	AY640471	DQ400670	AY643131

reactions with approximately 10–50 ng of total DNA, 20 mM Tris buffer (pH 8.3, with 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% Tween 20), 0.15 mM of each dNTP, 0.5 μM of each primer, and 1 U of *Taq* polymerase. The ITS region was amplified using primers ITS4 and ITS5 (White et al., 1990). The primers NIA3F and NIA3R developed by Howarth and Baum (2002) were used to amplify the *NIA-i3* region. The *trnC-trnD* region was amplified with three pairs of primers: trnC and petN2R, petN1 and psbM2R, and psbM1 and trnD as described in Lee and Wen (2004). The *ndhF* gene and *trnL-F* regions were amplified using the methods of Olmstead and Sweere (1994) and Taberlet et al. (1991), respectively. The PCR products were electrophoresed in 1.0% low-melting-point NuSieveGTG agarose gels (FMC BioProducts, Rockland, Maine, USA) containing 0.5 μg/mL ethidium bromide, with one-tenth the standard EDTA concentration (Sambrook et al., 1989) in 1× Tris-acetate buffer (pH 7.8). The amplicons were cut from the gel and digested using the GELaseTM Agarose Gel-Digesting preparation and the "Fast Protocol" method (Epicentre Technologies, Madison, Wisconsin, USA).

Some *Pistacia* species have multiple sequence signals on the ITS and *NIA-i3* sequences directly obtained from purified PCR products, suggesting the presence of intraindividual polymorphisms. All these purified PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, California, USA). At least eight white colonies from the cloning reactions of each species were screened and amplified.

Sequencing reactions were performed in a final volume of 10 μL using the BigDye Terminator cycle sequencing kit (PE Applied Biosystems, Foster City, California, USA) and the manufacturer's instructions, then viewed with an ABI 3100 automated DNA sequencer (Applied Biosystems). The resulting sequences were aligned and edited using the program Sequencher (version 3.1.1, Gene Codes Corp., Ann Arbor, Michigan, USA). Alignments were further adjusted by eye in the program PAUP* version 4.0b10 (Swofford, 2003). All sequences have been deposited at GenBank (see Table 1 for accession numbers). DNA divergence was estimated using Kimura's (1980) two-parameter method in PAUP*.

Phylogenetic data analysis—For the ITS, the *NIA-i3*, and the combined plastid data sets (*ndhF*, *trnC-trnD* and *trnL-F*), parsimony analyses (Swofford et al., 1996) were performed using PAUP*4.0b10 (Swofford, 2003) with heuristic searches: tree-bisection-reconnection (TBR) branch swapping, MULPARS option, and 100 random taxon addition replicates. The tree topology did not change when gaps were included in the analyses; however, support along some branches was higher. Therefore, each gap was coded as a separate binary character using the method of Simmons and Ochoterena (2000). Internal branch support was estimated with 1000 bootstrap replicates (Felsenstein, 1985) using the same heuristic search strategy described earlier.

Phylogenetic reconstructions were also conducted using the maximum-likelihood (ML) method as implemented in PAUP* 4.0b10 (Swofford, 2003). The ML trees were used in the Shimodaira-Hasegawa test to evaluate the congruence among three different data sets. The Bayesian analyses were performed as implemented in MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001). The best-fit model for the ML and the Bayesian analyses was selected using a hierarchical likelihood ratio test conducted in MODELTEST version 3.06 (Posada and Crandall, 1998). GTR+G, GTR+I and GTR+I+G were the best-fit models for ITS, *NIA-i3* and the combined plastid data sets, respectively. The Bayesian analysis was conducted with variation in gamma-distributed rate across sites and an initial estimate of equal base frequencies. The Markov chain Monte Carlo (MCMC) algorithm was run for 2 000 000 generations with four incrementally heated chains, starting from random trees and sampling one out of every 100 generations. A 50% majority-rule consensus tree was calculated with PAUP* 4.0b10 from the last 18 001 of the 20 001 trees sampled. The first 2000 trees were discarded as burn-in when the chains became stationary. The posterior probability of each topological bipartition was estimated from the frequency of these bipartitions across all 18 001 trees sampled.

The independent length difference (ILD) test (Farris et al., 1994), the Templeton test (Templeton, 1983), and the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) were used to evaluate the congruence among the combined plastid data sets (*ndhF*, *trnL-F*, and *trnC-trnD*), ITS and *NIA-i3* data sets. The putative hybrid species *P. saportae* was excluded from the analyses. Some *Pistacia* species have more than one allele per clone of ITS and/or *NIA-i3*. Using the clonal 1 sequence of each of these species does not change the tree topology. To maintain the consistency among different data sets, we included only one sequence from each species in the analysis. *Rhus* was supported as one of the closest relatives of *Pistacia* among the outgroups tested in the current study (Yi et al., 2004, 2007); therefore, *Rhus* species were used as the outgroups in the phylogenetic analyses. Additional outgroup taxa were not included to prevent outgroup interrelationships from complicating the issues of data congruence. The incongruence length difference (ILD) test was conducted with PAUP* 4.0b10 (Swofford, 2003) with 100 replicates, TBR branch-swapping heuristic searches, and gaps treated as missing data. Topological congruence between the gene trees produced by parsimony was evaluated with the Templeton test as implemented in PAUP* 4.0b10. The SH test as implemented in PAUP* 4.0b10 was used to evaluate the topological congruence between gene trees produced by the likelihood method. The test distribution was computed using the reestimated log likelihoods (RELL) approximation with 1000 nonparametric bootstrap replicates.

