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Diversity in the Australian ant genus *Iridomyrmex* MAYR, 1862 (Hymenoptera: Formicidae): a critique of HETERICK & SHATTUCK (2011), with particular reference to *I. coeruleus* HETERICK & SHATTUCK, 2011

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Abstract

The uniquely dominant Australian ant genus *Iridomyrmex* has been estimated to contain about 350 species, but a recent morphologically based revision of the genus (HETERICK & SHATTUCK, 2011) recognises only 79 species. This issue has important implications for an understanding of the biogeography of, and speciation processes within, this arid continent. We aim to show that the revision does not adequately document true morphological variation within the genus, and that many of the recognised taxa are multiple and often distantly related species, with clear morphological differentiation between populations that are often sympatric. We illustrate this by documenting morphological and genetic diversity within a taxon described by HETERICK & SHATTUCK (2011) as a morphologically uniform species, *I. coeruleus*. We show that this "species" represents several clearly differentiated morphotaxa with congruent genetic divergence (up to 12%) based on CO1 analysis. These differences are maintained in sympatry, with three of the taxa recorded from a single locality despite one of them showing very limited morphological and genetic variation throughout its range right across northern Australia. We then discuss published morphometric, genetic and distributional evidence from other *Iridomyrmex* "species" to show that the revision widely under-reports morphological and genetic differentiation and sympatric associations. We argue that the revision therefore gives a misleading picture of true diversity in such an ecologically dominant genus that should be so informative for the biogeography of a continent.

Key words: Ant taxonomy, CO1 sequencing, morphological differentiation, sequence divergence, sympatric associations.

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Introduction

The ant genus *Iridomyrmex* MAYR, 1862 ubiquitously dominates Australian ant communities in a way that is unparalleled by any other ant genus on other continents, and in doing so matches Australian eucalypts in their unique dominance of vegetation across a continent (ANDERSEN 2000, 2003). *Iridomyrmex* is an arid-adapted taxon, and has proved highly informative for understanding selection processes and patterns of speciation in this most arid of habitable continents (GREENSLADE 1982, GREENSLADE & HALLIDAY 1982).

In a systematic overview of the genus, ANDERSEN (2007) estimated that it contains approximately 350 species, based on the > 200 morphospecies in the CSIRO Tropical Ecosystems Research Centre (TERC) collection in Darwin, Australia. However, a recent revision of the genus by HETERICK & SHATTUCK (2011) [hereafter referred to as H&S (2011)] recognises only 79 morphologically based species. H&S (2011) noted that many of the taxa they considered as single species were highly variable morphologically, and that some likely represent complexes of cryptic species. We fully acknowledge that *Iridomyrmex* is an extremely challenging genus taxonomically and that species boundaries are often unclear (ANDERSEN 2007). However, our assessment is that many taxa considered as species by H&S (2011) show very clear rather than cryptic morphological variation that was not appropriately reported. We are concerned that H&S (2011) under-reported the extent of morphological differentiation, provided no formal morphological analysis of variable "species" to support their conclusions, and did not report sympatric associations of morphologically differentiated populations considered conspecific.

Here we illustrate our concerns by documenting morphological and genetic variation within the taxon newly described by H&S (2011) as *Iridomyrmex coeruleus*. We focus on *I. coeruleus* because it lacks the allometric variation shown by many other *Iridomyrmex* species, and is presented by H&S (2011) as being morphologically uniform except for some variation in iridescence. We then dis-

cuss published morphometric, genetic and distributional evidence from other *Iridomyrmex* "species" to argue that H&S (2011) widely under-reports morphological differentiation and sympatric associations.

Throughout our paper we adopt a species concept based on the notion of reproductive isolation, with gene flow actually or potentially occurring between populations throughout a species' range. This is made operational through the recognition of clear morphological and genetic differentiation that is maintained when ranges overlap. For geographically separated populations, the taxonomic significance of any differentiation needs to be assessed on the basis of known variation in widely distributed species that have been well-studied taxonomically. We recognize that in practice decisions on species boundaries often involve subjective opinion; this highlights the need for full documentation of the morphological variation that occurs so that readers are in a position to make their own assessments of its taxonomic significance.

