PHYLOGENETICS AND RETICULATE EVOLUTION IN
Pistacia (Anacardiaceae)¹

Tinghuang Yi,² Jun Wen,³,⁴,⁷ Avi Golan-Goldhirsh,⁵ and Dan E. Parfitt⁶

¹ Key Laboratory of Plant Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650204 China; ²Department of Botany, National Museum of Natural History, MRC 166, Smithsonian Institution, Washington, D.C. 20013-7012 USA; ³Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Nanxincun 20, Xiangshan, Beijing 100093 China; ⁴Department of Plant Sciences MS2, One Shields Ave., University of California, Davis, California 95616 USA

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. chinesis (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. chinesis and P. weinmannifolia), and two species from the southwestern United States to Central America (P. mexicana and P. texana).

Pistacia chinesis 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, plume style, 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 Rhoideae, 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 Rhoideae members by having three syncarpous carpels, unilocular fruits, and a thin exocarp. Engler (1876) placed Pistacia in the tribe Rhoideae (= Rhoaceae). 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 Anacardioidae within molecular studies of Terrazas (1994) and Pell (2004). Pistacia was included in subfamily Anacardioidae in both studies.

On the basis of the morphology of leaves, leaflets, 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 Burn., and P. weinmannifolia Poisson; Butmela Zoh., including P. atlantica Desf.; and Eu Terebinthus Zoh., including P. chinesis Bge., P. khinjuk

¹ Manuscript received 19 March 2007; revision accepted 7 December 2007. This study was supported by the National Basic Research Program of China (973 program, project no. 2007CB411600, subproject no. 2007CB411601), the Natural Science Foundation of China (project no. 30770138), the MacArthur Foundation, the Institute of Botany of the Chinese Academy of Sciences, and the Smithsonian Institution (J.W.). The authors thank the Pritzker Laboratory for Molecular Systematics and Evolution of the Field Museum and the Laboratory of Analytical Biology of the Smithsonian Institution for support of laboratory work. The authors thank H. Li, Y. H. Ji, and S. Ickert-Bond for help in obtaining some samples.

