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The Megadiverse Australian Ant Genus *Melophorus*: Using CO1 Barcoding to Assess Species Richness

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Abstract: *Melophorus* is an exceptionally diverse ant genus from arid Australia that has received little taxonomic attention, such that just a fraction of its remarkable number of species is described. The Commonwealth Scientific and Industrial Research Organization's Tropical Ecosystems Research Centre (TERC) in Darwin holds by far the most extensive collection of *Melophorus*, and as of September 2016 this comprised >850 sorted morphospecies. However, the reliability of such morphospecies is open to question because species delimitation is extremely challenging due to highly generalized morphology and worker polymorphism. Here we use CO1 barcoding of 401 *Melophorus* specimens from 188 morphospecies in the TERC collection to determine the reliability of morphologically-based species delimitations as a basis for assessing true diversity within the genus. Our CO1 data confirm the extremely challenging nature of morphologically-based species delimitation within *Melophorus*, and suggest substantially higher diversity than that indicated by morphospecies. We found many cases where combinations of high (>10%) CO1 divergence, polyphyly, sympatric association, and morphological differentiation indicated that single morphospecies represented multiple lineages. Overall, our analysis indicates that the 188 morphospecies barcoded represent at least 225 independent CO1 lineages. We discuss these results in terms of both their limitations and implications for estimating the total number of species in this exceptionally diverse, arid-adapted ant genus.

Keywords: ant diversity; biological species; morphospecies; species delimitation; sympatric association

1. Introduction

Melophorus Lubbock 1883 is an exceptionally diverse ant genus endemic to Australia, occurring primarily in arid and semi-arid regions. Despite it being a dominant component of inland ant faunas throughout the continent [1,2], it has attracted little research attention from either ecologists or taxonomists. Ecological studies have focussed on a single species, the "honeypot" ant *M. bagoti* Lubbock, 1883 [3], documenting its extreme thermophilia [4,5] and other behavioural aspects [6–9]. A small number of other species have also been subject to behavioural studies [10–12].

As highly thermophilic, polymorphic formicines, many species of *Melophorus* are strongly convergent both morphologically and behaviourally with *Myrmecocystus* in North American deserts [13] and *Cataglyphis* in northern Africa, southern Europe, and the Middle East [14]. However, the generally conservative morphology of *Melophorus* belies its extremely diverse biology. Although most species are generalist predators and scavengers, the genus includes specialist predators of ant brood (from the *M. fulvihirtus* Clark, 1941 and *M. anderseni* Agosti, 1997 species groups [15,16]) and

specialist granivores (species of the *M. wheeleri* Forel, 1910 species group), an extremely unusual condition in formicines [17].

Only about 30 species of *Melophorus* have been described (Shattuck 1999), almost all prior to 1950 and involving many different authors, but this represents just a small fraction of even the known fauna [18]. For example, 45 *Melophorus* species were recorded from near Mt Isa in northwesten Queensland [19], 28 species from Purnululu National Park in northern Western Australia [20], 30 species from Uluru National Park in Central Australia [21], and 41 species from the Great Western Woodlands of southern Western Australia [22], with little overlap among these local faunas.

It has recently been suggested that *Melophorus* is likely to contain well over 1000 species, based on holdings in the Commonwealth Scientific and Industrial Research Organization's Tropical Ecosystems Research Centre (TERC) in Darwin where the >10,000 pinned *Melophorus* specimens have been sorted into >850 morphospecies [23]. This would make *Melophorus* one of the world's most-species rich ant genera, and by far the richest with such a limited distribution. However, the generalized morphology of most *Melophorus* species makes species delimitation extremely challenging, and so the reliability of the morphospecies sorting in the TERC collection requires validation. Integrated morphological, behavioural, and genetic analyses have consistently validated the sorting of morphospecies in the TERC collection from other highly diverse and taxonomically challenging Australian genera such as *Monomorium* [24–26] and *Iridomyrmex* [27]. Indeed, CO1 data from specimens from the *M. aeneovirens* group suggest that the sorting of *Melophorus* morphospecies in the TERC collection is in fact highly conservative [23].

In this paper, we use CO1 barcoding of hundreds of *Melophorus* specimens representing almost all known species groups to assess the reliability of morphologically based species delimitations in the TERC collection, as a basis for gauging true diversity within the genus. Ideally, other genes would also be included in addition to CO1 in order to provide more-definitive results. However, there is a trade-off between number of genes and number of samples that can be feasibly analyzed, and we wished to maximize the number of samples. We also provide images of representatives of all common species groups in order to illustrate morphological diversity within the genus.