Methods

Morphology

Iridomyrmex coeruleus is characterised by H&S (2011) as having "large eyes, broad head, short, bristly, whitish setae on the mesosoma, and antennal scapes that are paler than the head capsule", with most specimens having strong, blue iridescence (which is the basis for the species name). Our analysis of this taxon is based on the 234 pinned specimens in the TERC collection, collected from 76 sites across northern Australia (Fig. 1). There are only 17 other Australian records of *I. coeruleus* listed in H&S (2011). All the TERC specimens were examined by Brian Heterick during the preparation of H&S (2011), and, with the exception of one morphotaxon, all were identified as belonging to *I. coeruleus*. The exception was identified by Brian Heterick as a "variant" of *I. hertogi* HETERICK & SHATTUCK, 2011, which we consider to be a member of the unrelated *I. mjobergi* FOREL, 1915 group.

The TERC specimens of "*Iridomyrmex coeruleus*" had been sorted by one of us (ANA) into five morphotaxa (Tab. 1) based on body size, eye size, gastric pubescence and pilosity. A representative of each taxon was photographed using a Leica M205C digital camera and Auto-Montage software. Morphometric measurements were taken using a stereomicroscope with micrometer from specimens of the most similar morphotaxa: representative specimens from throughout the range of a morphotaxon that occurs across northern Australia (*I. coeruleus* complex; n = 28), and all available specimens of morphotaxa A (n = 10) and B (n = 3). The measurements taken were head length (along the mid-line in full face view) and scape length (excluding articulatory condyle). Differences between taxa in the ratio of scape to head length were tested using Kruskill-Wallis ANOVA.

Genetics

We used CO1 analysis to investigate patterns of genetic variation, which is a standard genetic approach for informing species boundaries (HEBERT & al. 2004a, b, WARD & al. 2005). There is no arbitrary level of CO1 divergence that can be used to define species, but the level of variation within an ant species is typically 1 - 3% (SMITH & al. 2005). DNA was extracted from 13 samples, both pinned

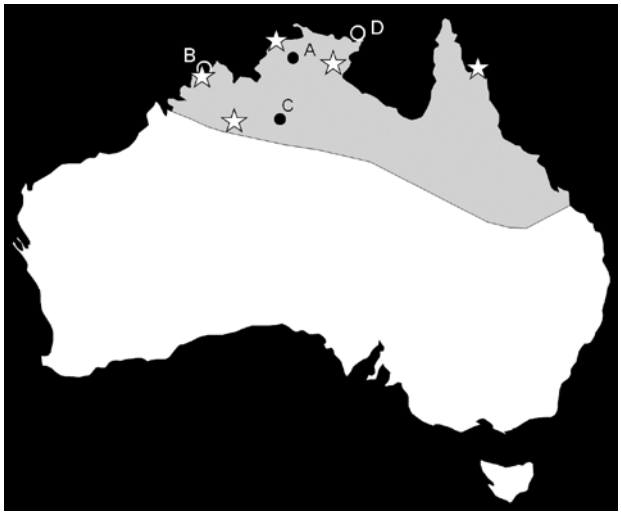

Fig. 1: Distribution of *Iridomyrmex coeruleus* complex (grey shade; stars represent the locations where specimens analysed for CO1 sequencing were collected) and morphotaxa A-D in northern Australia, based on specimens held in the TERC collection.

Tab. 1: Summary information on records of the *Iridomyrmex coeruleus* group from northern Australia in the TERC collection.

Morphotaxon	No. site records	No. pinned specimens
coeruleus complex	58	181
A	1	13
B	1	3
C	14	33
D	2	2

(up to ten years old) and ethanol-preserved, representing a range of *Iridomyrmex coeruleus* morphotaxa as well as *I. hertogi* (Tab. 2, Fig. 1). The two *I. hertogi* samples have been sorted by us as different species in the TERC collection, but were considered conspecific by H&S (2011), as well as being conspecific with the "variant" described above that we consider to be more closely allied to *I. coeruleus* (taxon C in Tab. 1). Where possible, we sequenced multiple individuals from the same sample (i.e., on the same pin or from the same vial). We were unable to extract DNA from taxon A as it was represented only by 15 year-old pinned specimens, and we were unwilling to risk damage to either of the two known specimens of taxon D given that it is so distinctive morphologically (as described below) that there is no reason to doubt its taxonomic status.

