

Using DNA barcodes to investigate the taxonomy of the New Zealand sooty beech scale insect

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ABSTRACT

It is currently proposed that there are two species of honeydew-producing sooty beech scale insects (*Ultracoelostoma* spp.) in New Zealand. It is thought that *U. brittini* lives exclusively on trunks of southern beech (*Nothofagus* spp.) trees, while *U. assimile* occurs mainly on branches. This study aimed to confirm this habitat specialisation by using a molecular genetic approach. We sequenced the c.650 base pair DNA 'barcode' region of the mitochondrial gene cytochrome c oxidase I (COI) from specimens collected from Mount Grey/Maukatere (North Canterbury), Greymouth, and the Nelson Lakes region. Although the COI sequences supported the existence of two species, there was no evidence of the two species specialising on trunk or branch microhabitats. The excess sugar that these insects excrete as honeydew is an important energy source upon which many native birds and insects depend. Further geographic sampling is needed to determine the distribution and extent of sympatry of the two species detected in this study, which might have implications for forest management decisions.

Keywords: sooty beech scale insect, Margarodidae, Coccoidea, DNA barcoding, DNA taxonomy, species identification, microhabitat specialisation

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1. Introduction

The sooty beech scale insect (*Ultracoelostoma assimile*; family Margarodidae) is a key driver of ecosystem processes in beech (*Nothofagus* spp.) forests in New Zealand. These insects imbibe carbohydrate-rich phloem and excrete excess sugar as a ‘honeydew’. These droplets of honeydew are an important energy source upon which many native birds and insects depend (Gaze & Clout 1983; Beggs 2001; Murphy & Kelly 2003). They are also important for sooty mould, which grows on the bark, branches and vegetation surrounding the tree and accelerates forest floor litter decomposition and nutrient turnover. Despite the importance of scale insects in beech forest ecology, very little is known about their basic biology. Up until 1991, all sooty beech scale insects were included in a single variable species, *U. assimile*. Morales (1991) re-described the genus and proposed a new species, *U. brittini*, based on morphology and apparent habitat preference. Morales noted that where collectors had recorded the region of the host plant from which type specimens had been collected, those conforming to her new *U. brittini* were found exclusively on trunks, whereas *U. assimile* was noted to occur mainly on branches. However, given that scale insects can disperse freely through forests (Chew 2003) and the habitats on trunks and branches appear to be qualitatively similar, it seems unlikely that trunks and branches would support populations of completely different species. Furthermore, both *U. assimile* and *U. brittini* have been recorded from at least three common host species of southern beech: red (*Nothofagus fusca*), black (*N. solandri*) and silver (*N. menziesii*).

We tested Morales’ (1991) hypothesis for trunk/branch specialisation by different species and also tested whether there was any genetic evidence to support the existence of two species of sooty beech scale insect in southern beech forests. A better understanding of the taxonomy and basic biology of these species is necessary for effective conservation and management of these species. Given the uncertain taxonomy of sooty beech scale insects and the difficulty with species identification based on morphological traits, we used a molecular genetic approach to investigate this. We used the c. 650 base pair DNA ‘barcode’ region of the mitochondrial gene cytochrome c oxidase I (COI) because of its high success rate in distinguishing species (Hebert et al. 2003; Hogg & Hebert 2004; Barrett & Hebert 2005; Ball & Armstrong 2006) and in flagging potentially new and morphologically cryptic species (Hebert et al. 2004). DNA barcoding (using short DNA sequences as species identification tags) offers a promising approach for identifying taxa for which morphology-based identification is problematic or impossible, as is the case for many invertebrate taxa, due to phenotypic plasticity, a lack of reliable diagnostic morphological characters, and a lack of availability of taxonomic keys for immature life stages.

If the hypothesis of different species inhabiting trunks and branches is supported, then we would expect to see substantial genetic divergence between specimens obtained from each microhabitat. Specimens collected from tree trunks should form one cohesive group while specimens collected from branches should form a separate cohesive group, with the genetic distances between the two groups being higher than that within groups.

2. Methods

Individual scale insects were collected from three different localities in the South Island, New Zealand: Mount Grey/Maukatere, North Canterbury, 9 November 2004; the ‘Loop Track’ along Lake Rotoiti, St. Arnaud, Nelson (N 59°32’800”, E 24°97’900”; elevation c.670 m), 12 November 2004; and Shellback Road (N 58°73’500”, E 23°87’300”; elevation 70 m) and Moonlight Road (N 58°74’200”, E 23°84’800”; elevation 90 m) at Greymouth, 16 November 2004. Scale insects were removed from the bark of tree branches and trunks and placed in 100% ethanol immediately.