2. Materials and Methods

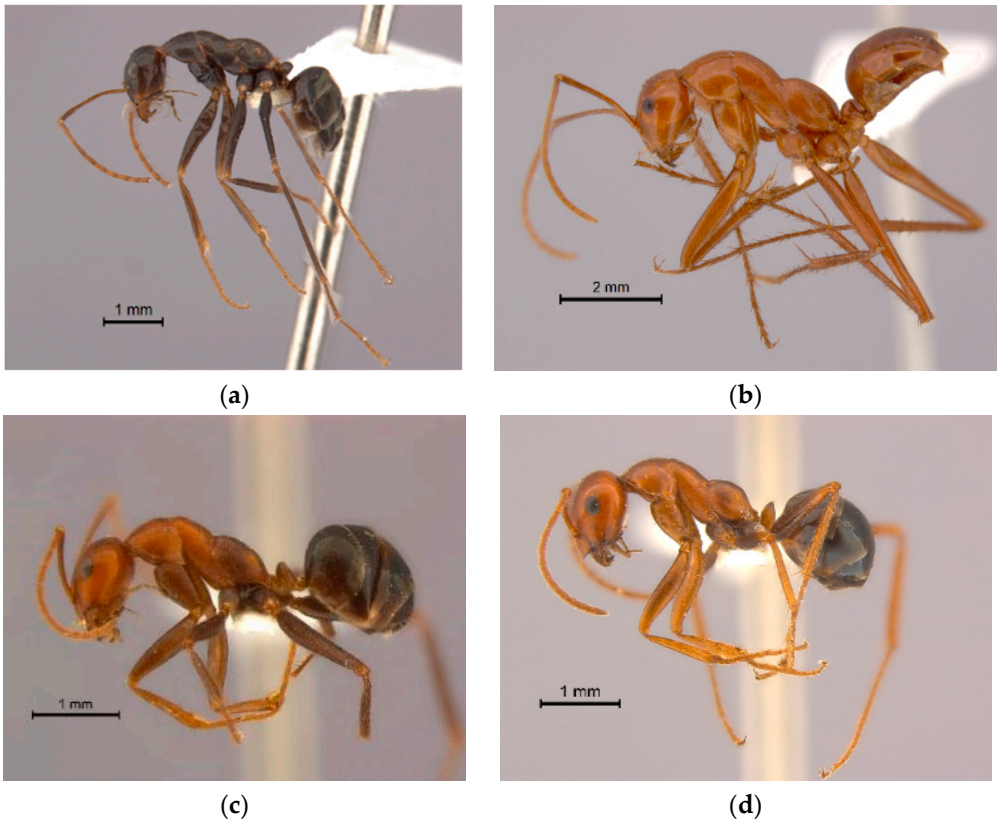
In 2015, 401 specimens representing 188 morphospecies from the TERC collection (Table S1) were sequenced for the CO1-barcoding region on the basis of DNA extraction and sequencing from either legs (larger species) or whole specimens (smaller species) through the BOLD Barcode of Life Data System (see [28] for extraction details). These specimens represent nearly all known species groups of *Melophorus*. Specimens of the melophorine genera *Notoncus* Emery, 1895 (single species) and *Prolasius* Forel, 1892 (13 species), along with a specimen of *Teratomyrmex greavesii* McAreavey, 1957, which the senior author suspected belongs to *Prolasius*, were similarly barcoded for use as outgroups (Table S2). A CO1 sequence of a species of the melophorine genus *Myrmecorhyncus* obtained from Genbank (DQ353336) was also incorporated into the analysis.

The DNA sequences were trimmed to 612 bases using Bioedit 7.0.9 [29] and translated into proteins using MEGA v.5 [30] to check for the presence of nuclear paralogues. PartitionFinder V1.1.1 [31] was used to determine the best evolutionary model partitioning scheme for codon positions 1, 2, and 3 using the Bayesian Information Criterion (BIC) and the "greedy" algorithm. Bayesian analysis was performed for 20 million generations, sampling every 1000 generations using MrBayes (3.1.2) through the CIPRES Science Gateway V. 3.1 [32]. Stationarity was assumed to have been reached once the standard deviation of split frequencies fell below 0.01. Additionally, TRACER 1.4 [33] was used to check for chain convergence and the first 25% of trees were discarded as burn-in. Genetic Distances were calculated in MEGA v.5 using the Kimura-2 parameter model.

There is no arbitrary level of CO1 divergence that can be used to define species; however, the level of variation within an ant species is typically 1%–3% [34], although it can be substantially higher [35]. We based our assessments of species delimitation on a combination of genetic divergence, morphological differentiation, phylogenetic structure, and sympatric association.

