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The genetic diversity, relationships, and potential for biological control of the lobate lac scale, *Paratachardina pseudolobata* Kondo & Gullan (Hemiptera: Coccoidea: Kerriidae)

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Abstract

The lobate lac scale *Paratachardina pseudolobata* Kondo & Gullan (Kerriidae) is a polyphagous pest of woody plants in Florida (U.S.A.) the Bahamas, Christmas Island (Australia) and it has been reported from Cuba. Its recent appearance as a pest in these places indicates that this scale is introduced; however, its native range is unknown. Until 2006, this pest species was identified mistakenly as *Paratachardina lobata* (Chamberlin) [now *P. silvestri* (Mahdihassan)], which is native to India and Sri Lanka. Quarantine laboratory acceptance trials with Indian *P. silvestri* parasitoids indicated a strong immune response from *P. pseudolobata*. Gregarious development of encyrtid wasps was the only observed parasitism, but parasitization levels were below 3%. Identification of the native range of *P. pseudolobata* would facilitate the search for natural enemies better adapted to the scale. Sequence data from the D2–D3 region of the nuclear large subunit ribosomal RNA gene (LSU rRNA, 28S) and the mitochondrial gene *cytochrome oxidase I* (*COI*) distinguished *P. pseudolobata* from the morphologically similar species *P. silvestri* and *P. mahdihassani* Kondo & Gullan, and showed *P. pseudolobata* to be more closely related to these Indotropical species than to an Australian species of *Paratachardina* Balachowsky. *Paratachardina pseudolobata* was genetically uniform throughout its exotic range, consistent with a single geographic origin, although lack of variation in these genes is not unusual for scale insects. Molecular identification of morphologically similar *Paratachardina* species was possible using the D2–D3 region of 28S, despite its length variation, suggesting that this gene region might be suitable as a non-*COI* barcoding gene for scale insects.
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Keywords: Native range; Invasive; *Cytochrome oxidase I*; Nuclear large subunit ribosomal RNA gene (LSU rRNA, 28S); Parasitism; Encyrtidae

1. Introduction

The lobate lac scale, a serious plant pest, was first detected in the Bahamas in 1992 and later in South Florida, in Broward County in 1999 and in Miami Dade County in 2000, where it built up alarming densities in the following two years (Howard and Pemberton, 2003; Pemberton, 2003a). The invasive pest was identified mistakenly as *Paratachardina lobata* Chamberlin [now a junior synonym of *P. silvestri* (Mahdihassan)] in all literature until the taxonomic revision of the genus *Paratachardina* Balachowsky by Kondo and Gullan (2007), in which it was described as a new species, *Paratachardina pseudolobata* Kondo and Gullan. This pest is highly polyphagous: over 300 plant species in 58 different families, including native plants and cultivated fruit trees and ornamentals, are attacked by

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the invasive scale in southern Florida (Howard et al., 2006). Due to the lack of natural enemies, the scale increased and spread quickly in Florida and the Bahamas and has a high potential to spread in the Caribbean and elsewhere (Pemberton, 2003b). The scale was reported from the territory of Christmas Island, Australia (Kondo and Gullan, 2007; Abbott and Green, 2007), where it is a pest of carambola (*Averrhoa carambola* L.) and other plants (Pemberton, unpublished). Recently it was recorded in Cuba for the first time (Mestre et al., 2006). Efficient natural enemies are needed urgently to control the scale's damage and to limit its spread. Thus identifying the native range of this scale insect is a necessary first step. A biological control research program against the lobate lac scale was initiated in 2003 (Pemberton, 2003b). The first approach was to collect parasitoids from the con-familial and commercially reared *Kerria lacca* (Kerr), which is host to some parasitoid species known to attack *Paratachardina* species (Varshney, 1976; Pemberton, 2003b). Three chalcidoid species recorded as parasitizing both *K. lacca* and *P. lobata* (now *P. silvestri*) were collected from *K. lacca* in Thailand and exposed to *P. pseudolobata* in the USDA, ARS Invasive Plant Research quarantine facility in Florida, but the wasps failed to parasitize the lobate lac scales (Pemberton et al., 2006).

The second approach was to search for the lobate lac scale and its natural enemies in its native range. The search began in India, because the lobate lac scale was previously identified as *P. lobata*, which was known to be native to southern India and Sri Lanka (Varshney, 1977). Robert Pemberton and P. Selvaraj located kerriid scales in India in 2005 at 14 sites. Living kerriid specimens were sent to the USDA, ARS Invasive Plant Research quarantine facility (Schroer et al., 2007). The scales from India were understood to be *P. lobata* as was the target pest in Florida. The Indian material was subsequently identified as *P. silvestri* and *P. mahdihassani* Kondo and Gullan, and the Florida pest as *P. pseudolobata* (Kondo and Gullan, 2007). Three primary parasitoids of *P. silvestri* and *P. mahdihassani* were isolated, and exposed to *P. pseudolobata* cultures reared from Florida collections. Despite the observed oviposition, only a few individual wasps were able to develop in lobate lac scales. Most parasitoid eggs were encapsulated by the host, indicating that the parasitoids are not well adapted to lobate lac scale (Schroer and Pemberton, 2007). Parasitoids that have evolved with particular hosts overcome the specific defensive reactions of their hosts, either by avoiding tissue with strong immune reactions or diminishing the chemical reactions with specific venoms or by abilities to disguise the alien tissue (Strand and Pech, 1995).

The apparent mismatch between the parasitoids and the lobate lac scale suggested that the Indian host of the parasitoids and the lobate lac in Florida (both identified as *P. lobata* prior to the taxonomic work of Kondo and Gullan (2007)) might be different species or biotypes. Kondo and Gullan (2007) re-examined the morphology of the invasive lobate lac scale from Florida, the Bahamas and Christmas Island, as well as other *Paratachardina* specimens from museum collections and recent Indian collections. They revised the taxonomy of the genus and determined that the lobate lac scale represented a distinct species on the basis of morphological comparisons. The specific name "*pseudolobata*" was coined in reference to the species with which it had been confused.