After a brief (1 - 2 min) wash in purified water to remove ethanol and glue residuals, we crushed each ant in a 1.5 ml centrifuge tube using a micropestle. DNA was isolated by using a modified Chelex protocol (WALSH & al. 1991), with 200 ml of 5% Chelex® 100 (BioRad) solution and 5 µl of Proteinase K (Qiagen). Samples were incubated at 56°C for 90 min, heated at 95°C for 20 min and then stored at -20°C. Genomic DNA was amplified by

Tab. 2: Specimens for which CO1 sequences were obtained. The locations where specimens of the *Iridomyrmex coeruleus* complex were collected are indicated in Figure 1.

Sample ID	Morphotaxon	Locality	Latitude	Longitude	GenBank accession #
Ir001	*I. coeruleus* complex	Humpty Doo, NT	12° 34' 27" S	131° 06' 05" E	KC160539
Ir004	*I. coeruleus* complex	Nhulunbuy, NT	12° 10' 54" S	136° 46' 48" E	KC160540
Ir012	*I. coeruleus* complex	Mitchell Falls Plateau, Kimberley, WA	15° 07' 14" S	125° 47' 39" E	KC160544
Ir016	*I. coeruleus* complex	Lizard Island, QLD	14° 40' 04" S	145° 27' 49" E	KC160541
Ir019	*I. coeruleus* complex	Lizard Island, QLD	14° 40' 04" S	145° 27' 49" E	KC160542
Ir023	*I. coeruleus* complex	Lost City, Limmen National Park, NT	16° 43' 05" S	134° 56' 52" E	KC160537
Ir026	*I. coeruleus* complex	Lost City, Limmen National Park, NT	16° 43' 05" S	134° 56' 52" E	KC160538
Ir029	*I. coeruleus* complex	Purnululu National Park, WA	17° 27' 31" S	128° 18' 39" E	KC160543
Ir032	*I. coeruleus* complex	Mitchell Falls Plateau, Kimberley, WA	15° 07' 14" S	125° 47' 39" E	KC160545
Ir038	Taxon B	Mitchell Falls Plateau, Kimberley, WA	15° 07' 14" S	125° 47' 39" E	KC160536
Ir041	Taxon C	Pigeon Hole stn., Victoria River Downs, NT	17° 02' 08" S	131° 17' 56" E	KC160546
Ir045	*I. hertogi* complex	Thylungra stn., QLD	26° 36' 48" S	144° 16' 07" E	KC160534
Ir047	*I. hertogi* complex	Maningrida Airport, NT	12° 02' 54" S	134° 13' 46" E	KC160535

polymerase chain reaction (PCR) using the CO1 primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAA TCA-3') (FOLMER & al. 1994). Each 25 µl reaction contained 2.5-5 µl of DNA, 0.5 µl (0.2 mM) of total dNTPs, 1.5 µl (0.6 µM) of each primer, 0.07 µl of 5 U / µl Robust DNA Taq polymerase, 5 µl of 5 × buffer A and 0.5 µl (0.5 mM) of $MgCl_2$, using the KAPA2GTM Robust PCR kit. PCR cycle conditions were: an initial 2 min denaturation step at 94°C, followed by 35 cycles of 50 s at 94°C, 2 min at 49°C, 1.5 min at 72°C, and finished with a 6 min final elongation step at 72°C. The PCR products were examined on a 1% agarose gel, and successful amplifications were purified with QIAGEN QIAquick PCR Purification kit and sent to Macrogene (Korea) for bidirectional sequencing.

Chromatograms were inspected for noisy and ambiguous base calling using the software Geneious Pro 5.5.6 (DRUMMOND & al. 2011). To decrease the risk of incorporating amplified pseudogenes, sequences were checked for pseudogenes and heteroplasmy and translated to amino acids. We did not find any evidence of pseudogenes, heteroplasmy, insertions or deletions in any of our sequences, and no stop-codons were observed after translation. The sequences produced averaged 670 bp in length, and the final alignment after trimming was 612 bp long. All sequences have been deposited in GenBank (accession numbers KC160534 - KC160546, Tab. 2). Multiple specimens from the same sample always gave identical sequences, and so we used only one specimen from each for subsequent analysis.

