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Mitochondrial DNA diversity of *Cleruchoides noackae* (Hymenoptera: Mymaridae): A potential biological control agent for *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae)

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ABSTRACT

Thaumastocoris peregrinus Carpintero and Dellapé (Hemiptera: Thaumastocoridae) is a native Australian *Eucalyptus* sap- feeding insect that has become invasive and seriously damaging to commercially grown *Eucalyptus* in the Southern Hemisphere. *Cleruchoides noackae* Lin and Huber (Hymenoptera: Mymaridae) was recently discovered as an egg parasitoid of the Thaumastocoridae in Australia. Mitochondrial DNA (cytochrome c oxidase subunit I, COI) sequence diversity amongst 104 individuals from these native *C. noackae* populations revealed 24 sequence haplotypes. The COI haplotypes of individuals collected from the Sydney and Southeast Queensland clustered in distinct groups, indicating limited spread of the insect between the regions. Individuals collected from Perth in Western Australia were represented by four COI haplotypes. Although this population is geographically more isolated from other populations, two COI haplotypes were identical to haplotypes found in the Sydney region. The results suggest that *C. noackae* has recently been introduced into Perth, possibly from the Sydney area. The high mtDNA diversity and limited spread that is suggested for *C. noackae* is in contrast to the lack of geographic associated mtDNA diversity and extensive spread of *T. peregrinus*. If implemented as a biological control agent, this factor will need to be considered in collecting and releasing *C. noackae*.

KEYWORDS

egg parasitoid; Hemiptera; Hymenoptera; laboratory rearing; Mymaridae; Thaumastocoridae

INTRODUCTION

Biological control programmes can benefit substantially from an understanding of the population diversity of the target pests as well as of the biological control agents (Sha et al. 2006). The frequency of mitochondrial DNA (mtDNA) haplotypes within species is sensitive to establishment events and population size. This allows for the identification of genetic geographic structure, which might have relevance to host or environmental adaptation, as well as migration events and genetic bottlenecks that may affect the level of diversity (Roderick 1996; Roderick and Navajas 2003). In the pest, such data can be useful to match the invasive population to its source population, thereby guiding one to the region in which matching natural biological control agents can be collected (Hoelmer and Kirk 2005). In the parasitoid, it can help to ensure that the maximum diversity is collected and maintained during the quarantine and mass rearing, which could affect its adaptability in a new environment and on different host types (Gariepy et al. 2007; Roderick and Navajas 2003).

Apart from population studies, the correct identification of a target pest and its potential biological control agents is fundamentally important. This is particularly true given that many parasitoid groups, such as the tiny Mymaridae that are also known as fairy flies, include cryptic species that are difficult to identify (Gariepy et al. 2007; Sha et al. 2006). In this regard, molecular techniques such as the mitochondrial cytochrome *c* oxidase subunit I (COI) bar-coding gene have been useful in ensuring accurate identifications (Darling and Blum 2007; Dasmahapatra and Mallet 2006; Gariepy et al. 2007; Sha et al. 2006).

The *Eucalyptus* sap-feeding insect *Thaumastocoris peregrinus* Carpintero and Dellapé (Hemiptera: Thaumastocoridae) is native to Australia, and is a serious pest on commercially grown non native *Eucalyptus* plantations in Southern Africa and South America (Jacobs and

Neser 2005; Nadel et al. 2010; Noack and Coviella 2006). There are currently no commercially viable control options for *T. peregrinus* infesting commercial *Eucalyptus* plantations. Biological control is widely accepted to be the most feasible control mechanism for *T. peregrinus*, but until recently no parasitoids were known that could be used for this purpose. In 2002, an egg parasitoid was reared from *T. peregrinus* eggs in Sydney and subsequently named as *Cleruchoides noackae* Lin and Huber (Hymenoptera: Mymaridae), (Noack 2002, Lin et al. 2007). This insect is currently the only available potential biological control agent for *T. peregrinus* and there is consequently an urgency to study its biology and diversity.

The diversity of native and invasive populations of *T. peregrinus* has recently been determined using mitochondrial COI bar-coding sequence data (Nadel et al. 2010). These data have helped to distinguish *T. peregrinus* from related cryptic species in its native range, and confirmed its identity in the invaded range. Furthermore, it revealed a great deal about the origin and invasion pathway of this insect. For example, the dominant haplotypes found in outbreak populations of *T. peregrinus* in Sydney and Brisbane overlapped with the haplotypes found in the invaded regions, suggesting these major cities to be the source of the invasions. It also confirmed that at least three independent introductions have occurred into South Africa and South America. There would be significant value in achieving the same level of understanding of the diversity of its potential biological control agent *C. noackae*.

