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Molecular phylogenetic analysis of a scale insect (*Drosicha mangiferae*; Hemiptera: Monophlebidae) infesting mango orchards in Pakistan

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Abstract. Mango orchards in Pakistan are attacked by the scale insect, *Drosicha mangiferae* (Hemiptera: Monophlebidae), commonly called the "mango mealybug". This insect is univoltine, active from December through May and targets multiple host plants. We used DNA nucleotide sequences to characterize and determine the phylogenetic status of *D. mangiferae*. Mango mealybugs were collected from several tree species from different localities and patterns of phylogenetic and genetic diversity were examined at both nuclear (18S, ITS1) and mitochondrial (COI) genes. Phylogenetic analysis confirms that the mango mealybug belongs to the family Monophlebidae. Minor genetic differences in both the ITS1 and the COI barcode region were noted among *D. mangiferae* collected from different geographic localities. These genetic differences revealed the existence of two genotypes of *D. mangiferae* that are region specific but not host-specific.

INTRODUCTION

Mango orchards in Pakistan are attacked by an insect belonging to the superfamily Coccoidea (Ben-Dov et al., 2006) called the "mango mealybug" which results in heavy fruit losses every year. Gravid females of this univoltine insect leave the trees in May–June, laying their eggs in the soil near the tree trunks and in December–January newly hatched nymphs crawl up the trees to feed. Nymphs and adults damage plants by sucking sap from inflorescences, shoots and fruit peduncles. This results in shriveled twigs and leaves and heavy fruit drop. The major target of mango mealybug is mango trees, but it has also become a pest on citrus and several other tree species.

Four coccid species have been reported as pests on mango trees including *Drosicha mangiferae*, *D. stebbingi*, *R. iceryoides*, and *Rastrococcus invadens* (Latif, 1961; Tandon & Lal, 1978; Williams, 1986; Willink & Moore, 1988). *R. invadens* is a serious pest of mango and other fruit trees in Africa (Agounké et al., 1988; Moore, 2004), whereas *R. iceryoides* has been reported on ornamental and forest trees as well (Sundararaj & Devaraj, 2010). *D. mangiferae* and *D. stebbingi* have been reported as mango pests in Pakistan (Latif, 1949, 1961). Due to their morphological similarity, *D. mangiferae* and *D. stebbingi* have often been treated as synonyms. Beeson (1941) stated that *D. stebbingi* is largely a pest of forest trees while *D. mangiferae* is a pest of fruit trees, but other authors have concluded that fruit trees are common hosts for both *D. mangiferae* and *D. stebbingi*. This confusion provoked re-examination of the mango mealybug status. Latif (1961) concluded that *D. mangiferae* and *D. stebbingi* not only share food plants, but that it was highly probable that *D. mangiferae* was synonymous with *D. stebbingi*. However, confusion still prevails as some authors continue to report mealybugs from forest trees as *D. stebbingi* (Gul et al., 1997).

The identification of scale insects to a species level is often challenging due to their reduced morphology, and the high similarity of their immature stages (Miller, 2002; Watson & Kubiriba, 2005). Because the use of standard taxonomic characters for identification has proven difficult (Danzing, 1997; Gullan & Cook, 2007), various molecular markers have been used to differentiate species and to ascertain evolutionary relationships (Beuning et al., 1999; Mowry & Barbour, 2004; Gadagkar et al., 2005; Gariepy et al., 2007). In some cases, molecular phylogenetic analyses have been combined with morphological characters to assess taxonomic relationships (Hardy et al., 2008). The most commonly used genes for the differentiation of insect species have been ribosomal RNA (18S, 28S, internal transcribed spacers) and mitochondrial cytochrome *c* oxidase I (COI) (Simon et al., 1994; Li et al., 2005). In fact, sequence diversity in the 5' region of the COI gene has been established as the standard for species identification across the animal kingdom (Hebert et al., 2003; Miller, 2007; Linares et al., 2009). Several prior studies (Hardy et al., 2008; Rung et al., 2008; Ashfaq et al., 2010) have examined mealybug classification based on sequence variation in ribosomal and mitochondrial DNA. Similarly, sequence data of nuclear, mitochondrial and endosymbiotic genes have been used to estimate the phylogenies of scale insects (Cook et al., 2002; Morse & Normark, 2006; Gullan & Cook, 2007). These studies have established the value of molecular techniques in both species identification and in assessing phylogenetic relationships.