3. Results

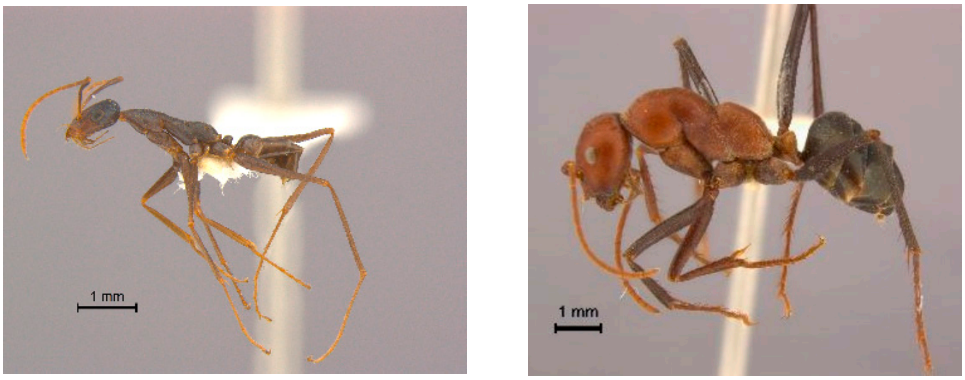
Our CO1 tree shows all *Melophorus* specimens forming a well-supported (PP = 0.95) clade (Figure S1). *Teratomyrmex greavesii* is embedded within the *Prolasius* clade. Within *Melophorus*, the CO1 tree shows a primary division (PP = 1) between species from what is described as the *M. aeneovirens* radiation by [18], including the *M. bagoti* group (Figure 1a,b), the *M. froggatti* group (Figure 1c), Group A (Figure 1d), Group O (Figure 1e) and the *M. aeneovirens* gp. (Figure 1f), and all other species (Figure 2). This second branch of the primary division, containing all species groups outside the *M. aeneovirens* radiation, is very poorly resolved with low branch support at the base (PP = 0.59). However, specimens from the *M. potteri* group form a well-defined clade (PP = 1) within it, and the two complexes recognized by [18] are recovered as well-supported (PP = 1) sub-clades. One of these is characterised by a projecting clypeus and dentate mandibles (specimens 490, 721 and 723; complex 1, Figure 3a), and in the other the clypeus is not projecting and the mandibles are massive and edentate except for a large apical tooth (specimens 064 and 722; complex 2, Figure 3b). In both these complexes the mandibular and maxillary palps are highly vestigial. There is a third complex within the group that was not sequenced, known from two morphospecies from the central and southern arid zones. These have a typical mesosoma for the *M. potteri* group, and the head similarly lacks dorsal setae, but neither the clypeus nor mandibles are modified, and the palps are relatively short but not vestigial (complex 3, Figure 3c). Interestingly, the *M. potteri* group is shown as containing an additional clade (complex 4; specimens 066—068), consisting of a recently discovered species from southwestern Western Australia known only from reproductives that have a unique, horseshoe-shaped petiolar node (Figure 3d). An absence of workers and the peculiar morphology of the female (Figure 3d) suggest that this species is a social parasite. The CO1 tree recovered none of the *M. fulvihirtus* (Figure 4), *M. ludius* (Figure 5), *M. wheeleri* (Figure 6) or *M. fieldi* (Figure 7) radiations of [18], or many of their component species groups (Figure 2).



(**a**) (**b**)

(**c**) (**d**)

Figure 1. *Cont.*



(**e**) (**f**)

Figure 1. Representatives of the *M. aeneovirens* radiation. (**a**) *M. iridescens*; (**b**) *M. bagoti*; (**c**) member of the *M. froggatti* group; (**d**) member of Group A; (**e**) member of Group O; (**f**) member of the *M. aeneovirens* group. All specimens are minor workers.

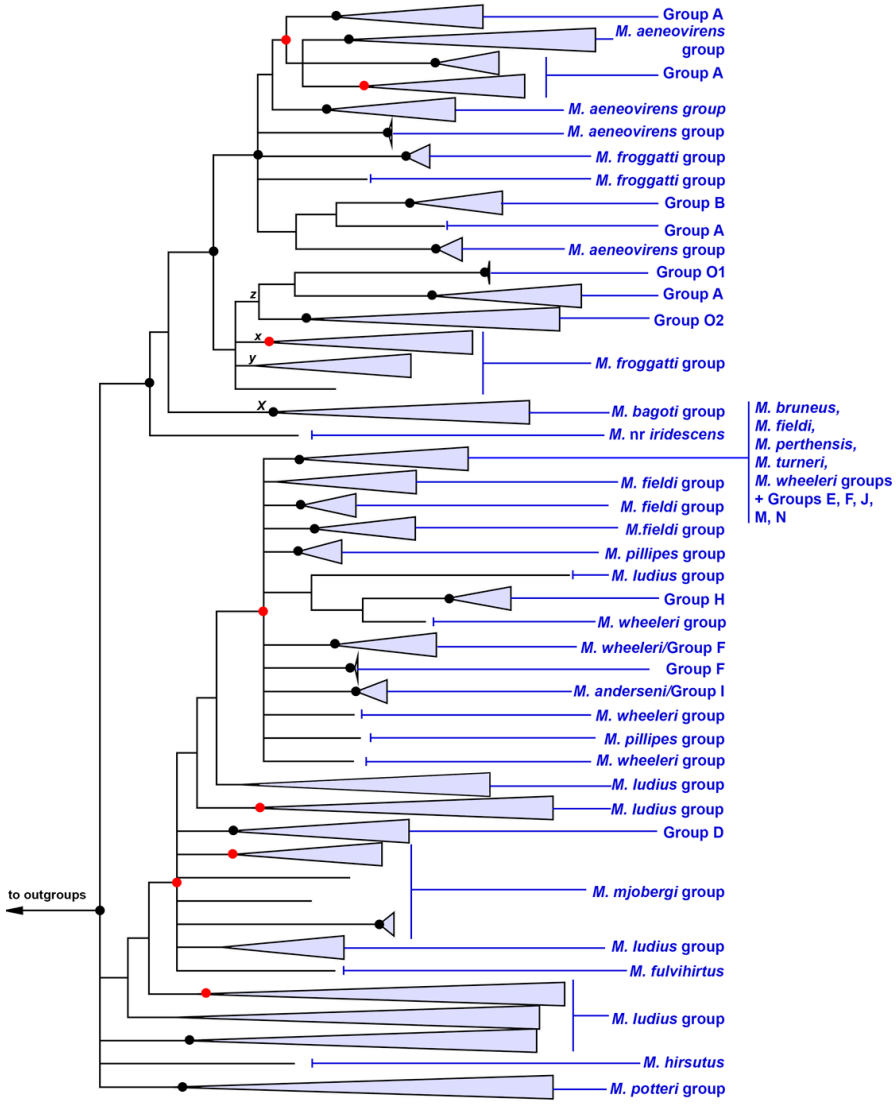


Figure 2. Bayesian 50 percent majority rule summary tree. The tree shows the overall clade structure for the 401 sequenced specimens of *Melophorus*. Nodes with ≥95 and ≥70 Bayesian posterior probabilities are indicated by black and red circles, respectively. The full tree is provided in Figure S1.