For the present study, specimens of *P. pseudolobata* from Florida and the Bahamas were collected to document levels of scale infestation and to record the presence of any parasitoids. Specimens from Florida, the Bahamas, Cuba and Christmas Island were obtained for molecular analysis to estimate the genetic diversity of the populations. We also used DNA sequence data to test the species status of *P. pseudolobata* and to place it in a phylogenetic context by comparison with other species of *Paratachardina* and with other genera of Kerriidae. Knowledge of the relationship of *P. pseudolobata* to other members of *Paratachardina* could narrow the search for the native range of the lobate lac scale based on the known distribution of its closest relatives, as done for the pestiferous Chinese wax scale (Qin et al., 1994). In contrast to the morphologically similar *P. silvestri*, the lobate lac scale is exclusively parthenogenetic (Pemberton, 2003a; Howard et al, 2006; Kondo and Gullan, 2007). We could expect that if the populations of *P. pseudolobata* originated from a single geographic region, or represent a single origin of an invasive form, there should be low (possibly zero) genetic variation within pest populations and among different geographical regions of its invasive range (e.g. Downie, 2002; Scheffer and Grissell, 2003; Grapputo et al., 2005). This study also provides the opportunity to evaluate the two genes used here for their usefulness as non-*COI* barcode regions for molecular identification of scale insects. To date, the typical DNA barcode region (5′ region of *CO1* (Herbert et al., 2003)) has not been reliably or successfully amplified for scale insects using universal or modified primers. Therefore, alternative barcode regions need to be explored. To be useful as a DNA barcode, a region needs to be easily and reliably amplified for the group of interest with universal primers, and to readily distinguish among species.

2. Materials and methods

2.1. Specimen collections

Specimens were obtained from as many populations and species of *Paratachardina* as possible (Table 1). Specimens of *P. pseudolobata* were collected from Florida, three islands of the Bahamas, Christmas Island (Australia) and Cuba. Specimens of other *Paratachardina* species, including *P. silvestri* and *P. mahdihassani* were obtained from several localities in India. Also, because the monophyly of *Paratachardina* has not been tested and the relationships within the genus are uncertain, sampling included an Australian species of *Paratachardina* and other lac scales from

Table 1
Specimens of Kerriidae used in the phylogenetic study (collectors' names are abbreviated as follows: L.G.C., L.G. Cook; T.K., T. Kondo; P.J.G., P.J. Gullan; R.W.P., R.W. Pemberton; S.S., S. Schroer); DNA vouchers of L.G.C. are in ANIC and those of T.K. are in the BME; T.K. and P.J.G. identified the *Paratachardina* species, P.J.G. identified the kerriid outgroup species

Scale species	Voucher code	Host plant	Collection data			GenBank Accession 28S, D2–D3	GenBank Accession *COI*
			Location	Date	Collector		
Paratachardina							
P. near *decorella* (Maskell)	TK0415	*Callitris glaucophylla*	Australia, N.S.W., 1 km N Gummin Gummin	14.viii.2004	PJG		
P. mahdihassani Kondo & Gullan	TK0396	*Pongamia pinnata*	India, Karnataka, Bangalore, Big Banyan Park	1–5.v.2006	P.Selvaraj & RWP		
	TK0409	*Pongamia pinnata*	India, Karnataka, Bangalore, Bannerghata Nat. Park	vi.2006	P. Selvaraj & RWP		
	TK0411	*Pongamia pinnata*	India, Karnataka, Bangalore, Jarakabande State Forest	vi.2006	P. Selvaraj & RWP		
P. pseudolobata Kondo & Gullan	TK0117	*Clusia* sp.	USA, Florida, Davie, University of Florida Fort Lauderdale	20.xi.2002	TK		
	TK0391	*Celtis timorensis*	Australia, Christmas Island, Dolly Beach Track	15.vi.2006	K. Retallick		
	TK0392	*Celtis timorensis*	Australia, Christmas Island, Daniel Roux Road	15.vi.2006	K. Retallick		
	TK0393	*Celtis timorensis*	Australia, Christmas Island, Winifred Beach Track	19.vi.2006	R. Reeves		
	TK0388	*Melaleuca vinifera*	USA, Florida, CR997/US 41	8.vi.2006	SS & RWP		
	TK0398						
	TK0399	*Psychotria nervosa*	USA, Florida, Broward, Fern Forest,	14.vi.2006	SS & RWP		
	TK0400	*Myrica cerifera*	USA, Florida, Big Cypress Seminole Indian Reservation	8.vi.2006	SS & RWP		
	TK0402	Unidentified host	Bahamas, New Providence, Nassau,	viii.2006	SS & RWP		
	TK0403	*Tetrazygia bicolor*	Bahamas, Grand Bahamas, Rand Nature Center,	viii.2006	SS & RWP		
	TK0404	*Eugenia confusa*	Bahamas, Grand Bahamas,	viii.2006	SS & RWP		
	TK0405	Unidentified host	Bahamas, Andros	viii.2006	SS & RWP		
	TK0406	*Chrysobalanus icaco*	Bahamas, Andros,	viii.2006	SS & RWP		
	TK0407	Unidentified host	Bahamas, Andros	viii.2006	SS & RWP		
	TK0558	*Ficus benjamina*	Cuba, Universidad Central de Las Villas, Facultad de Ciencias Agropecuarias	vii.2007	H.G. Ravelo		
	TK0559						
P. silvestri Mahdihassan	TK0344	*Pongamia pinnata*	India, Karnataka, Bangalore, Malleshwaram Circle	1–5.v.2006	P. Selvaraj & RWP		
	TK0397						
	TK0408						
	TK0345	*Pongamia pinnata*	India, Tamil Nadu, Coimbator, Onapalayam	i.2006	P. Selvaraj & RWP		
	TK0410	*Pongamia pinnata*	India, Tamil Nadu, Coimbator, Onapalayam	vi.2006	P. Selvaraj & RWP		
Outgroup kerriids							
Austrotachardia acaciae (Maskell)	LGC00299	*Acacia, probably A. aneura*	Australia, Northern Territory, outside Alice Springs Desert Park	4.x.2004	LGC		
A. near acaciae	LGC00380	*Acacia papyrocarpa*	Australia, South Australia, 52 km E of Kimba, Polygonum Tanks	4.ix.2005	LGC		
Austrotachardia near *melaleucae* (Maskell)	LGC00395	*Calothamnus quadrifidus*	Australia, Western Australia, Mt Ragged camp site	10.ix.2005	LGC		
A. near *melaleucae*	LGC00206	*Melaleuca strobophylla*	Australia, Western Australia, Lake King to Southern Cross Rd	1.ix.2004	LGC & M.D. Crisp		
A. near *melaleucae*	LGC00249	*Melaleuca* sp.	Australia, Western Australia, 30 km NE of Lake King,	1.ix.2004	LGC & M.D. Crisp		
A. near *melaleucae*	LGC00432	*Melaleuca sparsiflora*	Australia, Western Australia, 51 km W of Kamarl	16.ix.2005	LGC		
A. near *melaleucae*	LGC00457	Unidentified	Australia, Western Australia, 76 km S of Newdegate along Lake Magenta Road	18.ix.2005	LGC		