There is little information on the molecular identification, genetic relationships and species composition in mango mealybugs from different geographical areas or on various host plants in Pakistan. In fact, as already noted, confusion prevails about the species of mealybug attacking mango and other fruit and forest trees. The present study seeks to genetically characterize the mango mealybug to investigate the species composition by examining genotypic variation amongst its populations. The resultant information may aid pest management programs by allowing the implementation of a species-specific biological control strategy on mango mealybug.

MATERIAL AND METHODS

Both adult females and nymphs of the mango mealybug were collected during April–May 2008/2009 from fruit and forest trees. DNA was either extracted immediately or specimens were preserved in 95% ethanol and held at –20°C until use. DNA was extracted from the heads of the fresh/preserved specimens as described earlier (Erlandson et al., 2003). Briefly, insects were homogenized individually in Lifton buffer (100 mM Tris-HCl, pH 7.5, 50 mM EDTA, 0.5% SDS, and 0.2 M sucrose), proteins were precipitated by 8 M potassium acetate, and finally DNA was purified by repeated phenol-chloroform extractions. Precipitated DNA pellets were re-suspended in 50 µl of distilled water with 0.5 µl of 10 mg/ml of RNase A.

PCR, cloning and sequencing

Partial fragments of 18S rRNA and internal transcribed spacer (ITS1), and the 3' end of mitochondrial COI were amplified using primers and PCR conditions as described earlier (Ashfaq et al., 2010). PCR products were visualized under UV light on 1.2% agarose gels and excised and purified using a QIAquick gel extraction kit (Qiagen Inc. USA) following the manufacturer's protocols, and subsequently cloned into the pTZ57R/T vector (InsTAclone PCR Cloning Kit, Fermentas, Inc. USA). Recombinant colonies were inoculated into 4 ml of LB/ampicillin cultures and plasmid DNA was extracted using GeneJET Plasmid Miniprep Kit (Fermentas, Inc. USA). At least two cloned PCR product plasmids from the DNA of individual insects from various localities were sequenced commercially (Macrogen, Inc. South Korea). Amplification and sequencing of the COI barcode region were performed at the Canadian Centre for DNA Barcoding (CCDB) at the Biodiversity Institute of Ontario following standard protocols (Hebert et al., 2003).

Nucleotide sequence alignments and phylogenetic analysis

PCR amplicons were bidirectionally sequenced and contigs were assembled and edited using EditSeq (DNAStar, Madison, WI). Nucleotide sequences of 18S rRNA and COI of additional mealybug and scale species obtained from GenBank (NCBI) were aligned to determine evolutionary relationships of *D. mangiferae*. Multiple alignments were carried out under the profile alignment option with ClustalW using the default parameters. Phylogenetic and molecular evolutionary analyses were conducted and dendrograms constructed using MEGA version 4 (Tamura et al., 2007). Patterns of sequence divergence among taxa were visualized using the neighbor-joining method. Evolutionary distances were computed using the Maximum Composite Likelihood method based upon the number of base substitutions per site after all positions containing gaps and missing data were eliminated from the dataset (Complete Deletion model).

TABLE 1. Nucleotide variation in the barcode region of COI among specimens of *Drosicha mangiferae* collected from different host-plants in three regions of Pakistan.