In this study we use the mtDNA cytochrome c oxidase subunit I (COI) bar-coding sequences to determine the level of genetic variation of *C. noackae* within and between native populations in Australia. These data are compared to mtDNA COI sequence data from the host species, *T. peregrinus*. We subsequently consider the effect of the quarantine rearing

process on diversity by determining the mtDNA COI diversity in two laboratory reared populations of *C. noackae.*

MATERIALS AND METHODS

Insect collection

Cleruchoides noackae individuals emerged from eggs collected in and around three urban centres of Southeast Queensland, Perth and Sydney. In May 2008, *C. noackae* individuals were collected from several trees and from numerous egg masses that were grouped per sampling site, one site was in Southeast Queensland (n = 15), one site in Perth (n = 16) and two sites (n = 45) in the Sydney region. A second sampling to collect individuals for quarantine parasitism experiments in South Africa was made in May 2009, where *C. noackae* individuals were collected from only a single tree at one site (n = 28) in the Sydney region. All wasps collected in the 2008 sampling were allowed to oviposit on *T. peregrinus* eggs in a quarantine laboratory in South Africa. The second generation of these wasps that emerged from the parasitized eggs (n = 22) were also collected. All adults of *C. noackae* that emerged from parasitized *Thaumastocoris* sp. and *T. peregrinus* eggs from 2008 and 2009 collections were placed in labelled containers filled with 100 % ethanol for subsequent DNA analysis. Voucher *C. noackae* specimens for each of the sampled localities, were deposited in the Tree Protection Co-operative Programme (TPCP) insect collection, Pretoria, South Africa (ES-Nr 525 – 566).

DNA extraction, PCR and Sequencing

Total genomic DNA was extracted from 126 adult *C. noackae* individuals using Chelex® Ultra 100 molecular biology grade resin (Bio-Rad Laboratories, South Africa). Mitochondrial cytochrome *c* oxidase subunit I (COI) gene PCR amplifications were initially undertaken

using the primer pairs C1-J-2183 and TL2-N-3015 (Simon et al. 1994). From 10 resulting sequences, a consensus sequence was produced, with a new set of primers developed using the software Primer 3 (Rozen and Skaletsky 1998) and Net Primer (PREMIER Biosoft International) so as to increase sequencing efficiency. A 803 base pair fragment of the mitochondrial COI gene was amplified using PCR with this newly developed primer pair Tpara 2205F (5' -GGTCATCCAGAAGTTTATATT- 3') and Tpara 3008R (5' -ATCTGCCATATTAGAAATGA- 3').

PCR reactions included 4 µl of genomic DNA (50 – 100 ng/µl), 10x PCR buffer (containing 100mM Tris-HCl, 500mM KCl, pH 8.3 at 20°C) (Roche Diagnostics), 3 mM $MgCl_2$, 1mM dNTP's, 0.4 µM of each primer, 1 U Taq polymerase with 10 µl of Sabax water to bring the total volume of the reaction to 25 µl. The PCR cycling regime was as follows: initial denaturation at 95 °C for 1 min followed by 30 cycles of 30 sec at 95 °C, 45 sec at 54 °C, 45 sec at 72 °C, and a final elongation for 10 min at 72 °C. The PCR products were cleaned using the 3M NaOAc (pH 4.6) and Ethanol (CH_3CH_2OH) precipitation method prior to being visualized under UV on an Ethidium Bromide stained 2 % agarose gel.

All 126 collected specimens mentioned earlier were sequenced using the same primers described above for PCR. Sequencing reactions were performed in 10 µl with 4 µl of cleaned PCR product, 0.5 µM of the primers, 2 µl 5 x sequencing buffer and using the BigDye® Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, Warrington, UK). PCR conditions were 25 cycles of 10 sec at 95 °C, 5 sec at 50 °C, 4 min at 60 °C. PCR products were purified by precipitation using 3M NaOAc (pH 4.6) and ethanol (CH_3CH_2OH) followed by vacuum drying and sequencing using the ABI Prism™ 3100 Genetic Analyzer (Applied

Biosystems). From a total of 126 sequences, 24 representative sequences were deposited in GenBank (GU945400 – GU945423).

mtDNA sequence analyses

Sequence alignments were done using Vector NTI 9.1 (Invitrogen Corporation, 2004). Phylogenetic neighbour- joining and distance analyses were conducted using MEGA version 4 (Tamura et al. 2007). The haplotype diversity was determined using DnaSP ver. 4.5 (Rozas et al. 2003). A parsimony haplotype network with 95 % statistical support was obtained using TCS 1.21 (Clement et al. 2000) to determine the relationship among *C. noackae* haplotypes.