Austrotachardiella colombiana Kondo & Gullan	TK0122	*Psidium guajava*	Colombia, Valle, Santander de Quilichao	15.vii.2003	A. Delgado
Kerria (*Chamberliniella*) *greeni* Chamberlin	TK0121	*Ficus pumila*	Hong Kong, Pak Sha O,	vi.2005	C. Barthelemy
Tachardiella cornuta (Cockerell)	TK0413	*Viguiera* sp.	USA, Texas, Franklin Mts. (El Paso), Thom Mays Recreation Reserve	2.vi.2004	C.M. & R.L. Unruh
Tachardina aurantiaca (Cockerell)	TK0390	*Inocarpus fagifer*	Australia, Christmas Island, Daniel Roux Road	15.vi.2006	R. Reeves
Tachardina minor (Brain)	TK0416	*Elytropappus rhinocerotis*	South Africa, Northern Cape, Kamiesberge, Langkloof Pass	7.x.2005	PJG
T. minor	TK0418	*Elytropappus rhinocerotis*	South Africa, Eastern Cape, outside Thomas Baines Nature Reserve, roadside	11.xi.2005	PJG
Tachardina near *minor*	TK0417	*Felicia filifolia*	South Africa, Eastern Cape, ca. 20 km S of Whittlesea, roadside on R67	3.xi.2005,	PJG

Australia and Africa, which served as outgroups for phylogenetic analysis (Table 1).

For the lobate lac scale and the Indian *Paratachardina* species, infested twig segments were clipped from the plants and samples of about 10–30 specimens were stored in each of 70% and 95% ethanol for transportation to the Department of Entomology, UC Davis. Specimens of *P. silvestri* and *P. mahdihassani* were taken from shipments sent bimonthly from 14 sites in southern India from August 2005 until July 2006 (Schroer et al., 2007). Lobate lac scales from Christmas Island were provided by Dennis O'Dowd (Monash University, Australia) and Mick Jeffery (Parks Australia North, Christmas Island), from Cuba by Horacio Grillo Ravelo (Universidad Central de Las Villas, Cuba). In Florida, *P. pseudolobata* was collected at 12 sites. The sites were selected from a list of lobate lac scale collection localities provided by Greg Hodges (Florida Department of Agriculture and Consumer Services, Gainesville, FL). In the Bahamas, *P. pseudolobata* specimens were collected from three islands: Grand Bahamas, New Providence and Andros.

The density of *P. pseudolobata* infestations in Florida and the Bahamas was determined per plant with the highest infestation at each site, by estimating the number of lobate lac scales per 30 cm of three randomly chosen twig segments with $\leqslant$20 mm cross-section. Infestations of $\leqslant$10 female scales were rated as low, $\geqslant$11–100 as medium, and >100 as high (Pemberton, 2003a). From each collection site, scales on 30 cm twig segments with highest infestation were examined for the presence of parasitoid emergence holes. The percentage parasitization was determined by counting the number of emergence holes relative to the number of scales. Data were compared using one tailed Mann–Whitney *U* test for two independent samples. Due to the low percentage of parasitization we did not arcsine transform these data; significant levels were at 5%. If the tests had emergence holes, the specimens were removed and preserved in ethanol, and remains of parasitoids inside the scales were studied under the dissecting microscope.

2.2. Identification and morphological observations

Identifications and morphological observations of all Kerriidae available for this study were made from adult females mounted on microscope slides and examined under a compound microscope. Specimens were slide-mounted in Canada balsam using the method described in Williams and Granara de Willink (1992), except that xylene was used instead of clove oil. Specimens intended for molecular work were preserved in 95–100% ethanol and stored below 4 °C. After DNA extraction, these specimens were slide-mounted as vouchers. All vouchers of *Paratachardina* species are deposited in the Bohart Museum of Entomology (BME), University of California, Davis, USA, although other specimens from these same collections are deposited in a range of institutions (Kondo and Gullan, 2007). Vou-

cher specimens for outgroup taxa will be deposited either in BME or the Australian National Insect Collection (ANIC), CSIRO Entomology, Canberra. The collection data and depositories for voucher specimens are listed in Table 1.

2.3. Molecular comparisons

DNA was extracted from whole, ethanol-preserved adult female specimens using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. The cuticle of each adult female was recovered after the first incubation step and stored in 70% ethanol until being slide-mounted. Two partial gene regions were amplified; the 3′ region of the mitochondrial protein-encoding gene *cytochrome oxidase I* (*COI*) was amplified with an annealing temperature of 50 °C using CI-J-2183 (Jerry) (Simon et al., 1994) and C1-N-2568 (Ben) (Brady et al., 2000), and the D2–D3 region of the nuclear large-subunit ribosomal RNA gene (28S) was amplified with an annealing temperature of 55 °C using primers S3660 (28SF, Dowton and Austin, 1998) and A335 (28Sb, Whiting et al., 1997). The *COI* barcode region (Herbert et al., 2003) was not used because it has failed to amplify in most scale insects tried to date, including lac scales. Purified PCR products were sequenced in both the forward and reverse directions at the UC Davis genomics facility (CGF, UC Davis) or by Macrogen Ltd. (Korea).