RESULTS

ITS data—The aligned matrix of the ITS1, 5.8S, and ITS2 regions had a length of 748 bases, with 263 variable and 173 parsimony-informative sites. The sequence divergence among *Pistacia* species (excluding *P. saportae*) varied from 0.00 to 6.90%. Sequence divergence between *Pistacia* and outgroup taxa varied from 6.88 to 16.02%. The ITS sequence divergence between *P. palaestina* and *P. terebinthus* varied from 0.00 to 0.61%. Two accessions of *P. texana* had identical ITS sequences, and the divergence between *P. mexicana* and *P. texana* was only 0.73%.

Maximum parsimony (MP) analyses produced 16 maximally parsimonious trees (MPTs) with a consistency index (CI) of 0.65, a retention index (RI) of 0.82, and a length of 554 steps. The 50% majority-rule consensus of 18 001 trees from the Bayesian analysis was largely congruent with the trees of the parsimony analysis except that three accessions of *P. atlantica* did not form a monophyletic group in the Bayesian analysis.

The ITS data strongly supported the monophyly of *Pistacia* (Fig. 2). Several copies of ITS sequences detected from *P. weinmannifolia* formed a monophyletic group, which was resolved to be the sister to the remainder of the genus (Fig. 2), followed by the *P. mexicana-P. texana* clade. The monophyly of the *P. mexicana-P. texana* clade with all other *Pistacia* except *P. weinmannifolia* was strongly supported in the Bayesian analysis (with posterior probabilities or PP = 99%); however, only weak support was provided by the parsimony analysis (with the bootstrap support or BS = 68%). The remaining species were resolved into two subclades: the *P. lentiscus* subclade and the clade consisting of the remaining species. *Pistacia atlantica*, *P. khinjuk*, and *P. vera* formed a clade. The three accessions of *P. atlantica* constituted a monophyletic group, which was sister to the *P. khinjuk-P. vera* clade. Another clade included *P. chinensis*, *P. integerrima*, *P. khinjuk*, *P. palaestina*, and *P. terebinthus*. Separate accessions of *P. chinensis* formed a monophyletic group. The positions of *P. palaestina* and *P. terebinthus* were not well resolved (Fig. 2) in the consensus tree. Some alleles of *P. palaestina* and *P. terebinthus* had identical sequences.

Analysis of all clonal ITS sequences of *P. saportae* (accessions #1 and #2) revealed two distinct types. The type 1 sequence was represented by 21 clonal sequences of #1 and #2, and the type 2 sequence was represented by only one clonal sequence of #2 (Fig. 2). *Pistacia saportae* type 1 sequences varied from 0 to 0.61%. *Pistacia lentiscus*, a putative parent of *P. saportae*, also had multiple forms of ITS sequences. Sequence divergences among clonal sequences of *P. lentiscus* varied from 1.23 to 2.19%. All clonal sequences of the two accessions of *P. lentiscus* formed a monophyletic group together with *P. saportae* type 1 sequences. The sequence divergences between