Population structure was analysed using the Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) and by calculating pairwise F_{ST} (Hudson et al. 1992) values between populations, using the Kimura 2- parameter distance method (Kimura 1980). The statistical significance of the fixation indices was assessed by performing 1000 permutations of the original data set in ARLEQUIN ver. 3 (Excoffier et al. 2005).

RESULTS

Consensus sequences of the amplified mtDNA COI gene region, revealed a 589 bp fragment for 126 *C. noackae* individuals from three native (n = 104) and one laboratory bred (n = 22) population. A total of 24 different haplotypes were identified for the individuals sequenced, by patterns of single nucleotide polymorphisms (SNPs) (Table 1) GenBank (GU945400 – GU945423). The haplotypes contained 23 polymorphic nucleotide sites of a total of 589 (3.90 % of total sites) (nucleotide diversity 0.00744 ± 0.00061). As haplotype diversity is a measure of the uniqueness of a particular haplotype in a given population (Nei 1987), no haplotype diversity was present in the Sydney (2009) population. In contrast, the haplotype

diversity was high for all populations collected in May 2008 and this ranged between 0.575 and 0.962 (Table 1), with the Southeast Queensland region having the highest and Perth region the lowest diversity.

The 24 mtDNA COI haplotypes were differentially distributed among the four native populations sampled (Table 1). Only two haplotypes (H_5 and H_6) were shared amongst the native Perth and Sydney (2008) populations and all other haplotypes were restricted to only one of these populations. Of the original 24 COI haplotypes present within the native populations, only 6 haplotypes were present in the laboratory bred populations. All haplotypes present in the laboratory bred population were also present in the native Sydney population (H_1, $H_3 – H_6$ and H_8) with two of these haplotypes also occurring within the native Perth population (H_5 and H_6). The haplotype diversity for the laboratory bred population (h = 0.831) was very similar to that obtained from the Sydney (2008) population (h = 0.873) (Table 1).

The relationship among mitochondrial haplotypes was reconstructed using a haplotype network (Fig 1). The largest number of individuals collected was found to belong to haplotype H_1. Samples collected from different geographical locations grouped separately (Fig 1), for which the pairwise genetic nucleotide distance calculations ranged from 0.49 – 0.83 % between geographical locations. The overall haplotype diversity was h = 0.827 for all samples, with Southeastern Queensland ($H_{13} – H_{24}$) samples grouping separately from other localities (Fig 1) having the highest haplotype diversity (h = 0.962) (Table 1). The haplotype diversity was h = 0.873 for the Sydney (2008) population and h = 0.575 for the Perth population (Table 1) with haplotype $H_5 – H_6$, grouping together and representing individuals collected in both Sydney and Perth (Fig 1).

An AMOVA performed on the mtDNA data on the native populations of *C. noackae* confirmed significant population genetic structuring between the Southeast Queensland, Sydney and Perth regions. The AMOVA revealed highly significant genetic structure with 66 % (Φ_{ST} = 0.662, *P* = 0.0001) of the variation being explained by the subdivision among populations and 33 % by the subdivision within populations (Φ_{ST} = 0.338, *P* = 0.0001). There were significant subdivisions between all populations with the highest being between Sydney and Southeast Queensland (Φ_{ST} = 0.744) and the lowest being between Sydney and Perth populations (Φ_{ST} = 0.448) (Table 2).

DISCUSSION

Sequencing a portion of the COI gene of 104 native *Thaumastocoris* egg parasitoids, from 3 regions in Australia, revealed an overall genetic divergence (0.49 – 0.83 %). This was indicative of a single species (Hebert et al. 2004), which is believed to be that of the newly described egg parasitoid, *C. noackae* (Lin et al. 2007). The genetic divergence falls within the mean mtDNA intra-specific divergence range (0 - 4 %) reported for Hymentoptera (Cognato 2006; Meier et al. 2008) and other Mymaridae parasitoids (de León et al. 2008). The level of nucleotide divergence was approximately the same as that of the host, *T. peregrinus* over the same area (Nadel et al. 2010).