Contigs were assembled and manually edited using Sequencher v4.5 (Gibbs and Cockerill, 1995; GeneCodes) and aligned by eye using Se–Al (Rambaut, 1996). Phylogenetic analyses and genetic distance measures were undertaken using PAUP* (Swofford, 2003). Genetic distance measures were calculated using the Kimura-2 parameter option. Length variation in the D2–D3 region of 28S among lac scale taxa resulted in regions of ambiguous alignment. These regions were excluded from analyses that included sampling across the whole of Kerriidae. There was less length variation within *Paratachardina*, resulting in a less ambiguous alignment, so all sites were included in a reduced-taxon, *Paratachardina*-only analysis. Two different weighting schemes were applied in maximum parsimony analysis (MP) of the *COI* data: equally-weighted (ewt MP, all sites weighted equally) and differentially-weighted (dwt MP, in which first and second codon positions were weighted three times that of third codon positions).

For a gene region to be of use as a DNA barcode, an unknown sample needs to be reliably and confidently matched to a known sample, typically using a phylogenetic approach such as neighbor-joining. Length variation among samples has the potential to mislead analyses if the phylogenetic result is sensitive to alignment. To test the utility of 28S, despite its length variation within scale insects, an automated alignment program (MUSCLE, Edgar, 2004) was used to determine whether the species clusters could be recovered using a default ambiguous alignment of the whole of the D2–D3 region. The MUSCLE alignment was analyzed using the neighbor-joining (NJ) option (Kimura-2 parameter) in PAUP*.

3. Results

3.1. Colonization and parasitism

In southern Florida most sampled host plants were heavily infested with *P. pseudolobata*. Infested plants were found over the entire southern peninsula from the east to the west coast between latitudes 25°00′N to 27°46′N, including seven counties. In Miami-Dade and Broward Counties susceptible plants were infested with >500 scales per 30 cm. Wax myrtle (*Myrica cerifera*) and redberry stopper (*Eugenia confusa*) were among the most susceptible plants to *P. pseudolobata* in Florida and usually were the first infested plant species at newly invaded sites. At the most northern site in Florida and the most eastern site on Andros Island, these two plant species had infestation levels of >300 scales per 30 cm. In the three islands of the Bahamas, the infestation was lower than in Florida, but occasional very high infestations were recorded (e.g., >400 adult scales per 30 cm were counted on a redberry stopper on Grand Bahamas Island). Plants infested to a medium level were found throughout the observed islands. Table 2 presents the infestation levels and the number of scales found with parasitoid emergence holes at all collection sites in the Bahamas and Florida.

The parasitization level of *P. pseudolobata* was very low. In the Bahamas only 2.29% (±3.31) scales ($n = 2557$ scales) were parasitized. and even less 0.22% (±0.37) were parasitized in southern Florida ($n = 10860$ scales), (Mann–Whitney Test, $P = 0.23$). Fully developed adults of *Metaphycus* spp. and *Ooencyrtus* sp. (Hymenoptera: Encyrtidae) were found inside scales both in Florida and the Bahamas. A *Metaphycus* male and female were found inside a single scale on the Bahamas, and two males and one female were collected in Florida. Only males were found of *Ooencyrtus*, two on the Bahamas and one in Florida. All collected parasitoids developed gregariously with two to four siblings as indicated by the number of emergence holes and parasitoid remains found in the scales.

3.2. Morphological characteristics

Morphologically, *P. pseudolobata* is similar to two Indian species, *P. mahdihassani* and *P. silvestri* (Kondo and Gullan, 2007). In each of these three species, the test of the adult female is resinous and four-lobed. In the other species of *Paratachardina*, the test of the adult female is more globular and has up to 16 ridges radiating from the top center of the test towards the venter, giving a ribbed appearance. The morphology of adult females of all *Paratachardina* species is quite similar in structure and reliable identification requires well-prepared microscope slide-mounts. The most important features for distinguish-

Table 2
Sites and host plants of *Paratachardina pseudolobata* collections in the Bahamas and Florida from east to west, indicating the level of scale infestation (Inf) per plant, counted as low (l), medium (m) or high (h), with 0 to 10, 10 to 100 and >100 mature females per 30 cm, respectively, and the total number of scales with parasitoid emergence holes (Eh) per stem segment