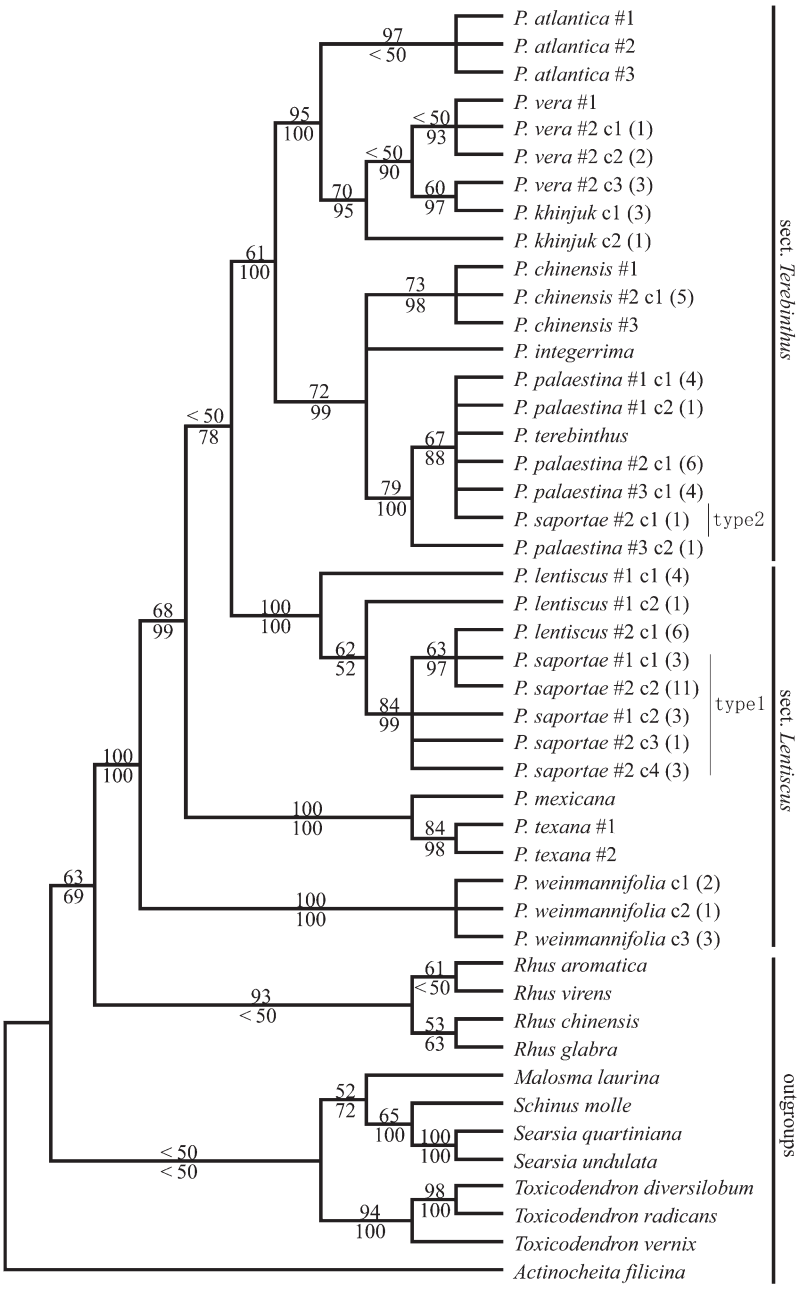

Fig. 2. The strict consensus tree of 16 most parsimonious (MP) trees for the ITS data of *Pistacia*, with each gap coded as a separate binary character (CI = 0.65 and RI = 0.82). The bootstrap values in 1000 replicates >50% are shown above the branches, and the Bayesian posterior probabilities are indicated below the branches. An asterisk (*) indicates the topological discordance of related clades between the MP and Bayesian trees. The sectional classification of *Pistacia* follows that of Parfitt and Badenes (1997) . # = accession numbers, c = clonal sequence, numbers in bracket = total numbers of certain clonal sequence.

P. lentiscus and *P. saportae* type 1 sequences were from 0 (between *P. lentiscus* #2 and *P. saportae* #1 clone1; *P. lentiscus* #2 and *P. saportae* #2 clone2) to 2.30% (between *P. lentiscus* #1 clone1 and *P. saportae* #2 clone4). *Pistacia palaestina* or *P. terebinthus* are also putative parents of *P. saportae*. *Pistacia palaestina* may need to be merged into *P. terebinthus*, only *P. terebinthus* was used to discuss the hybridization origin of *P. saportae*. One accession of *P. terebinthus* had only one allele. Sequence divergence between *P. saportae* type 2 sequences and *P. terebinthus* was 0.30% (between *P. terebinthus* and *P.saportae* #2 clone1).

NIA-i3 data—The aligned *NIA-i3* data matrix had 799 characters, 346 of which were variable, and 212 were parsimony informative. Excluding *P. saportae*, sequence divergences among *Pistacia* species ranged from 0.00 to 7.50%. Sequence divergence between *Pistacia* and outgroup taxa ranged from 9.01 to 21.27%. Divergence between *P. palaestina* and *P. terebinthus* ranged from 0.00 to 0.5%. The two accessions of *P. texana* had multiple *NIA-i3* copies. Sequence divergence among different alleles of *P. texana* was from 0.16 to 0.81%. The divergence between *P. mexicana* and *P. texana* ranged from 0.15 to 0.64%.

The MP analysis of the *NIA-i3* data set yielded 24 MPTs with a CI of 0.82, a RI of 0.93, and a total length of 510 steps. *Pistacia* was shown to be monophyletic. The *NIA-i3* data set resolved *Pistacia* into two major clades (Fig. 3). One clade included *P. atlantica*, *P. khinjuk*, *P. mexicana*, *P. texana,* and *P. vera*, within which two well-supported subclades were resolved: *P. atlantica-P. khinjuk-P.vera* and *P. mexicana-P. texana*. The other major clade comprised the remaining *Pistacia* species. *Pistacia weinmaniifolia* formed a sister clade to the *P. lentiscus-P. saportae* clade (type 2 sequences), and the *P. chinensis-P. integerrima-P. palaestina-P. terebinthus-P. saportae* clade (type 1 and type 3 sequences). The 50% majority-rule consensus of 18 001 trees from the Bayesian analysis was largely congruent with the MP trees except that *P. weinmaniifolia* was weakly supported as a sister clade to the *P. lentiscus-P. saportae* (type 2 sequences) clade.