² Author for correspondence (e-mail: wenj@si.edu)
Stocks, *P. palaestina* Boiss., *P. terebinthus* L., and *P. vera* L. On the basis of plastid restriction site analysis and morphological characters, Parfit and Badenes (1997) suggested the division of the genus into two sections, *Lentiscus* and *Terebinthus*. Section *Lentiscus* includes Zohary’s (1952) sects. *Butmela* and *Eu Lentiscus* and consists of the evergreen species with imparipinnate leaves and large 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 (Parfit and Badenes, 1998), 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). Parfit has hybridized a number of *Pistacia* species and has not seen evidence of genetic crossing barriers (Parfit, 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. lentiscus* 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 Parfit 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>
<thead>
<tr>
<th>Taxon</th>
<th>Voucher</th>
<th>Locality</th>
<th>Distribution</th>
<th>ITS</th>
<th>ndhF</th>
<th>trnC-trnD</th>
<th>trnL-F</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pistacia atlantica</em> Desf. #1</td>
<td>Golan 1.114 (F)</td>
<td>Israel (cult.)</td>
<td>W Asia to Mediterranean</td>
<td>EF193076</td>
<td>EF190543</td>
<td>EF193106</td>
<td>EF193140</td>
</tr>
<tr>
<td><em>P. atlantica</em> Desf. #2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. atlantica</em> Desf. #3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. chinensis</em> Bge. #1</td>
<td>Golan 1.412 (F)</td>
<td>Israel (cult.)</td>
<td>E Asia</td>
<td>EF193079</td>
<td>C1, EF190515</td>
<td>C2, EF190516</td>
<td>C3, EF190517</td>
</tr>
<tr>
<td><em>P. chinensis</em> Bge. #2</td>
<td>Ji 0174 (KUN)</td>
<td>China, Kunming</td>
<td></td>
<td>EF193080</td>
<td>EF190519</td>
<td>EF193110</td>
<td>EF193144</td>
</tr>
<tr>
<td><em>P. chinensis</em> Bge. #3</td>
<td>Wen 7090 (F)</td>
<td>USA, California (cult.)</td>
<td></td>
<td>DQ390466</td>
<td>DQ382323</td>
<td>DQ390462</td>
<td>DQ390470</td>
</tr>
<tr>
<td><em>P. integerrima</em> Parfitt D. E. &amp; Badenes M. L.</td>
<td>Parfitt 54 (F)</td>
<td>USA, California (cult.)</td>
<td>EN Africa</td>
<td>EF193081</td>
<td>EF190520</td>
<td>EF193111</td>
<td>EF193145</td>
</tr>
<tr>
<td><em>P. khhijak Stocks</em></td>
<td>Golan 1.149 (F)</td>
<td>Israel (cult.)</td>
<td>W Asia to Mediterranean</td>
<td>C1, EF193104</td>
<td>C1, EF190526</td>
<td>C2, EF190527</td>
<td>C3, EF190528</td>
</tr>
<tr>
<td><em>P. lentiscus</em> L. #1</td>
<td>Golan 1.1009 (F)</td>
<td>Israel (cult.)</td>
<td>Mediterranean</td>
<td>C1, EF193082</td>
<td>C2, EF193083</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. lentiscus</em> L. #2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. mexicana</em> H. B. K.</td>
<td>Partit 27 (F)</td>
<td>USA, California (cult.)</td>
<td>Mexico, Texas</td>
<td>DQ390468</td>
<td>DQ382325</td>
<td>DQ390464</td>
<td>DQ390472</td>
</tr>
<tr>
<td><em>P. palaestina</em> Boss. #1</td>
<td>Golan 1.222 (F)</td>
<td>Israel (cult.)</td>
<td>Mediterranean</td>
<td>C1, EF193084</td>
<td>C2, EF193085</td>
<td>C3, EF190536</td>
<td>C4, EF190537</td>
</tr>
<tr>
<td><em>P. palaestina</em> Boss. #2</td>
<td>Golan 1.202 (F)</td>
<td>Israel (cult.)</td>
<td>Mediterranean</td>
<td>C1, EF193097</td>
<td>C2, EF193096</td>
<td>C3, EF190538</td>
<td>C4, EF190539</td>
</tr>
<tr>
<td><em>P. saportae</em> Burnat #1</td>
<td>T. Yi 4 (US)</td>
<td>Israel (cult.)</td>
<td>Mediterranean</td>
<td>C1, EF193098</td>
<td>C2, EF193099</td>
<td>C3, EF190532</td>
<td>C4, EF190533</td>
</tr>
<tr>
<td><em>P. saportae</em> Burnat #2</td>
<td>T. Yi 10 (US)</td>
<td>Israel (cult.)</td>
<td>Mediterranean</td>
<td>C1, EF193100</td>
<td>C2, EF193101</td>
<td>C3, EF193102</td>
<td>C4, EF193103</td>
</tr>
<tr>
<td><em>P. terebinthus</em> L.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pistacia texana</em> Swingle #1</td>
<td>Golan 1.350 (F)</td>
<td>Israel (cult.)</td>
<td>Mediterranean</td>
<td>EF193086</td>
<td>EF190525</td>
<td>EF193119</td>
<td>EF193153</td>
</tr>
<tr>
<td><em>P. texana</em> Swingle #2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. vera</em> L. #1</td>
<td>Golan 1.539 (F)</td>
<td>Israel (cult.)</td>
<td>W Asia</td>
<td>AY677201</td>
<td>C1, EF193089</td>
<td>C2, EF193090</td>
<td>C3, EF193091</td>
</tr>
<tr>
<td><em>P. vera</em> L. #2</td>
<td>Wen 7099 (F)</td>
<td>USA, California (cult.)</td>
<td></td>
<td>DQ382327</td>
<td>DQ390465</td>
<td>DQ400564</td>
<td>DQ400561</td>
</tr>
<tr>
<td><em>P. weinmannifolia</em> Poisson</td>
<td>Li 1630 (KUN)</td>
<td>China, Yunnan</td>
<td>E Asia</td>
<td>C1, EF193092</td>
<td>C2, EF193093</td>
<td>C3, EF193094</td>
<td></td>
</tr>
<tr>
<td><em>Actinocheta filicina</em> (D. C.) Barkl.</td>
<td>Panero s. n. (CS)</td>
<td>Mexico</td>
<td>S Mexico</td>
<td>AY641509</td>
<td>DQ382321</td>
<td>AY640640</td>
<td>DQ400558</td>
</tr>
<tr>
<td><em>Malosma laurina</em> (Nutt.)</td>
<td>Miller 34 (CS)</td>
<td>Rancho Santa Ana Bot. Gard., CA (cult.)</td>
<td>S California and N Lower California E North America</td>
<td>AY641510</td>
<td>DQ382322</td>
<td>AY640461</td>
<td>DQ400559</td>
</tr>
<tr>
<td><em>Rhus aromatica</em> Ait.</td>
<td>Wen 7086 (F)</td>
<td>USA, Mexico</td>
<td></td>
<td>AY641493</td>
<td>C1, DQ382284</td>
<td>AY640447</td>
<td>DQ400535</td>
</tr>
<tr>
<td><em>R. chinensis</em> Mill.</td>
<td>Wen 6389 (F)</td>
<td>Morton Arb., IL (cult.)</td>
<td>E Asia to SE Asia North America</td>
<td>AY641480</td>
<td>C1, DQ382284</td>
<td>AY640435</td>
<td>DQ400536</td>
</tr>
<tr>
<td><em>R. glabra</em> L.</td>
<td>Wen 7171 (F)</td>
<td>USA, Alabama</td>
<td></td>
<td>AY641486</td>
<td>C2, DQ382284</td>
<td>AY640440</td>
<td>DQ400541</td>
</tr>
<tr>
<td>*R. virensi Lindl. ex Gray</td>
<td>Wen 7282 (F)</td>
<td>USA, Texas</td>
<td>SW America to N Mexico</td>
<td>AY641506</td>
<td>C3, EF193091</td>
<td>AY640458</td>
<td>DQ400557</td>
</tr>
<tr>
<td><em>Schinus molle</em> L.</td>
<td>Wen 6686 (F)</td>
<td>USA, Los Angeles, CA (cult.)</td>
<td>California and Texas</td>
<td>AY641512</td>
<td>DQ382333</td>
<td>AY640463</td>
<td>DQ400565</td>
</tr>
<tr>
<td><em>Toxicodendron diversilobum</em></td>
<td>Wen 6693 (US)</td>
<td>USA, California</td>
<td>W North America</td>
<td>AY677202</td>
<td>DQ382328</td>
<td>AY677208</td>
<td>DQ400568</td>
</tr>
<tr>
<td><em>T. radicans</em></td>
<td>Wen 6236 (US)</td>
<td>USA, Illinois</td>
<td>North America</td>
<td>AY677203</td>
<td>DQ382329</td>
<td>AY677207</td>
<td>DQ400569</td>
</tr>
<tr>
<td><em>T. vernix</em> (L.) Kuntze</td>
<td>Wen 7146 (F)</td>
<td>Morton Arb., IL (cult.)</td>
<td>E North America</td>
<td>AY541520</td>
<td>DQ382330</td>
<td>AY640471</td>
<td>DQ400670</td>
</tr>
</tbody>
</table>
reactions with approximately 10–50 ng of total DNA, 20 mM Tris buffer (pH 8.3, with 50 mM KCl, 1.5 mM MgCl₂, 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 Swoford (1994) and Taberlet et al. (1991), respectively. The PCR products were electrophoresed in 1% 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 intron/intragenomic 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 Seqencher (version 3.1. Gene Codes Corp., Ann Arbor, Michigan, USA). Alignments were subsequently analyzed as just described 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 built from 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 tree-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 non-parametric 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. saportaee) 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. chinesis, P. integerima, P. khinjuk, P. palaestina, and P. terebinthus. Separate accessions of P. chinesis 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. saportaee (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 saportaee type 1 sequences varied from 0 to 0.61%. Pistacia lentiscus, a putative parent of P. saportaee, 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. saportaee type 1 sequences. The sequence divergences between
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 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%.