In order to test our morphological species hypotheses, we used an unrooted neighbor-joining tree of nucleotide changes to display the data (SAITOU & NEI 1987), after calculating sequence divergence based on the Kimura-2-Parameter distance model (KIMURA 1980) using MEGA5 (Molecular Evolutionary Genetic Analysis) (TAMURA & al. 2011). Additionally, we explored phylogenetic relationships using *Froggattella latispina* (GenBank accession number: DQ353341, MOREAU & al. 2006) as an outgroup. *Froggattella* is considered to be the dolichoderinae genus most closely related to *Iridomyrmex* (see SHATTUCK 1992, MOREAU & al. 2006). We used the software JModelTest (POSADA 2008) to identify the best evolutionary model for our data, based on an AICc model selection procedure (AKAIKE 1974). The Generalised Time-Reversible model (TAVARE 1986) with a gamma-distributed rate of variation across sites (GTR + Γ) was selected. We performed a Bayesian inference analysis in MrBayes v3.1.2 (HUELSENBECK & RONQUIST 2001), specifying *Froggattella latispina* as the outgroup. This was implemented with a Markov Chain Monte-Carlo (MCMC) length 1.5×10^6 with the chain sampled every 100 generations after an initial burn-in of 3750 generations. Two runs were performed, and chain convergence was confirmed by the standard deviation of split frequencies (< 0.01) and the Effective Sample Size (ESS) (> 7000) given by the parameter estimation in Tracer v1.5 (RAMBAULT & DRUMMOND 2003). Phylogeny was estimated from the majority-rule consensus of the pooled post-burn-in trees from the two runs.

Results

Morphology

The majority of specimens in the TERC collection belong to a taxon that generally fits the description of *Iridomyrmex coeruleus* provided by H&S (2011), but shows systematic variation that was not reported. In most specimens from throughout the range, the gaster is covered in greyish pubescence, giving it a dull appearance, and in all these cases the mesosoma has the short, sparse setae outlined in the species description (Figs. 2a, b). However, there are similar forms from throughout the range that variably lack gastric pubescence, the gaster has pink rather than blue iridescence, and the mesosoma lacks pilosity. Such variation is indicative of a complex of cryptic species, and we refer to this taxon as the *I. coeruleus* complex.

Figs. 2a-f: Head and lateral views of the five morphotaxa within the *Iridomyrmex coeruleus* species group recognised in this study: *I. coeruleus* complex (a, b); taxon A (c, d); taxon B (e, f).

The four other morphotaxa are characterised as follows: Taxon A (Figs. 2c, d) has the same size and general appearance of members of the *Iridomyrmex coeruleus* complex, but has very feeble iridescence, and much larger eyes (outer margins reaching or exceeding lateral margins of the head in full-face view). It also has longer scapes relative to head length (mean ± SE: 0.93 ± 0.014) compared with the *I. coeruleus* complex (0.89 ± 0.016), but shorter than in taxon B (0.97 ± 0.09; post-hoc multiple comparisons of means (2-tailed) test, Kruskal-Wallis ANOVA: H = 19.6; P < 0.0001) (Fig. 3). It is known from a single locality in the Top End of the Northern Territory (Fig. 1), occurring on clay soils.

Taxon B (Figs. 2e, f) has the large eyes and feeble iridescence of taxon A, but has a broader (almost square) head and slightly longer scapes (Fig. 3). It is likewise known

Figs. 2g-j: Head and lateral views of the five morphotaxa within the *Iridomyrmex coeruleus* species group recognised in this study: taxon C (g, h) and taxon D (i, j).

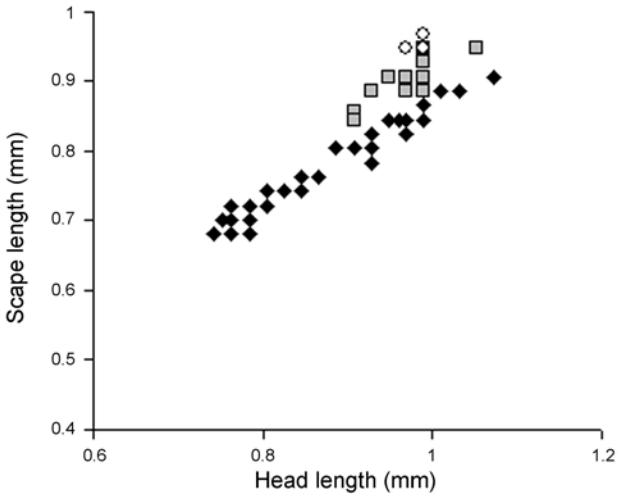

Fig. 3: Plots of scape length versus head length for the three most similar morphotaxa of the *Iridomyrmex coeruleus* group: *I. coeruleus* complex (diamonds), taxon A (squares) and taxon B (circles).

from a single locality, on sandy soil in the Mitchell Falls region of far northern Western Australia (Fig. 1).