Pistacia saportae had three distinct types of *NIA-i3* sequences. Its type 1 sequence had a close relationship to *P. terebinthus*, the type 2 sequence was similar to that of *P. lentiscus*, and the type 3 sequence was represented by *P. saportae* #1 clone2, which was sister to the *P. chinensis-P. integerrima-P. palaestina-P. terebinthus* clade. There was no sequence divergence between the two *P. saportae* type 1 sequences, as for the two type 2 sequences. Divergences between *P. saportae* type 2 sequences and *P. terebinthus* were from zero (between *P. terebinthus* and *P. saportae* #1 clone1) to 0.16% (between *P. terebinthus* and *P. saportae* #2 clone1). Divergences between *P. saportae* type 2 sequences and *P. lentiscus* varied from 0.16% (*P. saportae* #2 clone2 and *P. lentiscus* #2) to 2.93% (*P. saportae* #1 clone3 and *P. lentiscus* #1). The sequence divergence between the two accessions of *P. lentiscus* was 2.90%. The divergence between *P. lentiscus* and *P. terebinthus* varied from 6.06 to 6.83%. Sequence divergence between *P. saportae* type 1 and type 2 was from 7.04 to 7.18%.

Plastid DNA data—Because there is no recombination in the plastid DNA genome, we combined the three plastid DNA data sets in our analysis. The aligned matrix of combined plastid DNA data had 5438 characters with 491 variable and 175 parsimony-informative sites. Excluding outgroups, the aligned data matrix of *Pistacia* provided only 121 variable and 31 parsimony-informative characters. Within *Pistacia* (excluding *P. saportae*), sequence divergences varied from 0.02 to 0.64%. The sequence divergence between *Pistacia* and outgroup taxa varied from 0.81 to 2.51%. The MP analysis produced four MPTs (619 steps, CI = 0.85, RI = 0.85, and RC = 0.72). The strict consensus tree is presented in Fig. 4. The 50% majority-rule consensus of the 18 001 trees from the Bayesian analysis was congruent with the MPTs. The combined plastid tree strongly supported a monophyletic *Pistacia* genus. The *Pistacia* species were resolved into three clades including: the *P. weinmannifolia* clade, the *Pistacia mexicana-P. texana* clade, and the clade containing all other *Pistacia* species distributed from central Asia to the Mediterranean region (Fig. 4). Relationships among *Pistacia* species from central Asia to the Mediterranean region were not resolved. The data matrix including species of this clade had 85 variable and 20 parsimony-informative characters, and the sequence divergences varied from 0.02 to 0.5%. *Pistacia khinjuk* and the two accessions of *P. vera* constituted a clade, and *P. atlantica* #1 was strongly supported as a sister clade to the *P. khinjuk-P. vera* clade. Two accessions of *P. saportae* formed a well-supported clade together with one of its putative parents of *P. lentiscus* (#2). The sequence divergences between *P. lentiscus* #2 and *P. saportae* was only 0.10%. For each of the three species *P. atlantica*, *P. chinensis*, and *P. palaestina*, the three accessions sampled did not form a monophyletic group. The sequence divergences among the multiple accessions of each species (*P. atlantica*, *P. chinensis*, and *P. palaestina*) varied from 0.14 to 0.25%, from 0.31 to 0.48%, and from 0.16 to 0.33%, respectively.

Data incongruence—Significant incongruence between the ITS and the *NIA-i3* data sets was suggested by the ILD test ($P = 0.01$), the Templeton test ($P < 0.01$) and the SH test ($P < 0.01$). Significant incongruence was also detected between the combined plastid data set and the ITS data set and between the combined plastid data set and the *NIA-i3* data set, with a $P < 0.01$ the ILD, the Templeton, and the SH tests.

DISCUSSION

Discordance among molecular data sets—Topological incongruence among data sets may have either of the two sources. Sampling errors and/or use of inappropriate models of molecular evolution in phylogenetic analysis may cause discordance. This type of topological discordance often can be corrected by adding additional samples and modifying the model used in the phylogenetic reconstruction (Cunningham, 1997). Combined analysis of all data sets can give a better estimated phylogeny in this case (Barrett et al., 1991). The second type of discordance is caused by genealogical discordance, e.g., that caused by lineage sorting and hybridization (Hipp et al., 2004). Combined analysis of different data sets with genealogical discordance does not represent any one genealogy but a combined genealogy of several (Baum et al., 1998) different genealogical constructs.

In this study, significant incongruence was detected between the ITS and the *NIA-i3* phylogenies. The main difference between the ITS and the *NIA-i3* trees is the relative positions of the *P. atlantica-P. khinjuk-P. vera* clade and the *P. mexicana-P. texana* clade. In the ITS tree, *Pistacia weinmannifolia* was supported as the sister to the clade formed by the remaining *Pistacia* species; the next lineage was the North American *P. mexicana-P. texana* clade. The remaining *Pistacia* species included the *P. lentiscus* clade and the clade of *P. atlantica-P. khinjuk-P. vera* and close allies (Fig. 2). In the *NIA-i3* tree, the *P. atlantica-P. khinjuk-P. vera* clade and the *P. mexicana-P. texana* clade formed a monophyletic group, which was one of the two major clades resolved in the *NIA-i3* tree (Fig. 3). More than one accession for most *Pistacia* species was sampled, and PCR reactions of ITS and *NIA-i3* were conducted from the same DNA extraction. Therefore, sampling error is unlikely to explain the discordance between the two nuclear data sets. The discordance between the data sets was observed using both the parsimony and the Bayesian approaches, so different model assumptions probably do not explain the discordance between the ITS and the *NIA-i3* data sets.