Region	Specimen ID / Accession No. (GPS Coordinates) / Host	Nucleotide position[a]					
		36	110	186	258	417	463
		Consensus sequence					
		T	T	C	C	G	T
Southern	IMB452 / JF792876 (30°16N, 71°45E) / *Mangifera indica*	.	C	.	.	.	.
	IMB453 / JF792875 (30°16N, 71°45E) / *Ziziphus jujuba*	.	C	.	.	.	.
	IMB454 / JF792874 (30°16N, 71°45E) / *Mangifera indica*	.	C	.	.	.	.
	IMB214 / HM891565 (25°45N, 68°71E) / *Mangifera indica*	.	C	.	.	.	.
	IMB455 / JF792873 (25°41N, 68°53E) / *Citrus* sp.	.	C	.	.	.	.
Central	IMB210 / HM388808 (31°34N, 73°29E) / *Mangifera indica*	C	.	.	.	.	.
	IMB213 / HM891564 (31°33N, 72°81E) / *Mangifera indica*	.	.	.	.	.	.
	IMB211 / HM891563 (31°30N, 73°21E) / *Alstonia* sp.	C	.	.	.	.	.
	IMB448 / JF792879 (31°50N, 73°35E) / *Eugenia jambolana*	.	C	.	.	.	.
	IMB447 / JF792880 (31°33N, 72°81E) / *Citrus* sp.	.	.	.	.	.	.
	IMB446 / JF792881 (31°56N, 73°48E) / *Melia azedarach*	C	.	.	.	.	.
	IMB449 / JF792878 (31°53N, 74°31E) / *Mangifera indica*	.	.	.	.	.	.
	IMB450 / JF792877 (31°53N, 74°31E) / *Alstonia* sp.	.	.	.	.	.	.
	IMB215 / HM388809 (31°51N, 74°33E) / *Eugenia jambolana*	.	.	.	.	.	.
	IMB445 / JF792882 (31°60N, 74°21E) / *Citrus* sp.	.	.	T	T	A	C
Northern	IMB209 / HM388807 (35°53N, 72°45E) / *Ziziphus jujuba*	.	.	T	T	A	C
	IMB456 / JF792872 (35°53N, 72°45E) / *Ziziphus jujuba*	.	.	T	T	A	C
	IMB457 / JF792871 (35°53N, 72°45E) / *Acacia indica*	.	.	T	T	A	C
	IMB458 / JF792870 (34°32N, 71°32E) / *Ziziphus jujuba*	.	.	T	T	A	C
	IMB459 / JF792869 (34°32N, 71°32E) / *Acacia indica*	.	.	T	T	A	C

[a] Position in a 531bp 5'-end barcode fragment of COI. Dots indicate the consensus nucleotides.

RESULTS

PCR amplification of the genes

A 562 bp sequence of 18S rRNA was obtained after PCR amplification and cloning. This sequence is available in the DDBJ/EMBL/GenBank databases under accession number AB523733. The blast search (NCBI) revealed the highest nucleotide similarity with *Tessarobelus inusitatus* and *Monophlebulus* sp. (96%) followed by *Neohodgsonius* sp. (95%) of the family Monophlebidae.

Amplification of 3'-end of COI produced a PCR product of 857 bp. The sequence was deposited in GenBank under accession number AB523736. The gene was confirmed based on blast search and the nucleotide homologies with COI from other scale insects. Blast search of the COI sequence showed the highest (88%) nucleotide identity with that of the giant scale, *Drosicha corpulenta*.

Sequences for the barcode region of COI were obtained from 20 specimens of mango mealybug collected from various locations and plant hosts in Pakistan. Alignment of a 531bp fragment showed that all specimens from northern Pakistan showed diagnostic substitutions at four nucleotide positions from specimens collected in the southern and central regions (Table 1). Specimens from different hosts in the same region did not show any consistent sequence differences (Table 1).

A 510 bp fragment was produced using primers targeting ITS1 region that included partial sequences for 18S and 5.8S rRNA. The consensus ITS1 sequences of specimens collected from southern region (Sin-3) and northern region (Kar-5) were deposited in the DDBJ/EMBL/GenBank databases under acc. No. AB523735

TABLE 2. Host plants and geographic origin of scale and mealybug species used to determine the phylogenetic relationships of *Drosicha mangiferae*.