Location and habitat	GPS coordinates		Plant species	Plant family	Inf	Eh
	West	North				
Bahamas, New Providence						
Bahamas Trust retreat	077°18.63	25°03.83	*Guapira discolor*	Nyctaginaceae	l	3
			Croton eluteria	Euphorbiaceae	h	6
			Nectandra coriacea	Lauraceae	l	3
Roadside	077°19.28	25°04.51	*Chrysobalanus icaco*	Chrysobalanaceae	h	0
	077°26.76	25°03.19	*Exothea paniculata*	Sapindaceae	m	1
Bahamas, Andros						
Roadside	077°45.59	24°35.02	unknown species	Rubiaceae	h	0
Pineland	077°45.68	24°41.71	*Caesalpinia bahamensis*	Fabaceae	l	0
Roadside	077°47.40	24°41.95	*Chrysobalanus icaco*	Chrysobalanaceae	m	0
			Psidium sp.	Myrtaceae	l	1
			Hypelate trifoliata	Sapindaceae	m	3
			Chrysobalanus icaco	Chrysobalanaceae	l	0
	077°47.83	24°44.03	unknown species	Sapindaceae	l	0
	077°48.65	24°48.57	*Exothea paniculata*	Sapindaceae	m	0
	077°53.03	24°49.57	*Chrysobalanus icaco*	Chrysobalanaceae	h	0
	077°59.79	25°10.26	*Conocarpus erectus* var. *sericeus*	Combretaceae	l	1
Understory in forest	078°02.42	26°06.67	unknown shrub		l	0
Bahamas, Grand Bahamas						
Mangrove hammock	078°24.13	26°36.12	unknown tree		l	1
			Coccoloba diversifolia	Polygonaceae	m	0
Pineland	078°24.16	26°36.36	*Myrica cerifera*	Myricaceae	l	0
Rand Nature Centre, hardwood hammock	078°40.40	26°32.36	*Exothea paniculata*	Sapindaceae	h	0
			Lysiloma latisiliquum	Fabaceae	l	0
			Metopium toxiferum	Anacardiacaea	l	0
			Guettarda elliptica	Rubiaceae	l	0
			Tetrazygia bicolor	Melastomataceae	h	2
			Manilkara zapota	Sapotaceae	m	1
			unknown shrub		m	3
	078°40.42	26°32.30	unknown shrub		h	3
			Myrica cerifera	Myricaceae	m	0
			Lysiloma latisiliquum	Fabaceae	l	0
	078°40.50	26°32.24	*Myrsine floridana*	Myrsinaceae	l	0
			Schinus terebinthifolius	Anacardiaceae	m	0
			Metopium toxiferum	Anacardiaceae	m	1
Roadside	078°49.91	26°33.56	*Chrysobalanus icaco*	Chrysobalanaceae	m	1
	078°50.88	26°34.34	*Eugenia confusa*	Myrtaceae	h	1
Florida (County)						
Secret Woods, landscaped (Broward)	080°10.66	26°05.32	*Psychotria nervosa*	Rubiaceae	h	0
Fern Forest, hardwood hammock (Broward)	080°11.09	26°13.78	*Psychotria nervosa*	Rubiaceae	h	0
Simpson Park, landscaped (Miami Dade)	080°11.82	25°45.72	*Psychotria nervosa*	Rubiaceae	h	3
University of Florida, nursery plant (Broward)	080°14.40	26°04.97	*Randia aculeata*	Rubiaceae	m	0
Loxahatchee Wildlife Refuge (Palm Beach)	080°21.70	26°51.99	*Schinus terebinthifolius*	Anacardiaceae	m	0
Jungle Trail, landscaped (Indian River)	080°24.99	27°46.10	*Myrica cerifera*	Myricaceae	h	0
Melaleuca infested forest (Miami Dade)	080°29.20	25°45.93	*Melaleuca quinquenervia*	Myrtaceae	h	1
Everglades NP (Miami Dade)	080°46.03	25°45.41	*Chrysobalanus icaco*	Chrysobalanaceae	h	0
I–75, recreation area (Broward)	080°43.05	26°08.92	*Myrica cerifera*	Myricaceae	h	0
Big Cypress national preserve (Monroe)	080°55.50	25°50.80	*Myrica cerifera*	Myricaceae	m	3
Seminole Big Cypress Museum (Hendry)	081°00.90	26°21.73	*Myrica cerifera*	Myricaceae	h	0
Naples, shrub on side of road (Collier)	081°45.99	26°08.77	*Chrysobalanus icaco*	Chrysobalanaceae	m	0

ing the adult females of *P. pseudolobata* from those of *P. mahdihassani* and *P. silvestri* are (i) the distance between the two microduct clusters of the first pair of ventral duct clusters (vdc-1), and (ii) the total number of pairs of ventral duct clusters; see Kondo and Gullan (2007) for discussion of these features.

3.3. Molecular identification and relationships

Paratachardina was recovered as monophyletic in analyses of both gene regions but bootstrap support was weak (Figs. 1 and 2). *Paratachardina* sp. near *decorella* (Australia) was sister to the other three species (*P. pseudolobata,*