Species of the *P. atlantica-P. khinjuk-P. vera* clade share several morphological characters with species of the *P. chinensis-P. integerrima-P. palaestina-P. terebinthus* clade. Their similar morphological characters include deciduous leaves with much larger and fewer (1–5 pairs) leaflets and larger fruits in comparison with those of *P. mexicana* and *P. texana*. *Pistacia mexicana* and *P. texana*, however, have evergreen leaves with 6–20 pairs of much smaller leaflets. Close relationships among species of the *P. atlantica-P. khinjuk-P. vera* clade and the

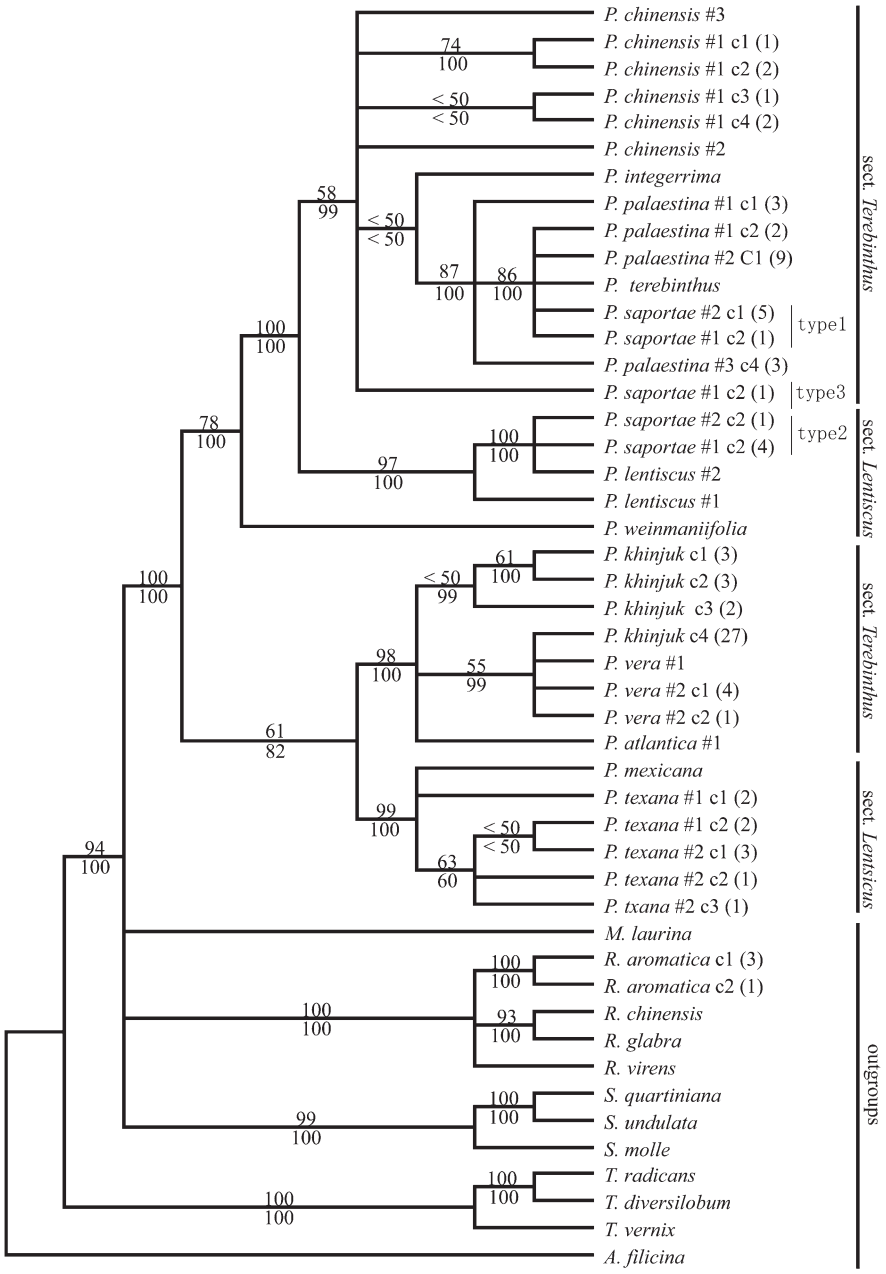

Fig. 3. The strict consensus tree of 24 most parsimonious trees for the *NIA-i3* data set of *Pistacia*, with each gap coded as a separate binary character (CI = 0.82 and RI = 0.93). The bootstrap values in 1000 replicates >50% are above the branches, and the Bayesian posterior probabilities are below the branches. The sectional classification of *Pistacia* follows that of Parfitt and Badenes (1997). # = accession numbers, c = clonal sequence, numbers in bracket = total numbers of certain clonal sequence.