Species name (Accession no.)	Host plant	Geographic origin
Erium globosum (AY426020)	*Acacia howittii*	Australia: Canberra
Pseudococcus calceolariae (AY426039)	*Citrus* sp.	Australia: Canberra
Trionymus frontalis (AY426059)	*Leymus arenarius*	USA: CA, Santa Barbara
Planococcus citri (AY426042)	*Citrus sinensis*	USA: CA, Davis
Dysmicoccus ryani (AY426035)	Ornamental juniper	USA: CA, Sacramento
Pseudococcus maritimus (AY426043)	*Vitis vinifera*	USA: WA, Witstrand
Dysmicoccus brevipes (AY426046)	under carton with ants	Bolivia: Santa Cruz
Paradoxococcus mcdanieli (AY426062)	grass roots	USA: FL, Payne's Prairie
Anisococcus adenostomae (AY426070)	*Adenostoma fasciculatum*	USA: CA, Mix Canyon
Anisococcus sp. (AY426017)	*Ephredra* sp.	USA: UT, Moab
Ferrisia malvastra (AY426019)	potato	USA: AZ, Tuczon
Ferrisia gilli (AY426067)	*Pistacio vera*	USA: CA, Tulare
Ferrisia virgata (AY426079)	*Mangifera indica*	Colombia: Cali, Valle
Phenacoccus solani (AY426058)	*Bidens* sp. crown	USA: FL, Vero Beach
Phenacoccus madeirensis (AY426025)	*Penstemon* sp.	USA: CA, Vacaville
Phenacoccus solenopsis (AB439210)	*Gossypium hirsutum*	Pakistan: Faisalabad
Heliococcus adenostomae (AY426071)	*Adenostoma fasciculatum*	USA: CA, Mix Canyon
Heliococcus clemente (AY426065)	*Gutierrezia* sp.	USA: CA, Cuyama Valley
Heliococcus bohemicus (HM156737)	*Vitis vinifera*	Italy: Torino
Maconellicoccus australiensis (AY426080)	*Acacia dealbata*	Australia: Tharwa, ACT
Maconellicoccus hirsutus (AY426033)	*Hibiscus* sp.	Thailand: Chiang Mai
Melanococcus albizziae (AF483205)	*Acacia* spp.	USA
Rhizoecus gracilis (AY426074)	*Artemisia tridentata*	USA: ID, Holbrook Pass
Geococcus coffeae (AY426066)	*Ptychosperma elegans*	USA: CA, Ramona
Rhizoecus hibisci (AY426053)	*Neodypsis decaryi*	USA: Hilo, Hawaii
Pulvinaria torreyae (AB439596)	*Torreya nucifera*	Japan
Icerya purchasi (AY426078)	*Nandina domestica*	USA: CA, Davis
Crypticerya townsendi (EU087715)	*Gutierrezia sarothrae*	USA: UT, Grand Co., BLM
Gigantococcus alboluteus (EU087723)	Palm	Ghana: Accra, Hotel Paloma
Crypticerya genistae (EU087719)	Legume	USA: FL, Broward Co., Hollywood
Icerya aegyptiaca (EU087753)	*Macaranga* sp.	Thailand: Patong Beach
Gueriniella serratulae (EU087754)	*Olea* sp.	Italy: Imperia
Laurencella sp. (EU087759)	Epiphyte mats	Costa Rica: Heredia la virgen
Neohodgsonius sp. (EU087758)	*Acacia* sp.	Belize: Cayo, Chiquibul F.R.
Tessarobelus inusitatus (EU087757)	*Tristaniopsis callobuxus*	New Caledonia: Plaine des Lacs
Monophlebus sp. (EU087756)	*Allocasuariana distyla*	Australia: NSW, Wattamolla
Nodulicoccus levis (EU087755)	*Eucalyptus pauciflora*	Ausstralia: NSW, Carwoola
Drosicha corpulenta (AB439510)	*Populus alba*	Japan
Drosicha howardi (AB439511)	*Viburnum odoratisimum*	Japan
Drosicha mangiferae (AB523733)	*Mangifera indica*	Pakistan: Faisalabad

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1   AGAGACATCGCGCTGTCTGCAAGTTGTCGGACTTTGAAATAGCGTTGATTCGATCGCTTAATTTTTTAAGCGCGTTTCAACGACTAAGTC Kar-5
1   AGAGACATCGCGCTGTCTGCAAGTTGTCGGACTTTGAAATAGCGTTGATTCGATCGCTTAATTTTTTAAGCGCGTTTCAACGACTAAGTC Sin-3