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 weinmannifolia* formed a sister clade to the *P. lentiscus*-*P. saportae* clade (type 2 sequences), and the *P. chinesis*-*P. integerrima*-*P. palaeastina*-*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. weinmannifolia* 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. chinesis*-*P. integerrima*-*P. palaeastina*-*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 *Pistacia 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. palaeastina*, 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. palaeastina*) 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. chinesis*- *P. integerrima*- *P. palaeastina*- *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
Pistacia was described as morphologically diverse (Zohary, 1952). Section Lentiscus (including

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
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*, *Mosquitoxylon*, 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

---

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.
that *P. atlantica* is nested within sect. *Terebinthus*. It formed a monophyletic group with *P. kihnuk* 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, oblanceolate or elliptical leaflets, fasciculate infrasubtance, 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 sect *Lentiscus* are evergreen and have paripinnate leaves, whereas section *Terebinthus* species are deciduous and have paripinnate 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. kihnuk*, *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. chinesis* 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 chinesis* (#2), *P. kihnuk*, *P. lentiscus* (#1 and #2), *P. palaestina* (#1, #2 and #3), *P. saporta* (#1 and #2), *P. vera* (#2), and *P. weinmannifolia* has multiple types of ITS sequences. *Pistacia chinesis* (#1), *P. kihnuk*, *P. palaestina* (#1, #2 and #3), *P. saporta* (#1 and #2), *P. texana* (#1 and #2), and *P. vera* (#2) each had multiple NIA-i3 sequences.

The two accessions of *P. vera* formed a clade with *P. kihnuk* 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 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 integrerrima* was described as a variety of *P. chinesis* by Zohary (1952). Parfitt and Badenes (1997) suggested that *P. integrerrima* should be viewed as a distinct species. Plastid and nuclear DNA data from this study showed that *P. integrerrima* had distinct plastid DNA, ITS, and NIA-i3 sequence profiles from *P. chinesis*, supporting a separate taxonomic classification for *P. integrerrima*. 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 analyses because of their ability to hybridize in common environments.

**Putative hybrid origin of *P. saporta*—**The divergent ribosomal DNA copies of *P. saporta* 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; Alvarez 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 saporta* 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 saporta* could be a hybrid between *P. lentiscus* and *P. terebinthus*. Different *P. saporta* 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. saporta* #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.,...
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 NA-i3 sequences (an example of a low-copy number gene) in the two accessions of P. saportaen one having a close relationship with P. terebinthus and the other with P. lentiscus (Fig. 3). The NA-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. palaeasina 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. saportaen (Fig. 4), confirming that the maternal parent of P. saportaen is probably P. lentiscus, and the paternal parent of P. saportaen should be P. terebinthus.

**LITERATURE CITED**


February 2008]  

YI ET AL. — PHYLOGENETICS AND RETICULATE EVOLUTION IN PISTACIA  

251