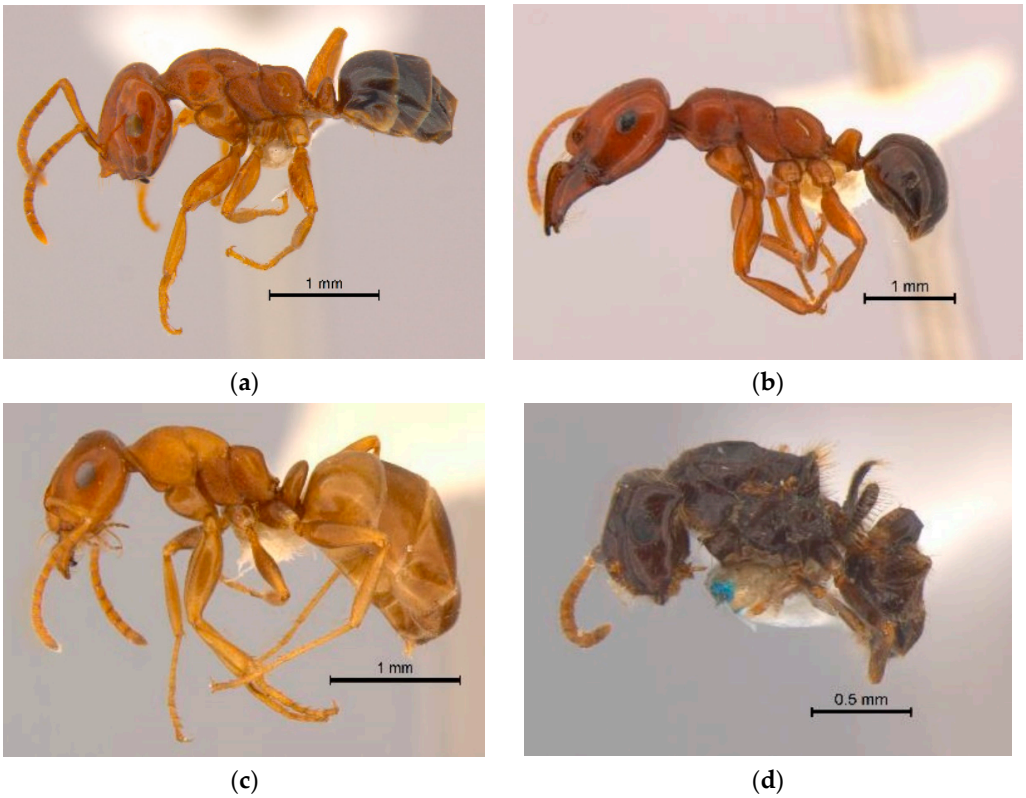


Figure 3. Representatives of the *M. potteri* group. (**a**) Member of complex 1; (**b**) member of complex 2; (**c**) member of complex 3; and (**d**) member of complex 4.

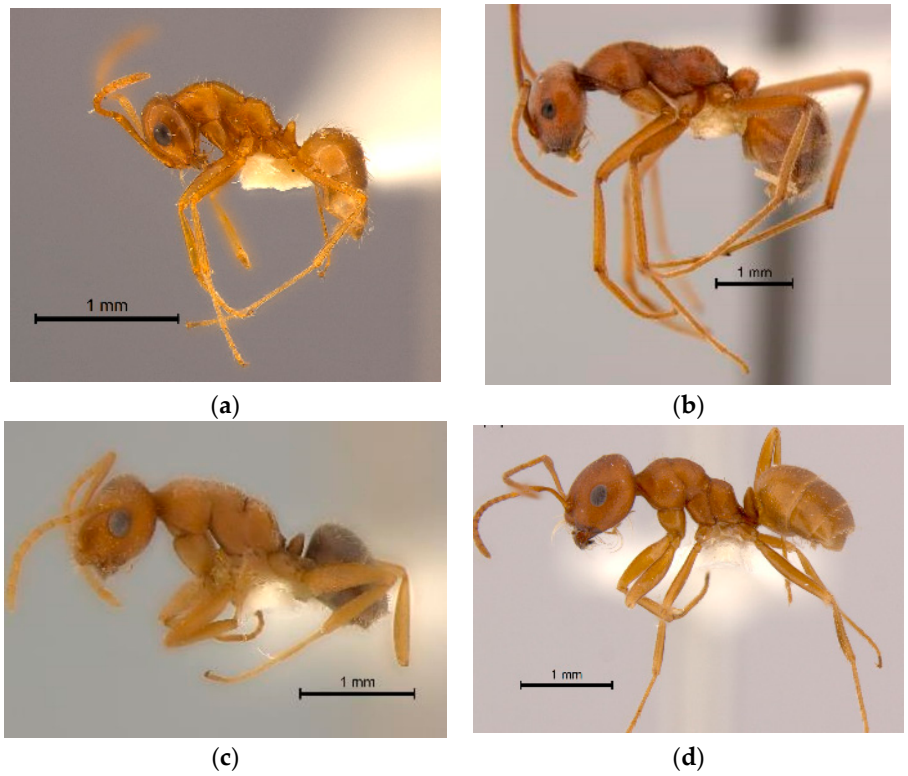


Figure 4. Representatives of the *M. fulvihirtus* radiation. (**a**) Member of the *M.* Group I; (**b**) *M. anderseni*; (**c**) *M. fulvihirtus*; and (**d**) member of *Melophorus* Group G.