91  CGACGACGCCCCCGTTTAAATTAATACACTATTCGTCTTTTTACGTAGCGGTTCTTGCGGCGTTACGCCTCTGCGTAGAGACGAAAAAAA Kar-5
91  CGACGACGCCCCCGTT--ATTTAATGCACTATTCGTCTTTTTACGAAGCGGTTCTTGCGGCGTTACGCCTCTGCGTAGAGACGAAGAAAA Sin-3

181 AGTATTACAAAATGCGACGCCGCGAAAATATTATAAAAATATTTATTATA------ATATATATTTGAAAAGAAAAA             Kar-5
179 GTATTACAAAATGCGACGCCGCGAAAATATTATAAAAATATTTATTATATGATGTATATATATTTGAAAAGAAAAA             Sin-3
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Fig. 1. Sequence alignment of ITS1 fragments of *Drosicha mangiferae* from northern (Kar-5) and southern (Sin-3) Pakistan. Nucleotide differences are boxed.

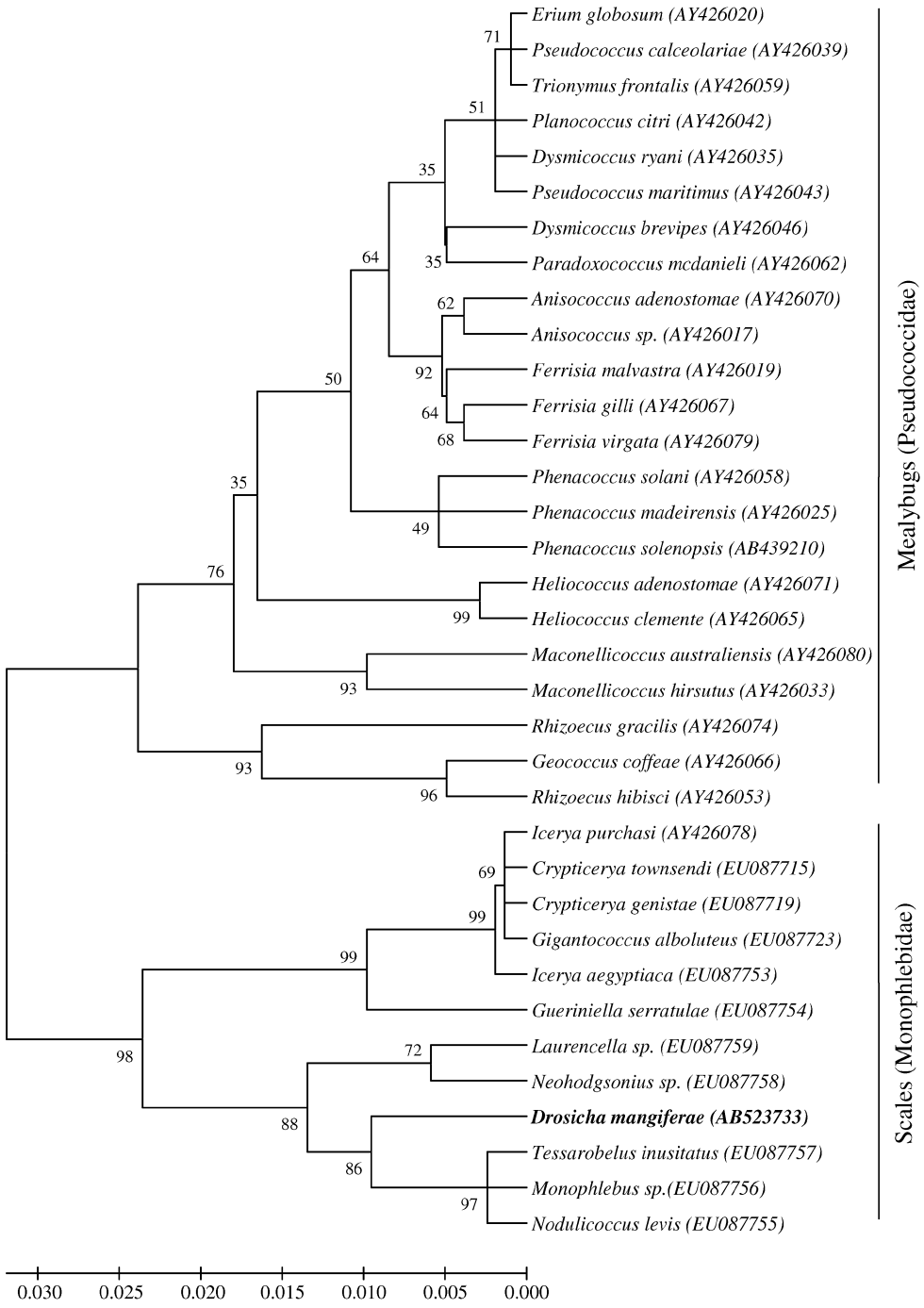

Fig. 2. NJ tree of *Drosicha mangiferae* with 35 taxa of scale and mealybug species based on 18S rRNA sequences. Bootstrap