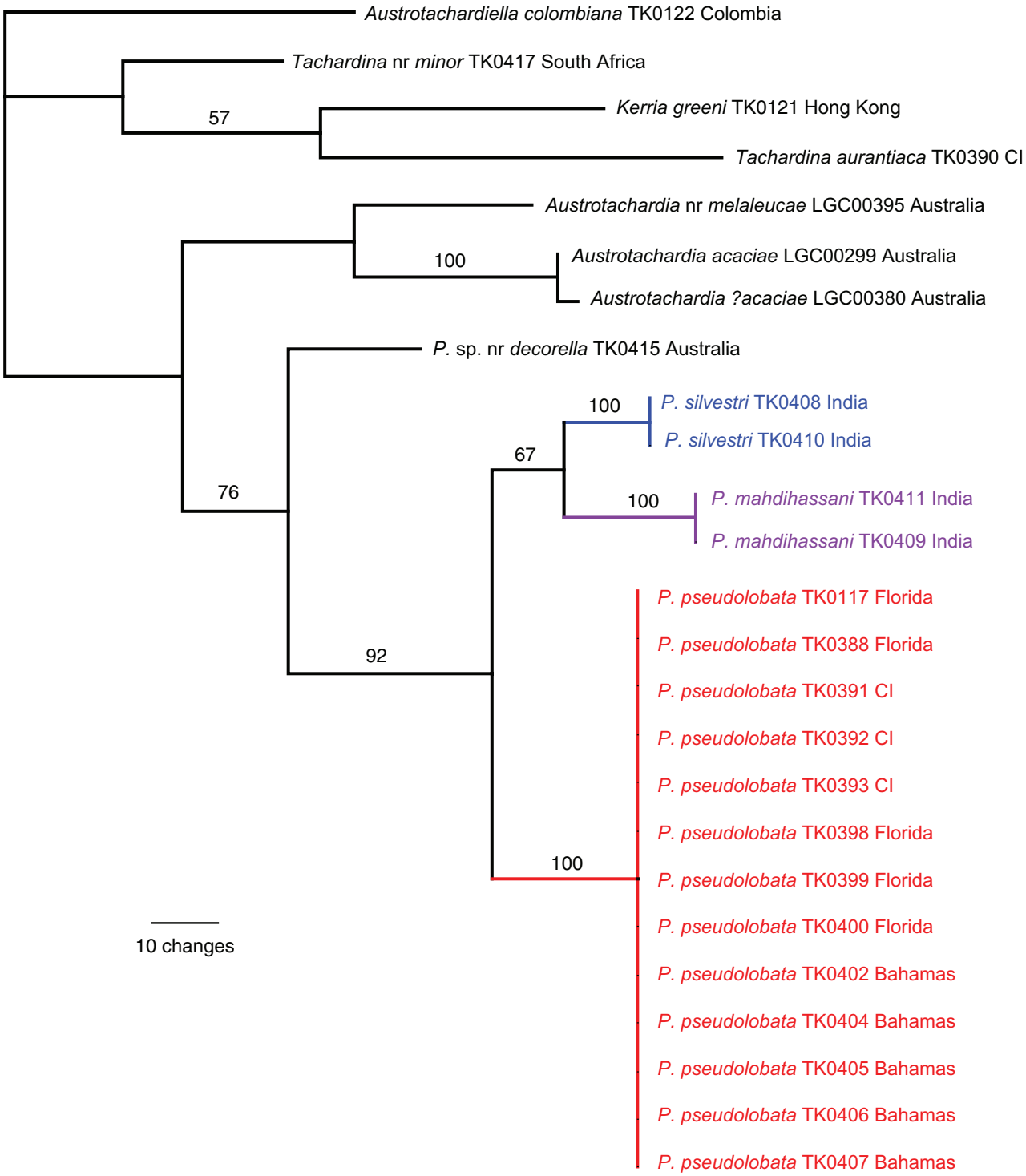

Fig. 1. Phylogenetic relationships of the lac scales (Kerriidae) based on maximum parsimony analysis of *COI* with codon positions weighted differentially (first and second positions weighted 3, third positions weighted 1). Bootstrap support from 1000 pseudoreplicates is shown above internodes. The specimen code and collection locality are shown after each species name. CI, Christmas Island (Australia).

P. silvestri and *P. mahdihassani*). All species for which multiple specimens were sampled were recovered as monophyletic. The relationships among *P. pseudolobata, P. silvestri* and *P. mahdihassani* differed among analyses. In dwt MP analysis of *COI* (Fig. 1), and in the *Paratachardina*-only analysis of 28S (Fig. 3), *P. silvestri* and *P. mahdihassani* were sister taxa. In the conserved region-only analysis of 28S, *P. silvestri* was sister to *P. pseudolobata*, whereas in the ewt MP analysis of *COI P. mahdihassani* was sister to *P. pseudolobata*. The NJ analysis of the MUSCLE alignment of 28S recovered all species, with each multiply-sampled species of *Paratachardina* receiving bootstrap values of 100.

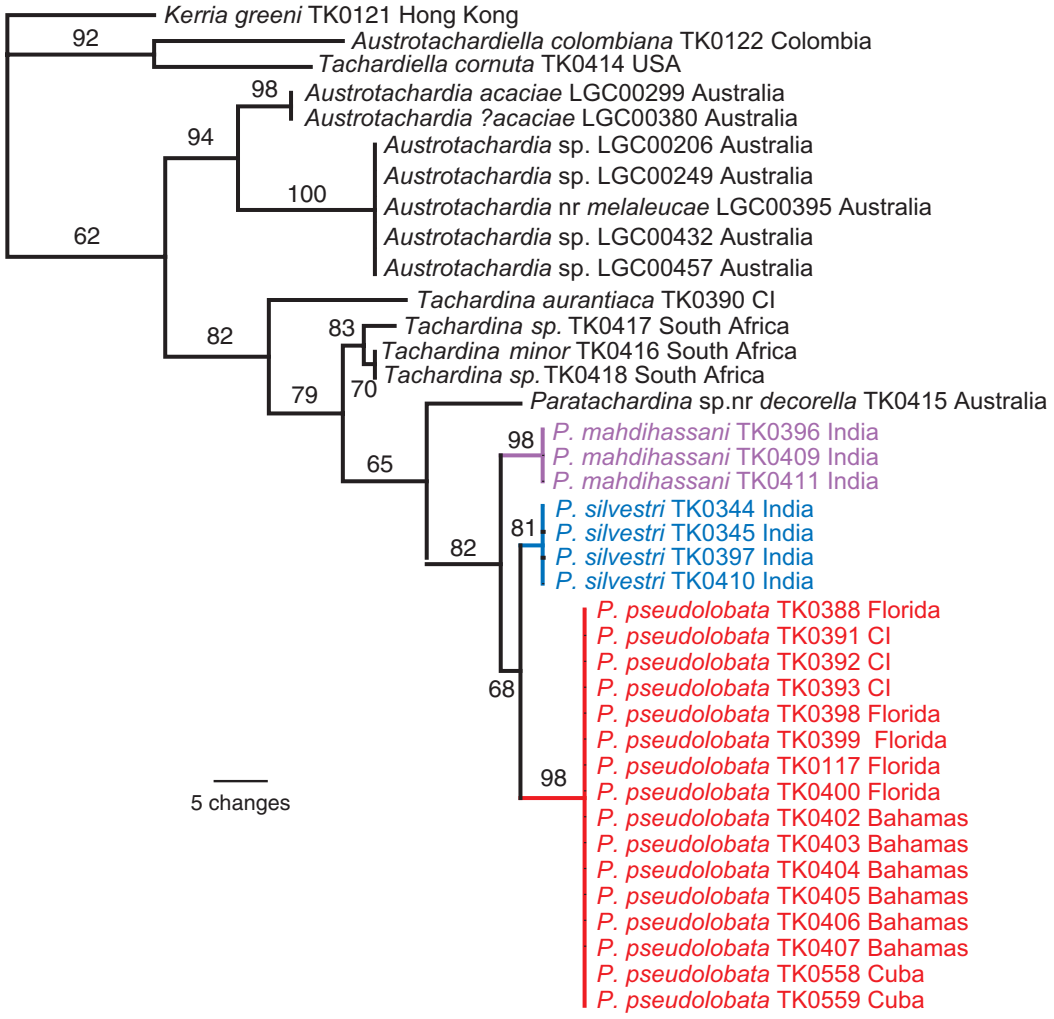

Fig. 2. Phylogenetic relationships of the lac scales (Kerriidae) based on maximum parsimony analysis of the conserved regions of the D2–D3 domains of 28S. Bootstrap support from 1000 pseudoreplicates is shown above internodes. The specimen code and collection locality are shown after each species name. CI, Christmas Island (Australia).