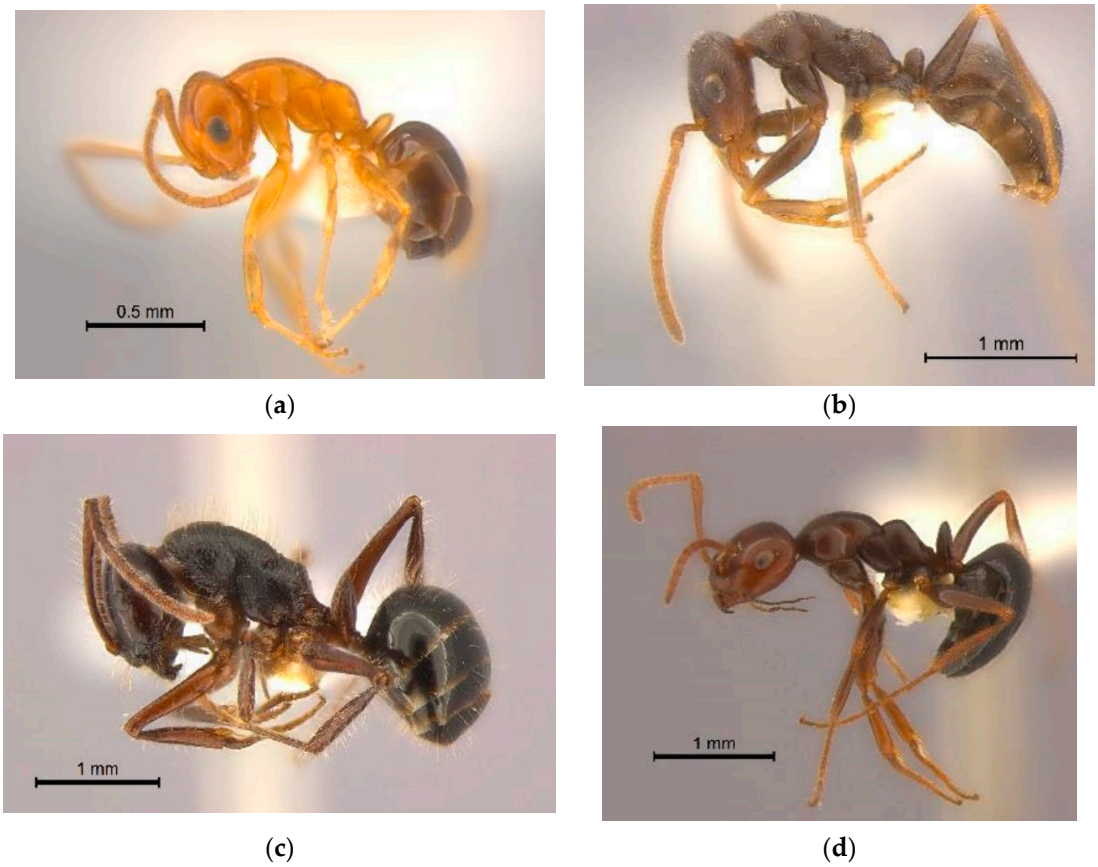


Figure 5. Representatives of the *M. ludius* radiation. (**a**) *M. ludius*; (**b**) *M. mjobergi*; (**c**) *M. hirsutus*; and (**d**) member of *Melophorus* Group D.

Figure 6. Representatives of the *M. wheeleri* radiation. (**a**) Member of *Melophorus* Group H; (**b**) Member of the *M. wheeleri* group.