values (500 replicates) for each node are shown next to each branch. All positions containing gaps and missing data were eliminated from the dataset and there were a total of 518 positions in the final dataset. 18S rRNA sequences of taxa other than *D. mangiferae* were obtained from GenBank.

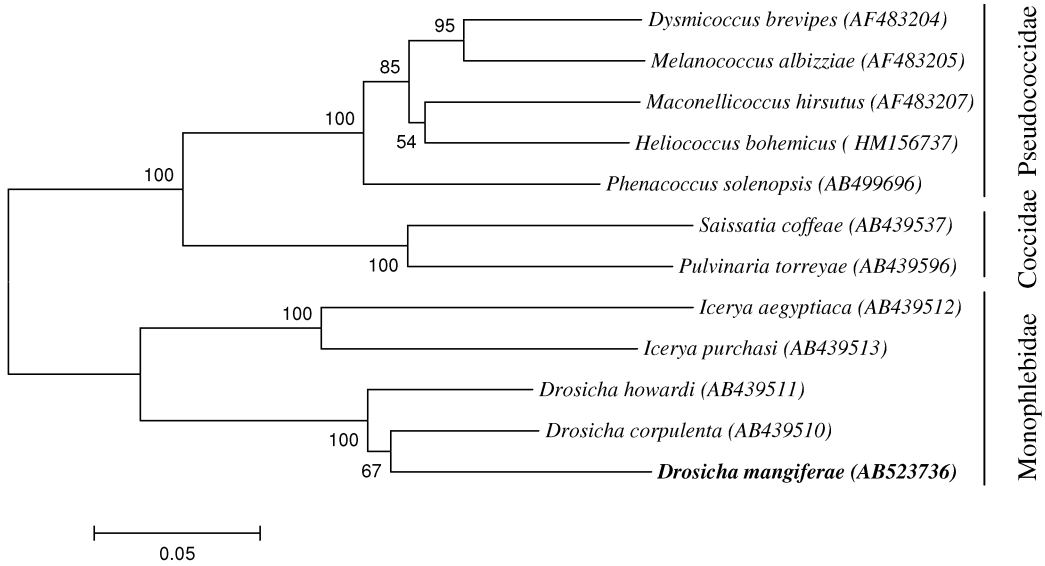

Fig. 3. NJ tree of *Drosicha mangiferae* with 11 other taxa in the superfamily Coccoidea based on COI sequences. Bootstrap values (500 replicates) for each node are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. All positions containing gaps or missing data were eliminated from the dataset and there were a total of 808 positions in the final dataset. COI sequences of taxa other than *D. mangiferae* were obtained from GenBank.