P. chinensis-P. integerrima-P. palaestina-P. terebinthus clade were also suggested in the plastid restriction site analysis (Parfitt and Badenes, 1997), the RAPD and the AFLP analyses (Golan-Goldhirsh et al., 2004) as well as our combined plastid data from the current study. Based on all available data, the ITS tree better reflects the species phylogeny of *Pistacia* than the *NIA-i3* tree. The close relationships between the *P. atlantica-P. khinjuk-P. vera* clade and the *P. mexicana-P. texana* clade in the *NIA-i3* data may be due to hybridization and/or lineage sorting. Many of these species have been shown experimentally to hybridize freely and produce fertile progeny (Parfitt, 2003). Therefore major crossing barriers are probably not genetic but are geographic or phenological as suggested by Parfitt and Badenes (1997). *Pistacia mexicana* and *P. texana* are distributed in North America, and *P. atlantica*, *P. khinjuk*, and *P. vera* are from central and western Asia. There are no paleobotanical data suggesting species of these two clades cooccurred in the same geographic region. Hybridization between these groups is thus an unlikely scenario for species from areas separated by such great distance.

Phylogenetic relationships—*Pistacia* was described as morphologically diverse (Zohary, 1952). Section *Lentiscus* (including

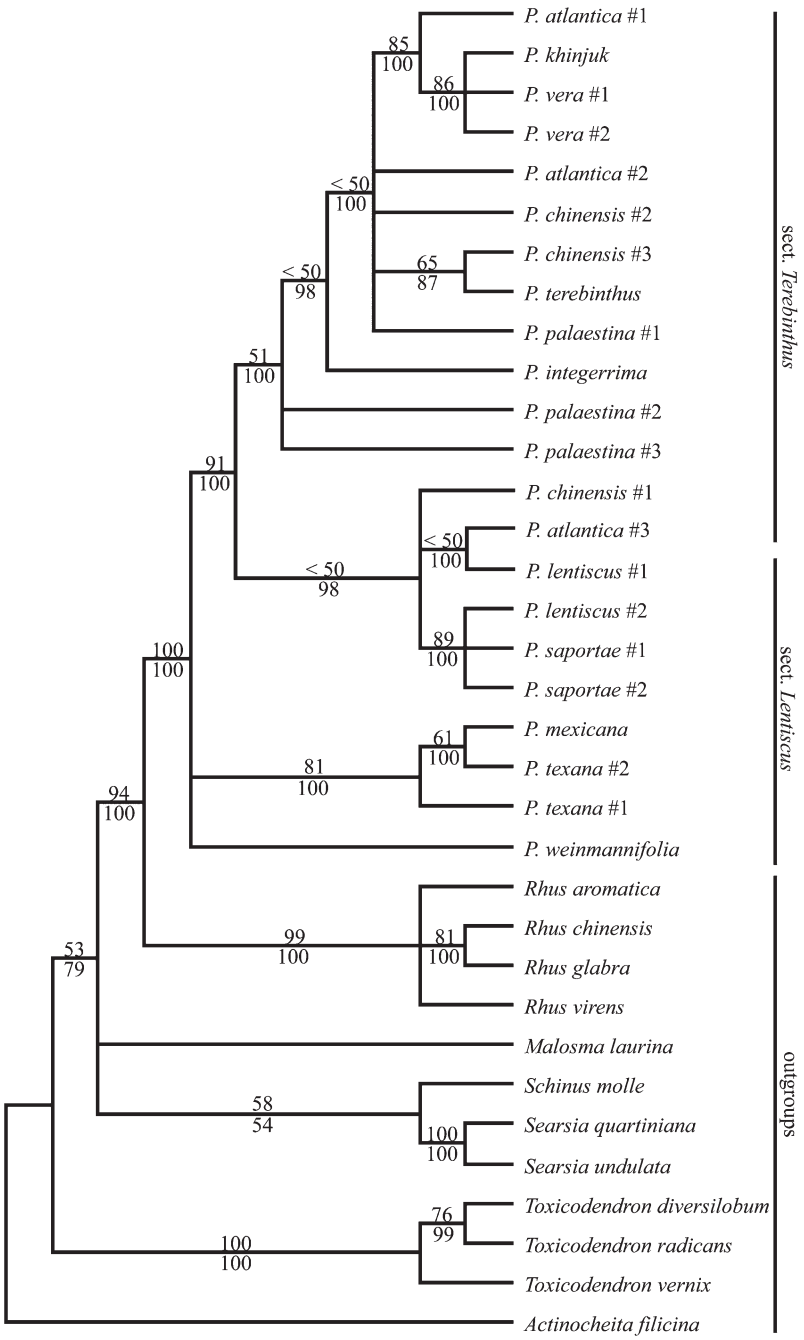

Fig. 4. The strict consensus tree of four most parsimonious trees from combined *ndhF*, *trnC-trnD* and *trnL-F* data sets of *Pistacia*, with each gap coded as a separate binary character (CI = 0.85 and RI = 0.85). The bootstrap values in 1000 replicates >50% are above the branches, and the Bayesian posterior probabilities are below the branches. The sectional classification of *Pistacia* follows that of Parfitt and Badenes (1997) . # = accession numbers, c = clonal sequence.