The levels of haplotype diversity reflected that which is expected for a native parasitoid population. From the 104 mtDNA COI *C. noackae* sequences, 24 haplotypes were recovered. Twelve haplotypes were found to be unique to Southeast Queensland, whereas 8 and 2 unique haplotypes occurred within the Sydney and Perth regions, respectively. In Southeast Queensland, the haplotype diversity (*h* = 0.962) was the highest, with twelve unique

haplotypes amongst 15 samples. The haplotype diversity was high as a result of several single individual haplotypes found within this population. Similar COI haplotype diversities have been published for other insect orders sampled from their native environment (Cai et al. 2008; Vandewoestijne et al. 2004). The levels of haplotype diversity for *C. noackae* was higher than the levels found for its host, *T. peregrinus*, over the same area (h = 0.52 - 0.75).

There was significant geographic structure in the populations of *C. noackae*, as illustrated by the large proportion of diversity (66 %) distributed between populations. Furthermore, the populations from Southeast Queensland and Sydney did not share any haplotypes, while all haplotypes within each region were related. These results suggest low or limited gene flow between these populations, and raise the possibility of local adaptations to host diversity (Hufbauer and Roderick 2005). This is not uncommon amongst parasitic wasps. For example, a study on the parasitoid wasp *Diaeretiella rapae* McIntoch (Hymenoptera: Braconidae) showed that genetic population structure occurred between populations more than 1 km away from each other, consistent with the limited movement of wasp (Vaughn and Antolin 1998). These same factors most likely affect the ability of *C. noackae* to spread given its extremely small size and the sparse distribution of *Thaumastocoris* eggs in natural environments. Fragmented or isolated environments can also lead to such geographically structured populations in insects (Chen and Ye 2008; Horn et al. 2006). Extensive sampling along the eastern coast of Australia may result in a better understanding of the regional structuring and distances between distinct *C. noackae* populations. As mtDNA is maternally inherited its application to ascertain gene flow must, however, be viewed with some caution as it does not indicate the movement of males between populations (Roderick 1996).

The geographically structured mtDNA diversity and limited spread of *C. noackae* is in contrast to the lack of geographic associated diversity and apparent extensive spread of *T. peregrinus*. *T. peregrinus* had a lower overall diversity ($h = 0.52 – 0.75$) and shared haplotypes between Southeast Queensland and Sydney (Nadel et al. 2010). It has also rapidly spread across vast areas in South Africa and South America, most likely in a stratified fashion, subsequent to its introductions into these regions (Jacobs and Neser 2005; Nadel et al. 2010; Noack and Coviella 2006). This suggests that the spread of the two insects is fundamentally affected by different factors such as *T. peregrinus* moving between areas by "hitch hiking" which is less likely for *C. noackae*, possibly their ability to fly, as well as size, differential host availability and host density. In certain parts of South Africa and South America where large populations of *T. peregrinus* occur on *Eucalyptus* in plantations that are more or less continuously distributed, the spread of *C. noackae* might be enhanced, although this would need to be closely monitored. Otherwise, for effective biological control, *C. noackae* would need to be introduced into smaller areas of the landscape and thus to ensure that it occurs in all areas where *T. peregrinus* is present.

In 2009, a relatively large number of *C. noackae* individuals were sampled, but only from a single tree at one site in Sydney. These individuals all represented a single haplotype, compared to the 10 haplotypes that were sampled in 2008 from several trees and two sites in Sydney. It has been suggested that for successful biological control programmes, conserving the highest genetic diversity of the biological control agent, over a wide geographic range, would allow for improved success (Lloyd et al. 2005). This is because increased genetic diversity in a population improves in the survival of haplotypes that are able to adapt, survive and breed within a new environment (Angalet et al. 1979; Grevstad 1999). Because *T. peregrinus* occurs over a wide range of climatic conditions in both South Africa and South

America (Nadel et al. 2010), *C. noackae* would require a high degree of adaptability to be an effective biological control agent in these areas. It would thus be advisable to collect *C. noackae* for biological control over as large an area as possible and thus to maximise opportunities to include insects with a wide range of possible adaptability.