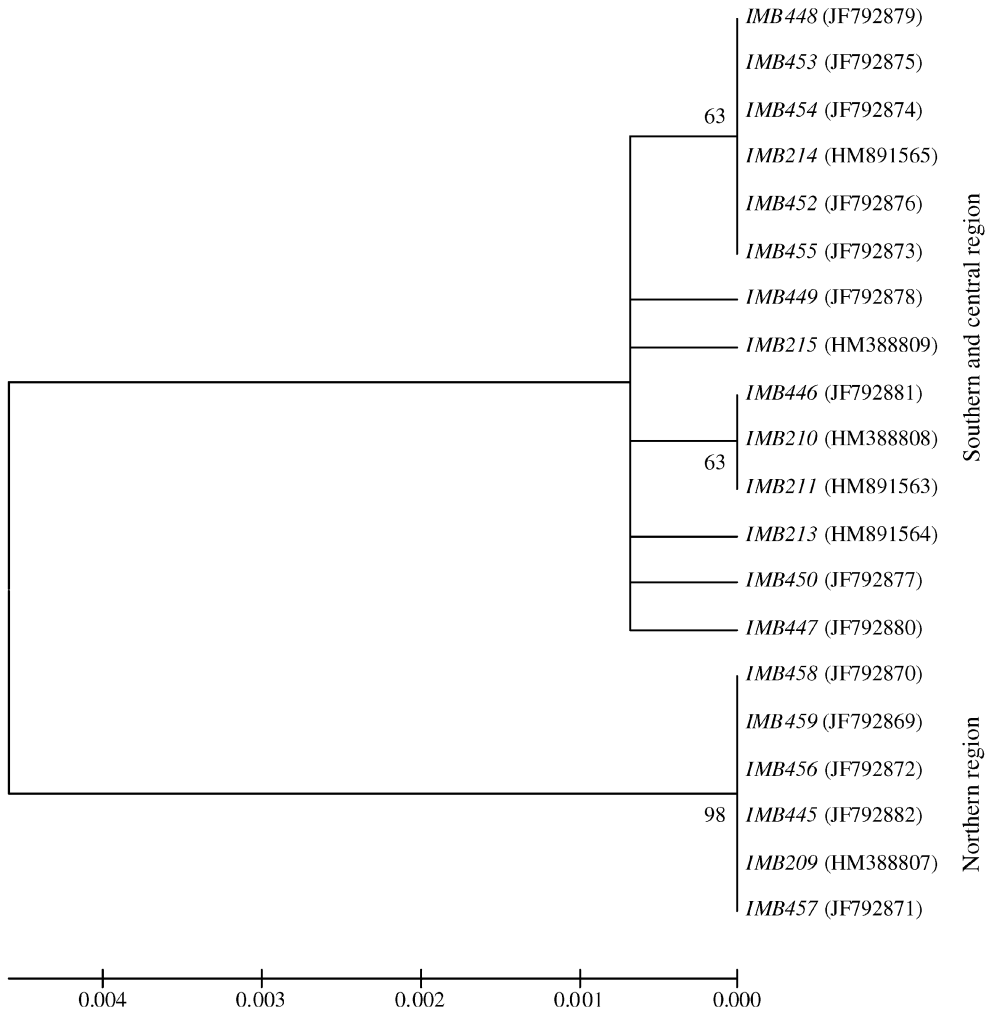

Fig. 4. NJ tree of *Drosicha mangiferae* specimens collected from different geographical regions of Pakistan. The tree was linearized assuming equal evolutionary rates in all lineages. Bootstrap values (500 replicates) for each node are shown next to the branches.

and AB623050, respectively. The ITS1 sequence of mango mealybug specimens from northern Pakistan showed several nucleotide substitutions from specimens collected in the central and southern regions. As well, specimens from the northern region (Kar-5) possessed a deletion of six nucleotides (Fig. 1).

Phylogenetic analysis and evolutionary relationships

18S rDNA

Sequence data for thirty five mealybug and scale insects (superfamily Coccoidea) were aligned to examine their phylogenetic relations. The resultant Neighbor-Joining tree was well resolved (Fig. 2) with scale and mealybug species forming two distinct clusters in the tree with strong bootstrap support, one of mealybugs (Pseudococcidae) and another of scales (Monophlebidae) (Fig. 2). The scale insects were strongly supported as monophyletic (98% bootstrap support) in this tree, but scales (Monophlebidae) split into two main clusters, one cluster mainly composed of the tribe Iceryini while the other includes species in four tribes (Llaveiini, Monophlebulini, Drosichini and Monophlebini). The tree indicates that *D. mangiferae* is most closely allied to *Tessarobelus inusitatus*, a member of the tribe Monophlebulini.