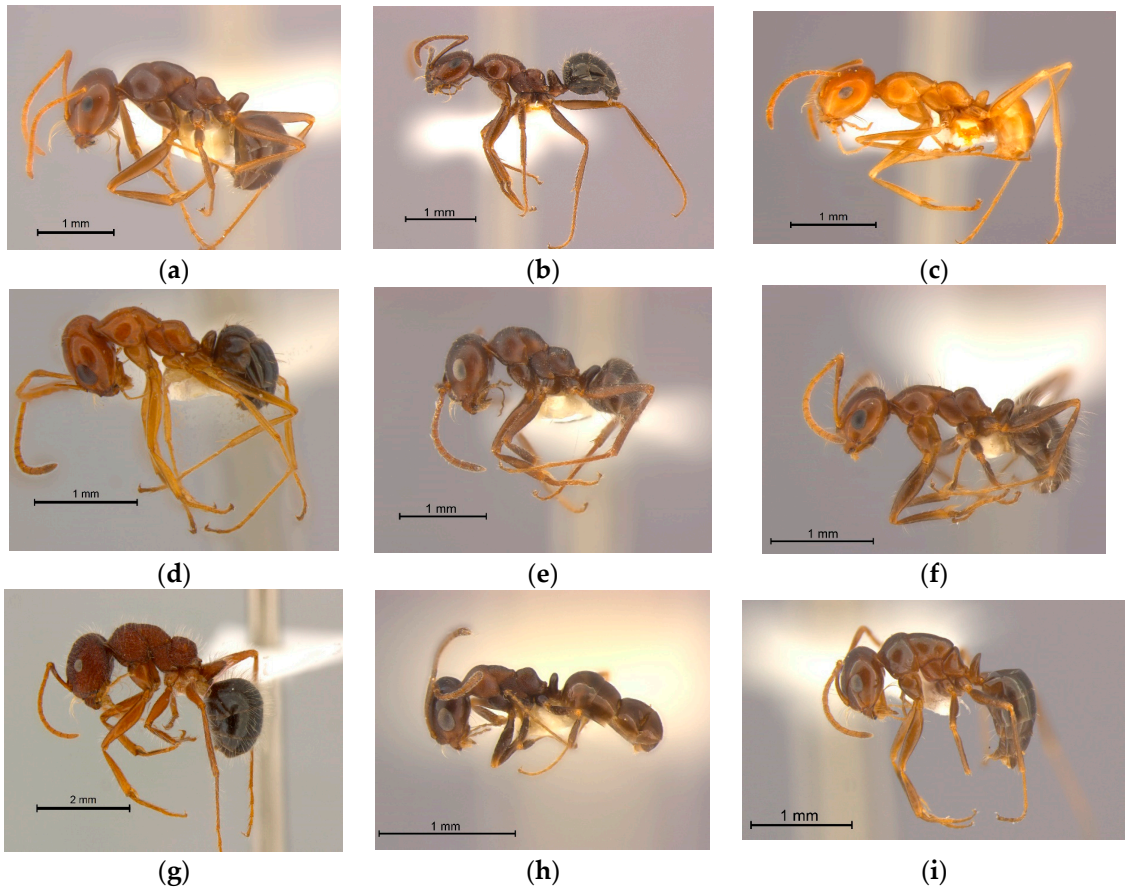


Figure 7. Representatives of the *M. fieldi* radiation. (**a**) Member of the *M. perthensis* group; (**b**) member of *Melophorus* Group J; (**c**) member of the *M. fieldi* group; (**d**) member of the *M. turneri* group; (**e**) member of the *M. bruneus* group; (**f**) member of the *M. pillipes* group; (**g**) member of *Melophorus* Group E; (**h**) member of *Melophorus* Group M; and (**i**) member of *Melophorus* Group N.

The CO1 tree revealed six cases where specimens sorted as two morphospecies are likely to be conspecific: sp. O (specimen 195) and GWW sp. Y (889 and 890) within the *M. turneri* gp.; GWW sp. CE (949) and GWW sp. BC (257) within the *M. fieldi* gp.; GWW sp. C (256) and sp. Eurardy 25 (259) within the *M. fieldi* gp.; NATT sp. AN (573) and NATT sp. AF (572) within Group A; sp. P (612) and sp. TREND AO (615) within Group B; and Eurardy sp. BB (074, 075) and Eurardy sp. M (076 and 077) within Group A (Figure S1). However, there were many more cases where a morphospecies was resolved as polyphyletic with high (>10%) genetic divergence, and often in sympatry.

The apparent occurrence of multiple independent lineages within a morphospecies was particularly prevalent in the *M. fieldi* group, which has especially conservative morphology. One example (*Melophorus* sp. 30) is a very gracile, glabrous, pale yellow morphospecies with a two-toned (paler anteriorly) head that occurs throughout arid and semi-arid Australia (Figure 6c). The CO1 data show that mean genetic distance among sequences in this morphospecies is 8.4% with a maximum distance of 18.8%, and indicate that it is highly polyphyletic; the nine specimens analysed are shown as representing six lineages (sp. 30 A–F in Figure 8), four of which occur at a single locality (Eurardy Station, WA; shown as sw WA in Figure 8). Morphological variation among the taxa is evident upon close re-inspection. For example, among the four species co-occurring at Eurardy Station, in one (sp. 30-A) the posterior face of the petiolar node is concave rather than straight in profile, another (sp. 30-D) has larger eyes, and another (sp. 30-E) has smaller overall body size. Another example is *Melophorus* sp. 82, a sparsely hairy, brownish morphospecies that also occurs throughout arid and semi-arid Australia; the CO1 data show the 11 analyzed specimens as occurring in at least six

polyphyletic lineages (sp. 82 A–F in Figure 8). Again, careful re-inspection of the specimens reveals previously undetected morphological variation relating to pilosity, relative scape length, and head shape. *Melophorus* sp. 11, occurring throughout the Top End of the Northern Territory, is shown as representing at least four lineages from four separate clades (sp. 11 A–D in Figure 8). Once again, close re-inspection of the specimens reveals a range of previously undetected morphological variation among the taxa.