DNA was extracted from specimens using the commercial kit, prepGEM (ZyGEM Ltd, Hamilton, New Zealand). A small amount of tissue (1–2 mm³) was removed from around the anal tube of each individual, to recover muscle tissue, which is rich in mitochondria. The tissue from each individual was added to 40 µl of prepGEM buffer + 1 µl prepGEM enzyme and incubated in thermocycler at 75°C for 15 min, followed by 5 min at 99°C. Between 0.7 µl and 1.0 µl of the DNA extract was used in the PCR. Details of the PCR are given in Ball & Armstrong (2006). PCR products were visualised on a 1% agar gel stained with SYBR safe™ DNA gel stain (Molecular Probes, Eugene, Oregon, USA). They were then cycle-sequenced with the forward (Folmer A) primer using methods described in Ball & Armstrong (2006). Unincorporated dyes were removed using the CleanSEQ® Dye-Terminator Removal kit (Agencourt Bioscience Corporation, Beverly, MA, USA) and cleaned products were sequenced on a 3100-Avant Genetic Analyzer (Applied Biosystems) automated sequencer. In total, 41 specimens were sequenced. The number of sequences obtained for each population and for each beech species within populations is given in Table 1. Several sequences from Family Pseudococcidae were added to the dataset to provide some taxonomic context to our sooty beech scale data. Pseudococcid sequences were generated at the Bio-protection Centre (Lincoln University) or were obtained from Genbank.

DNA sequences were aligned using Sequencher™ 4.2 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Sequence alignment was straightforward because of the absence of insertions or deletions. The alignment was pruned to 611 base pairs prior to analysis. We then created a neighbour-joining (NJ) tree based on Kimura-2-Parameter (K2P) distances (Nei & Kumar 2000), using MEGA v.3. Bootstrap analysis of the NJ tree was performed, using 1000 bootstrap replicates.

TABLE 1. NUMBER OF COI SEQUENCES OBTAINED FROM EACH POPULATION, FOR EACH BEECH (*Nothofagus*) SPECIES WITHIN EACH POPULATION AND FROM EACH LOCATION ON THE TREE.

POPULATION	BEECH SPECIES				TOTAL
	BLACK (<i>N. solandri</i>)		RED (<i>N. fusca</i>)		
	TRUNK	BRANCH	TRUNK	BRANCH	
Mount Grey/Maukatere	5	2	6	2	15
Greymouth	4	4	2	4	14
Nelson Lakes	1	0	1	3	5
Total	10	6	9	9	34

3. Results

Sequences from individuals collected from trunks and branches were intermingled, showing that there were no genetic differences between them (Fig. 1A). Instead, the NJ tree showed that COI sequences formed two major groups (I and II), which showed very large genetic divergence (12.8%) but did not correspond to tree microhabitat. Within each of these two groups, there were subgroups, which showed much smaller divergences between them (Table 2). Bootstrap support for these groupings was generally high (Fig. 1A). Within Species Group I, Mount Grey specimens formed a cohesive subgroup (A) in which all individuals were genetically identical. These included individuals collected from both red and black beech, and from trunks and branches. Specimens from Nelson Lakes and Greymouth formed a distinct subgroup (B), which was separated from group A by 0.7% sequence divergence. Mean COI divergence within group B was extremely small (0.1%). Species Group II contained three subgroups. Subgroup C was represented by a single sequence from Greymouth, which showed small divergence from subgroups D and E (Table 2). Subgroup D contained three sequences, which showed a mean sequence divergence of 0.1% and contained specimens collected in Nelson Lakes and Greymouth. Subgroup E consisted of specimens collected solely from black beech in Greymouth, all of which were genetically identical.

To provide some taxonomic context to our study, we also included sequences from several different genera and species of mealybugs (family Pseudococcidae) (Fig. 1B). Although they are in a different family from the sooty beech scale insects, they at least provide a very rough estimate of mean interspecific COI divergences within superfamily Coccoidea. Inclusion of nine mealybug species showed that mean COI divergence among species was 12.4% (range = 2.3%-17.1%). This mean interspecific divergence is very similar to the divergence found between our Groups I and II (12.8%), suggesting that these groups probably represent different species. Mealybugs showed a mean intraspecific divergence of 0.6%. This is similar to the mean divergences within each of the scale insect Groups I (0.3%) and II (0.2%).

TABLE 2. MEAN SEQUENCE DIVERGENCE BETWEEN THE SUBGROUPS SHOWN IN FIG. 1A, BASED ON K2P DISTANCES.