Taxon C (Figs. 2g, h) is the taxon considered by H&S (2011) to be conspecific with *Iridomyrmex hertogi*. It is a small species (head width < 0.6 mm) lacking iridescence, with a glabrous mesosoma that is flattened in profile. In full-face view the head is widest at its centre, and the scapes are relatively short, only very slightly exceeding the occipital margin. This taxon is known only from the Victoria River Region of the Northern Territory (Fig. 1), where it is common on clay soils.

Taxon D (Figs. 2i, j) is another small (head width < 0.6 mm) and mostly glabrous species, but with highly distinctive, yellowish green iridescence. Its propodeum is short, flattened and angular in profile, with a posterior cluster of short hairs. The eyes are strongly asymmetrical (narrowed posteriorly), the head is widest behind its centre, and the scapes fail to reach the occipital margin. It is known from only two specimens collected from the Gove Peninsula of northeastern Arnhem Land in the Northern Territory (Fig. 1).

Genetics

CO1 sequence divergences were highly congruent with our morphotaxa (Fig. 4). The *Iridomyrmex coeruleus* complex was recovered with 0 - 5% within-complex divergence, and included a specimen from the Mitchell Falls region of far northern Western Australia with 4 - 5% divergence from all other specimens in the complex sequenced, including another specimen from the same locality (Fig. 4,

Tab. 3: Pairwise distances between CO1 sequences, indicated as the average number of base substitutions per site using the Kimura 2-Parameter model. Divergences > 3% are indicated in grey.

	Ir045	**Ir047**	**Ir038**	**Ir023**	**Ir026**	**Ir001**	**Ir004**	**Ir016**	**Ir019**	**Ir029**	**Ir012**	**Ir032**	**Ir041**
Ir047 (*hertogi*)	0.07												
Ir038 (taxon B)	0.11	0.12											
Ir023 (*coeruleus* complex)	0.11	0.12	0.12										
Ir026 (*coeruleus* complex)	0.11	0.11	0.12	0.00									
Ir001(*coeruleus* complex)	0.11	0.11	0.11	0.01	0.01								
Ir004 (*coeruleus* complex)	0.11	0.11	0.11	0.01	0.01	0.00							
Ir016 (*coeruleus* complex)	0.11	0.11	0.12	0.01	0.01	0.00	0.00						
Ir019 (*coeruleus* complex)	0.11	0.11	0.12	0.01	0.01	0.00	0.00	0.00					
Ir029 (*coeruleus* complex)	0.11	0.10	0.11	0.01	0.01	0.01	0.01	0.01	0.01				
Ir012 (*coeruleus* complex)	0.12	0.10	0.11	0.02	0.02	0.01	0.01	0.01	0.01	0.01			
Ir032 (*coeruleus* complex)	0.11	0.11	0.10	0.05	0.05	0.05	0.05	0.05	0.05	0.04	0.05		
Ir041 (taxon C)	0.14	0.13	0.14	0.14	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.12	

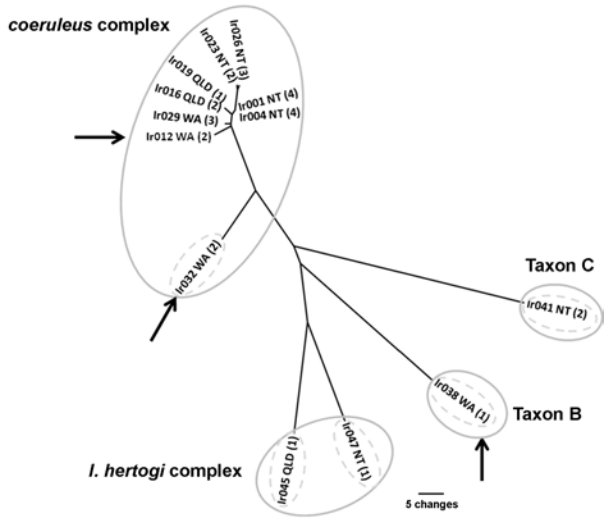

Fig. 4: Unrooted neighbor-joining tree of nucleotide variation in specimens CO1-sequenced during this study. Solid lines delimit morphotaxa described in this study, and dashed lines delimit taxa separated by more than 3% sequence divergence. Arrows indicate specimens collected from the same locality (Mitchell Falls region of far northwestern Australia). Numbers in parenthesis indicate the total number of individuals sequenced, with multiple specimens from the same sample giving the same sequence in all cases.