P. lentiscus) was suggested to be a distinct genus by Tournefort (1700). *Pistacia* was strongly supported as monophyletic in the *trnL-F*, the *rps16*, and the combined *trnL-F* and *rps16* parsimony analyses of Pell (2004) and by the results of the present analysis of nuclear and plastid DNA data sets. Most workers have placed *Pistacia* in the tribe Rhoeae. However, this genus occupies a relatively isolated position, and its sister genus is still unknown. *Rhus* was supported as a close relative among the outgroups selected in two previous molecular studies (Yi et al., 2004, 2007). *Astronium* was weakly supported as sister to *Pistacia* in the *rps16* likelihood tree (Pell, 2004). *Cotinus*, *Mosquitoxylum*, and *Rhus* also formed a weakly supported clade with *Pistacia* in the *trnL-F* likelihood tree (Pell, 2004).

Zohary (1952) divided *Pistacia* species into four sections, *Butmela*, *Eu Lentiscus*, *Eu Terebinthus*, and *Lentiscella*. Zohary's sect. *Butmela* is monotypic and includes only *P. atlantica*. This section was not supported by the present analysis because the plastid and two nuclear DNA data sets all suggested

that *P. atlantica* is nested within sect. *Terebinthus*. It formed a monophyletic group with *P. khinjuk* and *P. vera*, which is consistent with previous analyses (e.g., Parfitt and Badenes, 1997; Kafkas and Perl-Treves, 2002; Golan-Goldhirsh et al., 2004). Section *Terebinthus* formed a monophyletic group in the ITS tree; but this section was not resolved as a monophyletic group in the plastid and *NIA-i3* trees. Species of this section split into two distinct clades in the *NIA-i3* tree. *Pistacia lentiscus* is nested within this section in the plastid tree. Morphological data supported the merge of sects. *Butmela* and *Eu Terebinthus*. The main difference between these two sections is that sect. *Butmela* has a winged leaf rachis. Our results also support the merger of the two sections, as proposed by Parfitt and Badenes (1997).

Zohary's sect. *Lentiscella* was strongly supported as a monophyletic group by both plastid and nuclear DNA data sets. This section was established based on its isolated geographical distribution and larger number of smaller leaflets per leaf (Zohary, 1952). The sections of *Eu Lentiscus* and *Eu Terebinthus* sensu Zohary are not monophyletic in all three molecular data sets. Species of sect *Eu Lentiscus* have evergreen paripinnate leaves, winged rachis, oblong, lanceolate or elliptical leaflets, fasciculate inflorescence, fleshy or dry mesocarp, and bony or leathery endocarp. Parfitt and Badenes (1997) combined Zohary's (1952) sects. *Eu Lentiscus* and *Lentiscella* into a single section *Lentiscus*. Species of section *Lentiscus* are evergreen and have paripinate leaves, whereas section *Terebinthus* species are deciduous and have imparipinnate leaves. Section *Lentiscus* did not form a monophyletic group in the present analysis of the plastid and nuclear DNA data sets. In the plastid DNA and the ITS data sets, species of section *Lentiscus* were resolved into a few parallel clades with the clade of sect. *Terebinthus* nested within it (Figs. 2 and 4). In the *NIA-i3* data, species of sect. *Lentiscus* belonged to two distinct clades (Fig. 3).

Species delimitation—All *Pistacia* species except *P. khinjuk*, *P. mexicana*, and *P. weinmannifolia* were sampled with multiple accessions. The different accessions of each of the following species: *P. lentiscus*, *P. palaestina*, *P. terebinthus*, and *P. vera* did not form a monophyletic group in the combined plastid DNA data as well as in the two nuclear DNA data sets (Figs. 2–4). Different accessions of *P. chinensis* formed a clade in the ITS data set, but were not monophyletic in the *NIA-i3* and plastid DNA data sets. Different accessions of *P. atlantica* formed a clade in the ITS data but were not monophyletic in the combined plastid data. Each species of *Pistacia chinensis* (#2), *P. khinjuk*, *P. lentiscus* (#1 and #2), *P. palaestina* (#1, #2 and #3), *P. saportae* (#1 and #2), *P. vera* (#2), and *P. weinmannifolia* has multiple types of ITS sequences. *Pistacia chinensis* (#1), *P. khinjuk*, *P. palaestina* (#1, #2 and #3), *P. saportae* (#1 and #2), *P. texana* (#1and #2) and *P. vera* (#2) each had multiple *NIA-i3* sequences.