SUBGROUPS	A	B	C	D	E
A	-	0.7	12.7	12.9	12.9
B		-	12.5	12.7	12.7
C			-	0.5	0.2
D				-	0.4
E					-

4. Discussion

Our results did not support Morales' (1991) hypothesis that different species of sooty beech scale insect occupy separate trunk and branch microhabitats on southern beech trees. For all populations examined, specimens from trunks and branches were intermingled in the same groupings, showing that they were not genetically distinct. This result is not surprising, as sooty beech scale insects have a mobile 'crawler' stage and adult males are winged and able to disperse (Morales 1991).

The individuals examined did fall into two separate groups, however. These showed large COI divergence (12.8%), which is indicative of different insect species (Hebert et al. 2003; Hogg & Hebert 2004; Ball et al. 2005; Barrett & Hebert 2005). Although we do not have data on mean interspecific COI divergences for other Margarodid species, we did include a small number of Pseudococcidae sequences to provide at least some taxonomic context within the Coccoidea. These additional specimens, which spanned five genera and nine species, showed a mean interspecific divergence of 12.4%, which is very similar to the COI divergence between Groups I and II. This strongly suggests that these groups provisionally represent two different species, although more COI data on interspecific COI divergences in the Margarodidae is needed to validate this result. Similarly, reference sequences from both *U. assimile* and *U. brittini* are needed to determine whether the two species in this study correspond to these species or represent new, undescribed species. At MountGrey/Maukatere, only one species was found; however, at both Greymouth and Nelson Lakes two sympatric species were present. Although both species occurred on red and black beech, the data suggest the possibility of some host specialisation for Species Group II, as specimens from the Greymouth population found on black beech formed a distinct group, which showed small, but consistent genetic divergence from the Greymouth and Nelson specimens found on red beech. This tendency toward host specialisation occurred only with Species Group II specimens. In Species Group I, there was no genetic differentiation between specimens found on either black or red beech. This hypothesis of possible host specialisation could not be tested for the Nelson Lakes population, as sequence data were obtained from only one specimen on black beech. Further sampling is needed to rigorously test this hypothesis of possible host specialisation in Species Group II.

Two to three subgroups occurred within each major (species) group. These subgroups had high bootstrap support, but the COI divergences were small (0.2%–0.7%) and do not suggest the existence of different subspecies within each group (Hebert et al. 2003; Ball & Armstrong 2006). However, this does suggest the possibility of limited gene flow. For example, all Mount Grey specimens formed a single cohesive subgroup that was 0.7% divergent from the subgroup containing Nelson Lakes and Greymouth specimens. Similarly, specimens from Greymouth that were collected from black beech formed a cohesive subgroup showing small (0.2%–0.6%) divergence from specimens