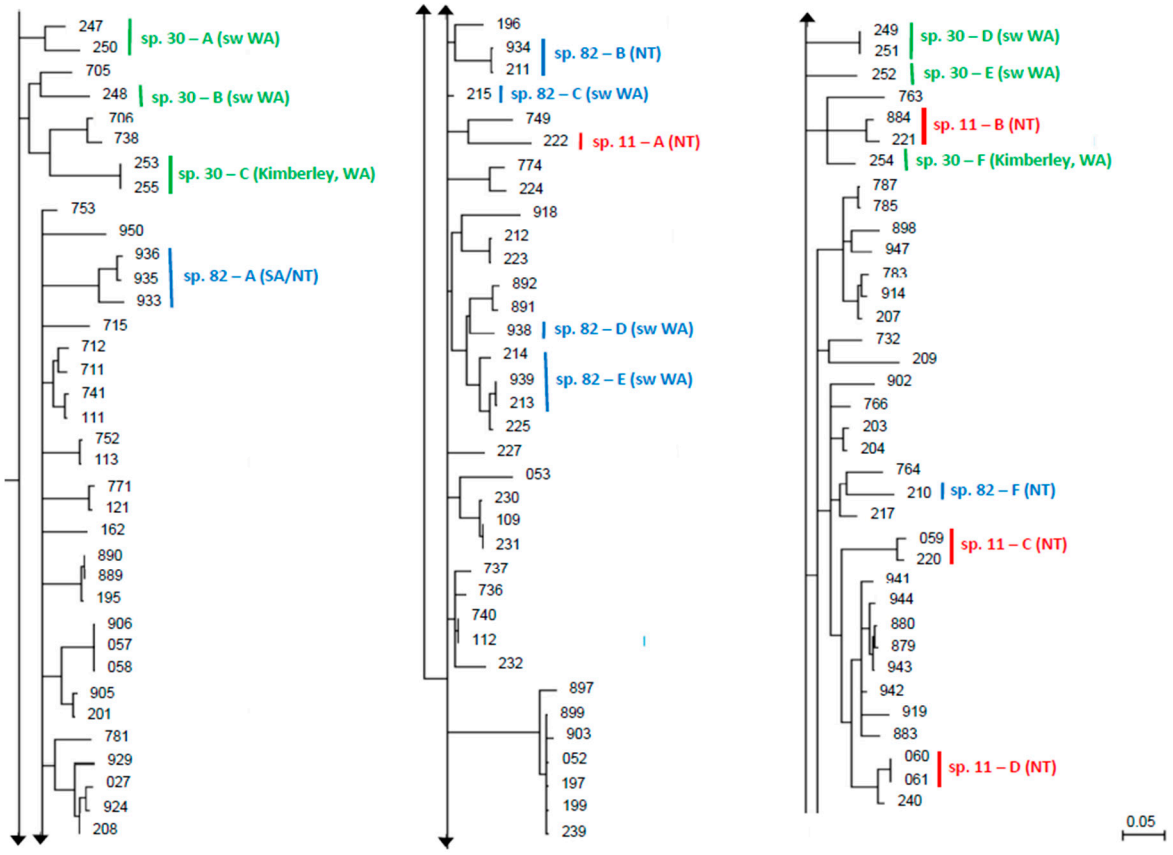


Figure 8. Extract from the full CO1 tree (Figure S1)—I. The tree indicates that sp. 11 (red), sp. 30 (green), and sp. 82 (blue) from the *M. fieldi* group each represent several species, as indicated by different letters. NT = Northern Territory; SA = South Australia; WA = Western Australia.

Species of the *M. ludius* group are the smallest (ca. 1.5 mm total length) within the genus, and also have very highly generalized (plagiolepidine-like) morphology (Figure 5a); it is therefore not surprising that there were several cases where CO1 data indicated multiple taxa within a morphospecies. For example, specimens identified as *Melophorus* sp. AJ from Eurardy Station in Western Australia occur in three clades at that locality, showing a mean K2P distance of 11% (Figure 9). Morphometric variation among the taxa, especially relating to head shape, is evident upon re-inspection of the specimens. Specimens from the related Group D sorted as a single morphospecies occurring in high rainfall areas of northwestern Australia are indicated as representing four species (Figure 10). *Melophorus bagoti* shows very high (up to 21%) CO1 variation that is not related to proximity between samples and is therefore suggestive of a species complex (Figure S1). Overall, the CO1 data from the 188 barcoded morphospecies are indicated as representing at least 225 species.

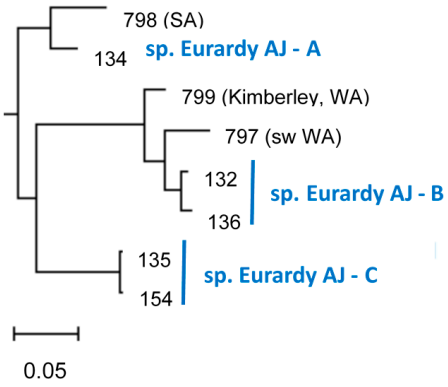


Figure 9. Extract from the full CO1 tree (Figure S1)—II. The clade represents species from the *M. ludius* group with reddish head and mesosoma and contrasting black gaster. Specimens sorted as *Melophorus* Eurardy sp. AJ (*ludius* gp.) appear to represent three species (A–C), all from Eurardy Station in Western Australia. SA = South Australia; WA = Western Australia.

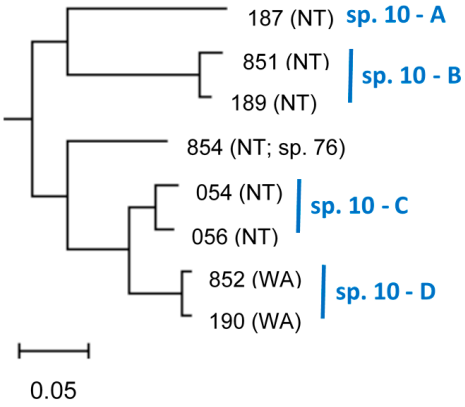


Figure 10. Extract from the full CO1 tree (Figure S1)—III. The clade represents species from Group D occurring in northwestern Australia. Specimens sorted as *Melophorus* sp. 10 (Group D) likely represent four species (A–D). NT = Northern Territory; WA = Western Australia.