More variation was found within and among taxa in 28S than in *COI* (Figs. 1 and 2). The length of the amplified D2-D3 region of 28S varied more than 30% among the kerriid specimens (748–1114 bp). There was 11% divergence in 28S between *P. pseudolobata* and *P. mahdihassani*, and 12% between *P. pseudolobata* and *P. silvestri*. *Paratachardina* sp. near *decorella* (TK0415) was the only individual displaying variation in 28S, with 5 polymorphic sites. At each polymorphic site, both peaks of the electropherogram were of equal intensity. There were both length variation and substitutions among individuals of *P. silvestri* from different localities, with 9 substitutions separating TK0410 from the other two populations. No variation was found within or among populations of either *P. pseudolobata* or *P. mahdihassani*.

There was no length variation in the *COI* region amplified and no intra-specific polymorphism (Fig. 1), but there was up to 15% difference within *Paratachardina* and 7–9% divergence between *P. pseudolobata* and its nearest relatives. There was 10% divergence in *COI* between *P. pseudolobata* and *P. mahdihassani*, and 8.5% between *P. pseudolobata* and *P. silvestri*. Of the lac scale insect species compared here, only *P. silvestri* exhibited intra-specific variation in 28S. None were found to have intra-specific variation in *COI*.

4. Discussion

The level and pattern of DNA sequence divergence between *P. pseudolobata* and other kerriid specimens distinguishes *P. pseudolobata* from the morphologically most similar species *P. silvestri* and *P. mahdihassani*. Both *COI* and 28S clearly distinguished each of the described species of Kerriidae and resulted in clustering of individuals by species in the phylogenetic analyses (Figs. 1 and 2). *COI* was less variable than 28S but easier to align and with less uncertainty. 28S was more variable than *COI* in proportion of substitutions, presence of indels and fragment length. Scale insect 28S appears to be more diverse and length-variable than that of most other insects examined to date (e.g. Dowton and Austin, 1998; Gillespie et al., 2004). This also is the case for the more conserved 18S region, in which

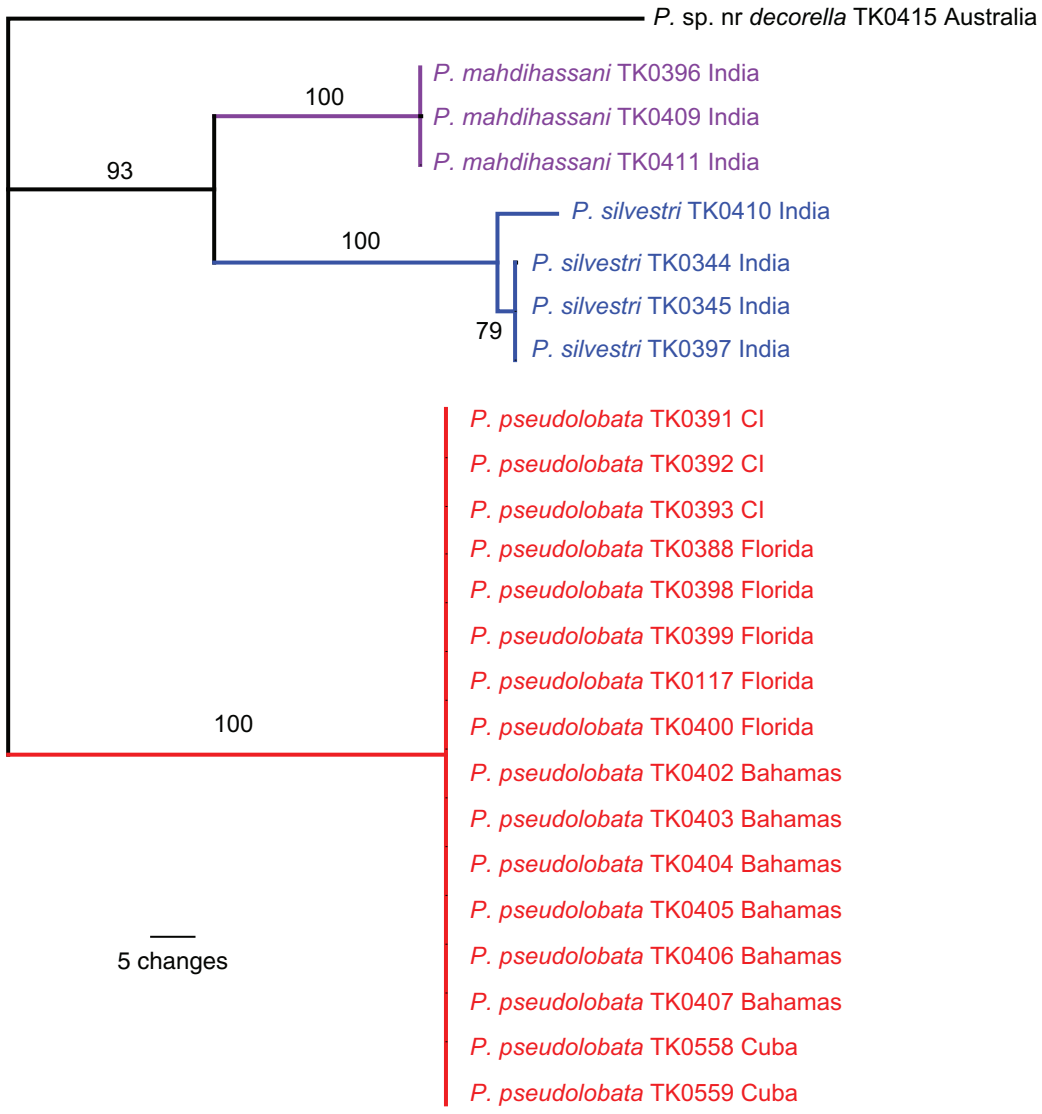

Fig. 3. Phylogenetic relationships within *Paratachardina* based on maximum parsimony analysis of the D2–D3 regions of 28S. Bootstrap support from 1000 pseudoreplicates is shown above internodes. The specimen code and collection locality are shown after each species name. CI, Christmas Island (Australia).