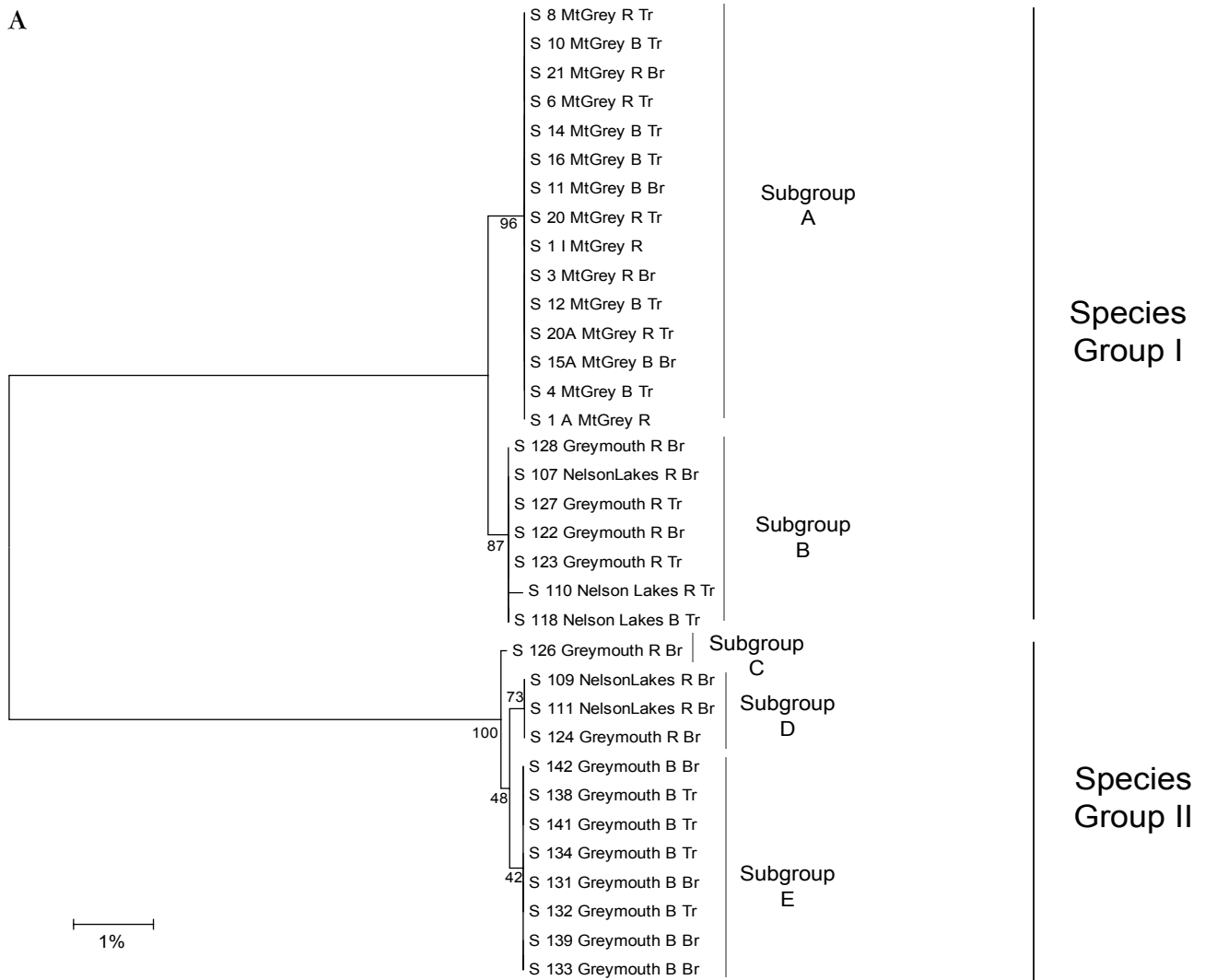
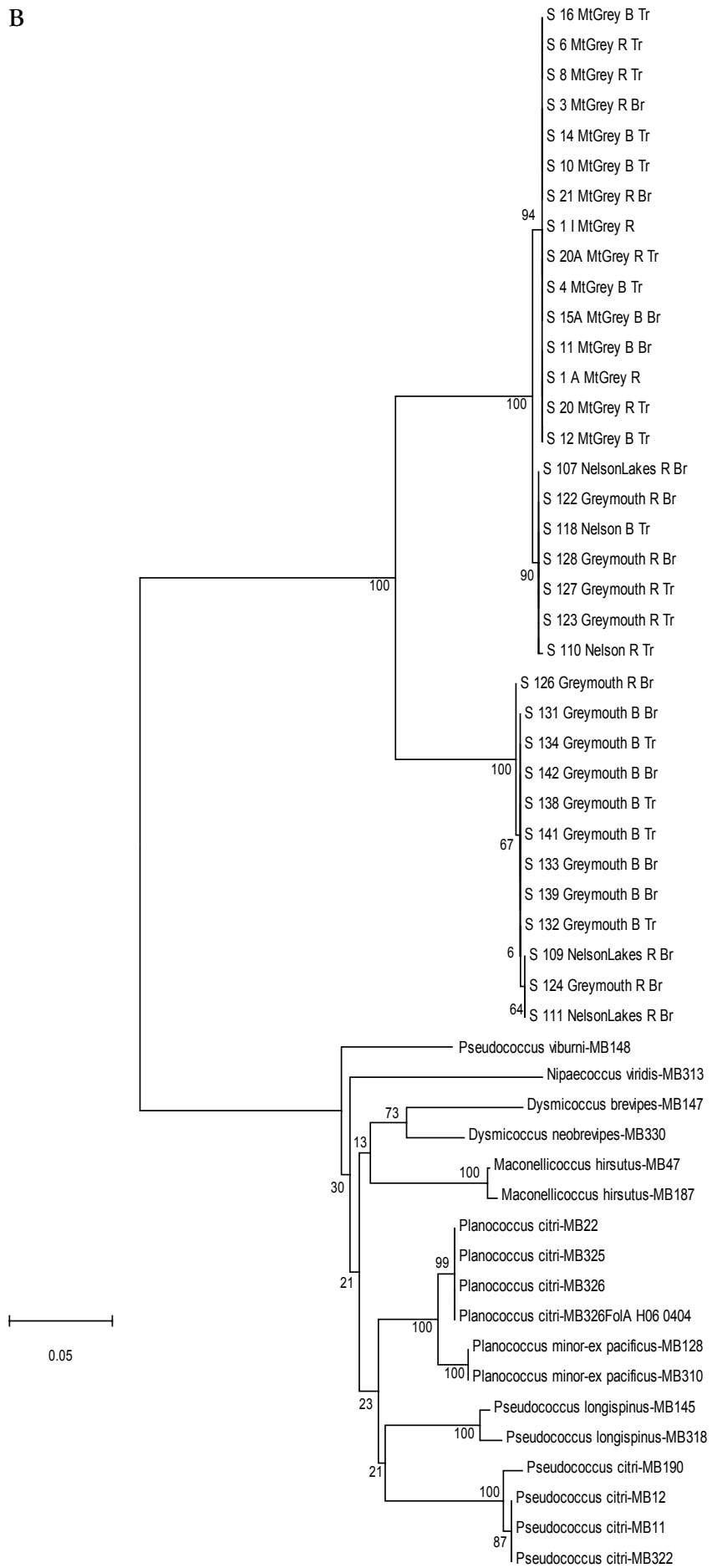