4. Discussion

The CO1 gene is used primarily to inform species boundaries and relationships among closely related species [36], and has limited reliability for informing deeper phylogenetic relationships [37,38]. The deeper clade structure of our CO1 tree, which showed generally poor recovery of the species groups recognised by [18], is therefore not reliable. However, our CO1 tree did recover the *M. aeneovirens* radiation of [18]. It also recovered the highly distinctive *M. potteri* group, and indicated that a newly discovered, apparently parasitic species belongs to it, despite having very different morphology. Given that ant social parasites and their hosts are typically closely related, we assume that this new species is a parasite of other members of the *M. potteri* group.

Our CO1 data strongly support the genetic differentiation of our morphospecies, showing that just a very small number of the 188 morphospecies analysed are likely to be conspecific with other morphospecies. Such "splitting" appeared to be far less of an issue than apparent "lumping", with our CO1 analysis indicating many examples of multiple lineages within single morphospecies. CO1 data are generally reliable in informing species boundaries [36], and commonly reveal that species previously considered variable and widespread are likely to represent multiple species [39,40]. In some cases, CO1 data can provide misleading results due to *Wolbachia*-associated divergence in the mitochondrial genome, incomplete lineage sorting, or introgressive hybridisation [41,42]. CO1 analysis has sometimes provided conflicting data in studies of ant genera, such as *Cataglyphis* [43].

However, we are unaware of any study showing a single ant species to include multiple sympatric and morphologically differentiated clades with very high CO1 divergence, as was often the case in our study for TERC morphospecies.

Analysis of nuclear markers is needed to provide more definitive results, especially where the results from mitochondrial DNA are in conflict with the morphology. However, the widespread pattern of high CO1 divergence, polyphyly, sympatric association, and morphological differentiation within morphospecies as sorted in the TERC collection make it most likely that such sorting has been conservative. It confirms the extremely challenging nature of morphologically-based species delimitation within *Melophorus*, which is to be expected when extreme diversity combines with highly generalized morphology and worker polymorphism. Useful morphometric characters such as relative scape length and head width show allometric variation within a species, and so without full nest series it can be highly problematic to determine whether or not similar taxa from separate collections are conspecific. This is especially the case when assessing specimens with slightly different morphology collected from different locations, where species differentiation needs to be disentangled from both allometric and geographic variation. Such issues have been well-documented in other polymorphic ant genera. For example, in many cases species delimitation is virtually impossible in the absence of major workers in the exceptionally diverse genus *Pheidole* [44]. Even in genera with complex morphology, such as *Atta*, it is often not possible to differentiate species based on minor workers, despite the species being highly divergent phylogenetically [45].

The TERC collection holds >850 *Melophorus* morphospecies. Our CO1 data indicate that the number of actual species in the collection is substantially higher. This is consistent with results from CO1 analyses of specimens of *Monomorium* from the TERC collection [23,24,26], as well as analyses of CO1 data from >1000 sequenced specimens from the TERC collection belonging to other diverse genera such as *Iridomyrmex*, *Camponotus*, *Rhytidoponera*, and *Tetramorium* (A. Andersen, unpublished data). Given that vast areas of inland Australia have never been surveyed for ants, we would not be surprised if the total number of *Melophorus* species is more than 1500. This is truly remarkable diversity for an arid-adapted ant genus confined to a single biogeographic realm. The number of *Melophorus* species is at least twenty times higher than the world's next richest specialist arid-adapted ant genus (*Cataglyphis*). It is also an order of magnitude higher than that for any other ant genus with such a biogeographically limited range. Indeed, *Melophorus* should be viewed as being among the top few of the world's richest ant genera, along with the cosmopolitan *Camponotus* and *Pheidole* that are found in most of the world's terrestrial habitats that support ants.

Many other ant genera are also exceptionally diverse in arid Australia, where *Monomorium*, *Camponotus*, *Pheidole*, *Tetramorium*, *Meranolus*, and *Rhytidoponera* are also all represented by hundreds of species [18,46]. Such remarkable diversity has been attributed to Australia's unique history of aridity and associated patterns of speciation and extinction during the Pleistocene glaciations, when massive movement of sand likely resulted in a highly fragmented biota confined to a very large number of isolated refugia [46].

Supplementary Materials: The following are available online at www.mdpi.com/1424-2818/8/4/30/s1. Figure S1: Fifty percent majority rule Bayesian consensus tree of Melophorus. Black circles indicate posterior probability (PP) > 0.95, green circles indicate PP > 0.7 < 0.95 and red circles indicate PP > 0.5< 0.7. Table S1: List of specimens of Melophorus that were CO1-barcoded for this study. Table S2: List of outgroup specimens that were CO1-barcoded for this study.

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Author Contributions: A.N.A. conceived the study, developed the TERC collection, and wrote the first draft of the manuscript; B.D.H. helped develop the TERC collection, and contributed to the writing of the paper; K.S. undertook the analysis of the CO1 data, and contributed to the writing of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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