The two accessions of *P. vera* formed a clade with *P. khinjuk* in all molecular data sets. Some of the ITS and *NIA-i3* sequences of these two species were identical, suggesting a close relationship of these two species. All the earlier molecular results also suggested a close relationship between these two species (Parfitt and Badenes, 1997; Kafkas and Perl-Treves, 2001, 2002; Golan-Goldhirsh et al., 2004). *Pistacia palaestina* was not well separated from *P. terebinthus* in either the plastid or nuclear DNA data sets. Close relationships between these two species were also suggested by the AFLP and the RAPD results (Golan-Goldhirsh et al., 2004; Kafkas, 2006). The present results are consistent with Engler (1936) and Yaltirik (1967), who merged *P. palaestina* and *P. terebinthus*.

Pistacia mexicana and *P. texana* were not distinguishable in the plastid restriction analyses (Parfitt and Badenes, 1997). The ITS data suggest that *P. mexicana* and *P. texana* are sister taxa; and the sequence divergence between these two species is low. The *NIA-i3* and the combined plastid DNA data cannot separate these two species. Sequence divergence among different clonal *NIA-i3* sequences of *P. texana* is higher than that between these two species. In comparison with *P. mexicana*, *P. texana* has smaller and fewer leaflets; less pubescence on its branches, rachis of leaves and midribs of leaflets; and it branches from the base whereas *P. mexicana* has a single trunk. Furthermore, *Pistacia texana* is evergreen, whereas *P. mexicana* is semideciduous, shedding its leaves in the spring. It thus seems to be justifiable to maintain them as two distinct yet closely related species. More accessions of *P. mexicana* and *P. texana* should be sampled in future studies to test the relationships of these two species. *Pistacia integerrima* was described as a variety of *P. chinensis* by Zohary (1952). Parfitt and Badenes (1997) suggested that *P. integerrima* should be viewed as a distinct species. Plastid and nuclear DNA data from this study showed that *P. integerrima* had distinct plastid DNA, ITS, and *NIA-i3* sequence profiles from *P. chinensis*, supporting a separate taxonomic classification for *P. integerrima*. These species are geographically disjunct and do not have a significant overlap in flowering period when grown in a common environment (D. Parfitt, personal observation). The delimitation of some *Pistacia* species requires careful morphological, ecological, and population genetic analysesbecause of their ability to hybridize in common environments.

Putative hybrid origin of P. saportae—The divergent ribosomal DNA copies of *P. saporate* may be due to different evolutionary trajectories before their merger into a single genome as a consequence of a reticulate event (Wendel, 2000). Under this scenario, different copies of rDNA are maintained, evolving independently without recombination. In this case, the ITS sequences may be used to infer the occurrence of an ancient hybridization event and the maternal and paternal progenitor lineages (Soltis and Soltis, 1991; Soltis et al., 1995; Baumel et al., 2001; Álvarez and Wendel, 2003). Most *Pistacia* species maintain different ITS alleles, suggesting that ITS may be a useful marker to detect hybridization events among *Pistacia* species. *Pistacia saportae* has been reported to be a putative hybrid of *P. lentiscus* and *P. terebinthus* (Zohary, 1952, 1972). This species (#2) has two types of ITS sequences, with one showing a close relationship with *P. lentiscus* and the other similar to that of *P. terebinthus* (Fig. 2). *Pistacia saportae* could be a hybrid between *P. lentiscus* and *P. terebinthus.* Different *P. saportae* ITS alleles would have been exposed to biased concerted evolution, resulting in the selection of alleles from one progenitor following hybridization. Among the 16 clonal ITS sequences from accession #2, only one sequence is type 1, showing a close relationship to *P. terebinthus*, while the other 15 clonal sequences belong to type 2 and form a clade with *P. lentiscus*. All six clonal sequences from *P. saportae* #1 belong to type 2. The low number of clonal sequences assayed or geographically biased samples are the probable reasons for failure to detect the type 1 sequence from #1. Biased concerted evolution may have eliminated most type 1 sequences. Similar results have been reported in other studies (e.g., Brochmann et al.,

1996; Ferguson et al., 1999; Franzke and Mummenhoff, 1999; Fuertes Aguilar et al., 1999a, b; Roelofs et al., 1997).

Low-copy number genes have been suggested as not being subject to concerted evolution (Cronn et al., 1999; Wendel, 2000; Zhang et al., 2002; Senchina et al., 2003), and were successfully used to identify several *Paeonia* hybrids (Sang and Zhong, 2000). There were two types of *NIA-i3* sequences (an example of a low-copy number gene) in the two accessions of *P. saportae*, one having a close relationship with *P. terebinthus* and the other with *P. lentiscus* (Fig. 3). The *NIA-i3* data are consistent with the hypothesis of a hybrid origin for this species with *P. lentiscus* and *P. terebinthus* as the parental taxa. This result was also supported by ITS data, which showed that *P. palaestina* kept both types of ITS profile from its putative parents: *P. lentiscus* and *P. terebinthus*. The plastid DNA data strongly suggested a sister relationship of *P. lentiscus* and *P. saportae* (Fig. 4), confirming that the maternal parent of *P. saportae* is probaly *P. lentiscus*, and the paternal parent of *P. saportae* should be *P. terebinthus*.

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