COI

Sequence data for twelve mealybug and scale insects in the superfamily Coccoidea were aligned and phylogenetic relations were determined using Neighbor-Joining analysis. The resultant tree (Fig. 3) is well resolved as the species in each family form a cohesive cluster. The Coccidae show closer affinity to species in the family Pseudococcidae than to members of the family Monophlebidae. Within the family Monophlebidae, *Icerya* and *Drosicha* form two clusters each with strong bootstrap support (Fig. 3). Sequence data of COI barcode region for twenty *D. mangiferae* specimens collected from different regions and host plants were aligned and phylogenetic relations were determined using neighbor-joining analysis. The resultant tree shows two clusters, one cluster represents the specimens collected from northern region and the second for the specimens from southern and central region (Fig. 4).

DISCUSSION

We sequenced nuclear and mitochondrial genes from mango mealybug populations, and compared these results with sequence data for other mealybug and scale species to assess phylogenetic relationships. Nucleotide data for all genes sequenced in this study differed from prior entries in GenBank, but the closest matches were to scale species in the family Monophlebidae. Phylogenetic analysis of 18S rRNA sequences from 35 mealybug and scale species in the families Pseudococcidae and Monophlebidae revealed that the mango mealybug was closest to *T. inusitatus*, a member of the Monophlebidae. When aligned with COI sequences from other scale and mealybug species, the mango mealybug showed greatest similarity with that of giant scale, *D. corpulenta*. COI-based phylogenetic analysis also placed the mango mealybug in the family Monophlebidae and showed that it is closer to the genus *Drosicha* than to *Icerya*.

Previous morphological studies have suggested that the mealybug damaging mango trees in Pakistan is *D. mangiferae* (Latif, 1961), but its close similarity to *D. stebbingi* has left uncertainty (Latif, 1949). Both of these species are endemic to Pakistan and India and have not been reported from any other part of the world. Another serious pest on mango, *R. invadens,* widely distributed in other parts of the world is a true mealybug belonging to the family Pseudococcidae.

Sequence analysis of the COI barcode region and ITS1 of mango mealybugs specimens was performed to study the species composition and to assess the extent of genotypic diversity in mealybug populations. The ITS1 region is highly variable and has previously been used to differentiate insect species (Erlandson et al., 2003; Gariepy et al., 2007) and subspecies (Beuning et al., 1999; Ashfaq et al., 2005). COI is also rapidly evolving and is commonly used to study the species relationships and genetic diversity among populations (Miller, 2007). In our studies we examined specimens of *D. mangiferae* from different geographic locations and from various host plants. Samples collected from northern Pakistan showed several diagnostic substitutions at COI from populations in the southern and central regions. Evidence of similar nucleotide differences were evident in the ITS1, suggesting the existence of two major lineages of mango mealybugs in Pakistan.

Two mealybug species, *D. mangiferae* and *D. stebbingi* lack diagnostic morphological differentiation between their nymphal instars (Latif, 1961). Researchers have argued that *D. mangiferae* targets fruit plants while *D. stebbingi* attacks forest trees (Beeson, 1941; Gul et al., 1997). We surveyed mealybugs on both fruit and forest trees at several locations. Sequence analysis of 18S rDNA and COI genes did not suggest the presence of two species with differing host plant preferences. COI and ITS1sequences revealed two clusters of genotypes that were region specific but not host-specific. A noticeable difference between northern and southern region of Pakistan is the high altitude and hilly terrain of north versus the lowlands of the south, factors that may have played a role in explaining the regional divergence we detected in *D. mangiferae*. Other members of the Monophlebidae are typically polyphagous (Unruh & Gullan, 2008) so the presence of *D. mangiferae* on multiple host plants is not unusual. We conclude that *D. mangiferae* and *D. stebbingi* (Latif, 1949) are simply host races of the same species. In conclusion, only one *Drosicha* species was found attacking both fruit and forest trees in Pakistan. However, the detection of genetic differences among mealybug populations from different regions suggests that mango mealybugs may show variation in important biological attributes across Pakistan.

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