scale insects also exhibit considerable length variation among taxa (Cook et al., 2002; Cook and Gullan, 2004).

Incomplete concerted evolution, or another process with a similar outcome, is apparent in the 28S sequence from *P.* nr *decorella* (TK0415), with five clear polymorphic sites in the one individual. The equal intensity of each polymorphism in the electropherogram suggests that each is present in equal copy number. This could indicate that there are two independent loci, each concertedly evolving within a locus but not across loci. Multiple copies of 28S have been reported for insects previously (e.g. Dowton and Austin, 1998; Gillespie et al., 2004) and have sometimes been attributed to multiple loci. An alternative explanation might be recent hybridization, with the TK0415 individual representing an F1-equivalent hybrid.

The combination of extreme length polymorphism among taxa and substitutional polymorphism within individuals suggests that the use of 28S as an alternative DNA barcode region in scale insects might not be straightforward. The variation leads to greater branch length between taxa but difficulty in aligning sequences across diverse taxa, even within Kerriidae. This might not be a major problem if specimen identification is the primary objective. Despite the length variation present within the kerriids included here, specimens were clustered by species with high confidence (bootstraps = 100) using an automated alignment program (MUSCLE) and neighbor-joining algorithm. This suggests that 28S, despite length variation, might be a suitable non-*COI* barcode for scale insects.

The lack of variation among all specimens of *P. pseudolobata* sampled to date is consistent with a single geographic, or genotypic, origin of the scale. Given the apparent parthenogenetic nature of the populations of *P. pseudolobata*, it is possible that each could have been founded by as few as one female. However, the DNA

sequence data do not preclude multiple introductions or origins. Although there was no variation among individuals of *P. pseudolobata* from across its sampled introduced range, this is not unusual compared with other scale insects. A lack of variation among populations in the same gene regions has been found also for some other recognized species of scale insects (L.G. Cook, unpublished data). This is despite some species apparently being obligatorily sexual and having been collected from disparate localities in their native range.

No genotype was sampled from India that matched or was conspecific with *P. pseudolobata*, indicating that *P. pseudolobata* may be native to an unsampled region within India, or to another country not yet sampled. In two of the analyses (Figs. 1 and 2) it is sister to species native to India. Thus, these molecular phylogenies are uninformative as to where its native range might be, leaving the whole of Southeast Asia and Australia (the apparent natural range of the genus) as possibilities. Takumasa Kondo examined insect collections of Kasetsart University, Bangkok, and the Department of Agriculture, Bangkhen, Thailand, in 2007. Few Kerriidae were in the dry collections, none slide-mounted, and the material mostly appeared to be *Kerria* species. No specimens of *Paratachardina* were found in any collections or in field searches.

In the conserved region-only analysis of 28S (Fig. 2), *P. pseudolobata* is nested within the Indian clade, suggesting this area as the possible native range. Sampling has not yet been extensive across India and Sri Lanka, and it is possible that there might be more species of *Paratachardina*. For instance, *P. silvestri* had considerable variation in 28S and the levels, up to 9 substitutions, suggest that further cryptic species may exist in this taxon. The introduction of the lobate lac scale into Florida and the Bahamas is very likely due to the importation of plants infested with this inconspicuous species. The time of the introduction of *P. pseudolobata* in Christmas Island is unknown. It might have been introduced with plants from Asia, where a high proportion of the human population of Christmas Island originate (Heng and Forbes, 2006). Furthermore, distribution within the New World tropical regions, particularly in the proximate West Indies, might be promoted by further movement of infested horticultural plants, as well as by wind and vectors such as wild animals and humans (Gullan and Kosztarab, 1997).

Despite the close similarity to *P. silvestri* and *P. mahdihassani*, the invasive *P. pseudolobata* is equipped with a different immune response. Parasitoids reared from Indian *Paratachardina* spp. developed successfully in *P. pseudolobata* only when multiple parasitoid eggs per scale were laid (Schroer and Pemberton, 2007). In Florida and the Bahamas, the same gregarious parasitoid development was observed for both the *Metaphycus* and *Ooencyrtus* spp. These parasitoid species are currently undescribed and their biology is not known; they might be local species that have shifted hosts. Similarly, *Ammonoencyrtus carolinensis* (Meyer) (Hymenoptera: Encyrtidae), a parasitoid species that was isolated from *P. pseudolobata* in Florida (Schauff, 2005), is known to attack the coccid *Mesolecanium nigrofasciatum* (Pergande) and may have shifted recently to the lobate lac insect. The parasitization levels of *P. pseudolobata* in the Bahamas and in Florida are very low. The slightly higher parasitization level in the Bahamas might be due to greater parasitic wasp occurrence, promoted by favorable habitats and more wild areas, untreated with pesticides. However, the parasitization levels are insufficient to influence the abundance of *P. pseudolobata* populations in either region. Further research to investigate the origin and biology of the parasitoids and the habitats where they are most successful parasitizing the lobate lac scale are necessary for the success of future biological control strategies. This effort, although challenging because the native range of *P. pseudolobata* is unknown, will be facilitated by the correct identification of the pest, provided by both the morphological data (Kondo and Gullan, 2007) and the molecular research reported here.

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