The first generation of *C. noackae* emerging from *T. peregrinus* infested eggs under quarantine conditions included 6 haplotypes. This amounts to a haplotype diversity of h = 0.831, which is the same as that of the original populations. This is despite the fact that this first generation emerging in quarantine was fairly small (n = 22). This is encouraging for future breeding in quarantine although it will be necessary to increase population sizes of the subsequent generations. Otherwise, small populations would increase the chances for stochastic events causing genetic drift and loss of diversity (Grevstad 1999).

Two mtDNA haplotypes (H_5 and H_6) were found to occur in *C. noackae* collected in both the Sydney and Perth regions. The large distance (± 4054 km) between Sydney and Perth, separated by both semi-arid and desert regions, suggest it is unlikely that this was due to the natural movement of *C. noackae* between these regions. Furthermore, the lack of shared mtDNA haplotypes between Southeast Queensland and Sydney, although closer in proximity compared to Sydney and Perth, suggests an introduction of *C. noackae* into Perth. The lack of diversity in Perth compared to populations from eastern Australia, further supports a founder effect following an introduction of a small number of individuals. The introduction of plant material for the rapid expansion of *Eucalyptus globulus* plantation forestry in south-western Australia, since the mid 1990's (Burgess and Wingfield 2002; Loch and Floyd 2001), is the most likely pathway by which *C. noackae* was introduced into Western Australia. This mirrors the increased incidence of pests and pathogens in this region (Burgess and Wingfield

2002; Loch and Floyd 2001). Interestingly, *T. peregrinus* was not found in Perth (Nadel et al. 2010).

Cleruchoides noackae is not species specific and possibly has a broad host range within the Thaumastocorinae and possibly with other Hemiptera associated with *Eucalyptus* in Australia. The two shared *C. noackae* haplotypes found in Perth and Sydney (2008) emerged from two different hosts, namely *Thaumastocoris peregrinus* in Sydney and a newly discovered *Thaumastocoris* sp. in Perth (Nadel et al. 2010). Furthermore, the host of the *C. noackae* Holotype, *Braclozygum depressum* Bergroth (Hemiptera: Thaumastocorinae) was collected in Tasmania (Lin et al. 2007). *C. noackae* has not been found on any other hosts outside the Thaumastocorinae in Australia, which will enhance the opportunity for its use as a potential in a biological control program in South Africa and South America, as no native Thaumastocorinae occur in these regions. Host specificity testing with bugs from related families is, however, essential prior to the release of *C. noackae* as a biological control agent in these invaded regions.

mtDNA COI gene sequence analysis provided interesting and potentially useful information regarding *C. noackae* and its potential use in biological control programmes for *T. peregrinus*. The data confirmed that a single parasitoid species parasitizes *Thaumastocoris* eggs over a very large area of Australia. The substantial diversity and geographic structure in the *C. noackae* populations also suggest that it might have local adaptations and a limited ability to spread naturally. This suggests that the collection of individuals over a broad geographic area will be necessary to enhance genetic diversity and the chances of successful establishment in the diverse environments of South Africa and South America where *T. peregrinus* occurs.

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LIST OF FIGURES

TABLES

Table 1: *Cleruchoides noackae* mtDNA COI haplotypes, representing the number of sequenced individuals (*N*) and the number of each haplotype (H_{1-24}), haplotype diversity ($h \pm$ SD) for each of the populations sampled.

		Haplotypes																								
Populations	*N*	H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8	H_9	H_{10}	H_{11}	H_{12}	H_{13}	H_{14}	H_{15}	H_{16}	H_{17}	H_{18}	H_{19}	H_{20}	H_{21}	H_{22}	H_{23}	H_{24}	Haplotype diversity (*h*)
Southeast Queensland	15	-	-	-	-	-	-	-	-	-	-	-	-	2	3	1	1	1	1	1	1	1	1	1	1	0.962 ± 0.040
Perth	16	-	-	-	-	4	1	-	-	-	-	10	1	-	-	-	-	-	-	-	-	-	-	-	-	0.575 ± 0.115
Sydney (2008)	45	12	3	9	6	1	7	2	1	3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.873 ± 0.031
Sydney (2009)	28	28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Laboratory bred	22	8	-	6	2	3	2	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.831 ± 0.060

Table 2: Pairwise F_{ST} values between *C. noackae* populations based on the Kimura 2- parameter distance between mtDNA haplotypes (*indicates significants at the 1% level)

Populations	Perth	Southeast Queensland	Sydney
Perth	-		
Southeast Queensland	0.664*	-	
Sydney	0.448*	0.744*	-