Figure 1. COI neighbour-joining (NJ) tree of sooty beech scale insects A. excluding mealybugs, and B. including mealybugs. NJ tree based on K2P distances of sooty beech scale insects collected from three South Island populations (Mount Grey/Maukatere, Greymouth and Nelson Lakes) on two different species of beech (*Nothofagus*) tree (B: black *N. solandri*; and R: red *N. fusca*) and in two different localities on the tree (Tr: trunk; and B: branch). Numerical values are bootstrap support, based on 1000 replicates. Scale bar shows % sequence divergence.

B



in the other two subgroups, although bootstrap support for this subgroup was low.

Our data clearly indicate the presence of two distinct species. However, further work is needed to determine whether these two species represent *U. assimile* and *U. brittini* or whether one or both represents a previously undescribed species. Although morphological examination of specimens is currently underway (Rosa Henderson, Landcare Research, pers. comm.), morphological identification of these species is extremely difficult. Reference sequences from *U. assimile* and *U. brittini* specimens of unambiguous identity need to be included to place the sequences obtained in our study in a valid taxonomic context. Therefore, subsequent work will focus on sequencing and identifying specimens from the type locality (Maruia) of *U. assimile*. In addition, further geographic sampling of sooty beach scale insects is planned to investigate the distribution of the two species.

The development of DNA barcoding offers an approach that complements the traditional morphology-based approach to species identification and discrimination. This is particularly important for invertebrates, for which the morphology-based approach, although clearly fundamental, is subject to several limitations. Firstly, significant variation in taxonomically important traits, whether environmentally or genetically controlled, can make accurate identification difficult or impossible. Secondly, some life stages (e.g. eggs and young larvae/nymphs) lack any distinguishing features, meaning that a morphology-based approach to species identification will not work. Thirdly, even where taxonomically important traits are reliable, dichotomous keys are often difficult for non-experts to use. Finally, taxonomic keys often exist only for certain life stages (adults, pupae, late instar larvae), making identification of eggs and young larvae impossible. DNA-based identification methods can overcome these limitations and provide a reliable means of identification when morphological identification is difficult or impossible.

The success of DNA-based species identification and discrimination depends fundamentally on the existence of taxonomically diverse DNA sequence databases that also include samples from throughout the species' range. Development of such databases is currently underway (e.g. The Barcoding of Life Database (BoLD), Guelph University, Canada). A global initiative to develop this for scale insects is also underway under the auspices of the Consortium for the Barcoding of Life (CBOL). It is not intended that DNA-based species discrimination will supplant traditional morphological taxonomy; instead, it will complement it. Recently, 'integrative taxonomy', which combines complementary approaches such as morphology, genetics, ecology, behaviour, development and phylogeography, has been suggested as a new approach to taxonomy (Dayrat 2005). This presents a holistic and powerful approach to species discrimination and a new approach to taxonomy in the 21st century.

5. Conclusions and recommendations

Our data support the existence of two distinct species. However, these species do not correspond to branch and trunk microhabitats on southern beech trees. The authors make the following recommendations for further research:

- The specimens sequenced in this study should be analysed morphologically, to determine whether species names can be associated with individual sequences. Alternatively, unambiguously identified specimens of *U. assimile* and *U. brittini* should be included as reference sequences for these species, to assess the genetic similarity between these species and the species in our study.
- Further geographic sampling of sooty beech scale insects should be carried out to determine the distribution and extent of sympatry of the two species detected in this study. This should include increasing the sample sizes of specimens collected from the two host species, red and black beech.

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