Tab. 3). Such divergence suggests that the Mitchell Falls specimen represents a distinct species, although it shows very little morphological differentiation from others in the complex, except that the three available specimens all have a very broad head. Other specimens in the complex exhibited patterns of genetic differentiation related to sampling location, and this is associated with consistent variation in gastric pubescence, iridescence and pilosity. For example, all specimens from the Mitchell Falls population lack gastric pubescence, the gaster has pink rather than blue iridescence, and the mesosoma is glabrous. However, the level of genetic divergence between all these other specimens is relatively low (< 2%), and further sampling and analysis are required before this complex can be fully resolved.

The large-eyed taxon B (10 - 14% divergence from other specimens sequenced), and taxon C (12 - 14% divergence) were also strongly supported by the sequence data (Fig. 4, Tab. 3). CO1 analysis also supports the view that taxon C is only distantly related to the two specimens from the *Iridomyrmex hertogi* complex, and that the latter two specimens themselves represent different species (7% divergence, Tab. 3).

Our phylogenetic analysis (Fig. 5) shows the *Iridomyrmex coeruleus* group as recognised by us as a single clade that is distinct from that of the *I. hertogi* complex. Taxon C belongs to the *I. coeruleus* clade (with 90% Bayesian probability), despite being considered by H&S (2011) to be conspecific with *I. hertogi*. The *I. coeruleus* complex is also shown as a single clade, but with only 60% Bayesian probability; this reflects the lack of samples with intermediate divergence between this complex and Taxon B. Within the *I. coeruleus* complex, there is strong support (Bayesian probability of 1) for the Mitchell Falls specimen discussed above representing a distinct clade. All other specimens from the complex cluster according to geographic location.

Discussion

Iridomyrmex coeruleus was described by H&S (2011) as being morphologically uniform, but we have demonstrated that it actually represents a range of morphologically differentiated taxa, and that these morphotaxa are congruent with very high (up to 12%) levels of genetic divergence based on CO1 analysis. Notably, a taxon that shows little morphological and genetic (< 2% CO1 divergence) variation throughout its range right across northern Australia is sympatric with more localised taxa that are very clearly differentiated morphologically and genetically (up to 12% CO1 divergence). Three such differentiated taxa occur in the one locality (the Mitchell Falls region of far northern Western Australia). *Iridomyrmex coeruleus* as defined by H&S (2011) clearly represents several species. We have

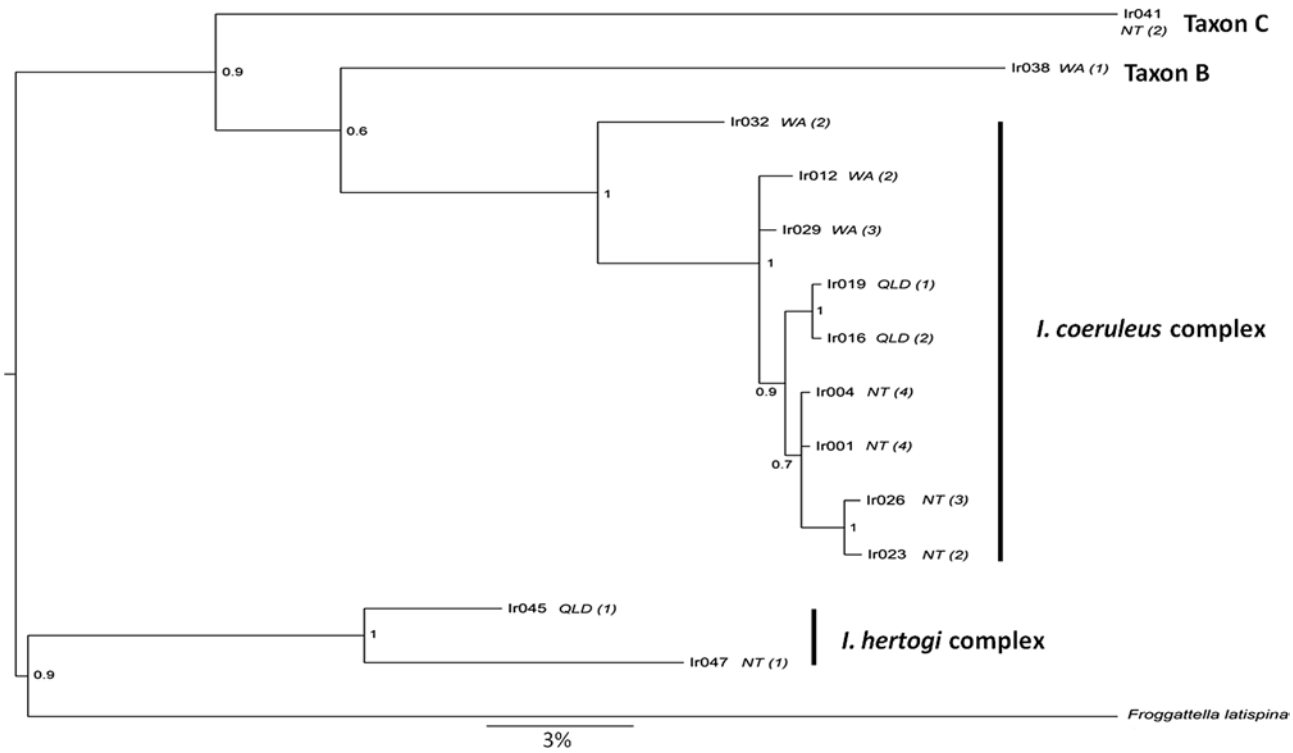

Fig. 5: Bayesian inference tree for CO1-sequenced specimens of "*Iridomyrmex coeruleus*", based on a GTR + Γ model of evolution. Values at nodes represent Bayesian posterior probabilities. Numbers in parenthesis indicate the total number of individuals sequenced, with multiple specimens giving the same sequence in all cases.

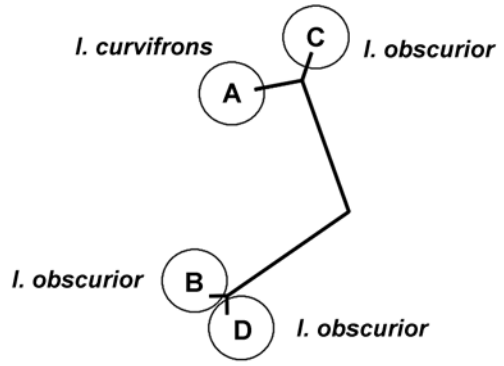

Fig. 6: Unrooted neighbor-joining tree of species of the *Iridomyrmex obscurior* complex attending the lycaenid butterfly *Jalmenus evagoras* in southeastern Australia, based on CO1 divergence (modified from Fig. 2 of EASTWOOD & al. 2006). Letters refer to morphotaxa identified by one of us (ANA) prior to genetic analysis (based on 2, 4, 31, and 7 records of A-D respectively), and species names follow H&S (2011).

shown that this is also the case for *I. hertogi*, which encompasses morphologically differentiated taxa with levels of CO1 divergence (14%) that are several-fold higher than is typical for intraspecific variation, and with specimens occurring in different phylogenetic clades.

We have elsewhere documented clear morphometric differentiation in the taxon presented by H&S (2011) as *Iridomyrmex anceps* (ROGER, 1863), occurring from northern Australia to China, and on many islands of the southwest Pacific (HOFFMANN & al. 2011). Multiple morphotypes occur together in particular regions (e.g., Timor, North Queensland), and the taxon is likely to include at least six species (HOFFMANN & al. 2011). In addition, it is evident from EASTWOOD & al. (2006) that what H&S (2011) present as *I. obscurior* FOREL, 1902 (complex A in Fig. 2 of EASTWOOD & al. 2006) includes multiple morphologically and genetically differentiated taxa. Moreover, analysis of genetic divergence shows that a taxon identified by H&S (2011) as *I. obscurior* is much more closely related to another species (sp. A in EASTWOOD & al. 2006, newly described by H&S (2011) as *I. curvifrons*) than it is to its supposedly conspecific taxa (spp. B and D in EASTWOOD & al. 2006; Fig. 6).

H&S (2011) described morphological variation in all the above "species" as being relatively limited. Many other "species" were described as being highly variable, and often with continent-wide distributions. In our view all these are multiple and in some cases very many species, often occurring in sympatry. For example, one of us has recognised three clearly differentiated morphotypes that co-occur on Wilgena Station in the Kingoonya region of South Australia, all of which are considered by H&S (2011) to be *I. dromus* CLARK, 1938 (see HOFFMANN & JAMES 2011).

Conclusion

Iridomyrmex is a highly diverse but morphologically conservative genus that is extremely challenging taxonomically. In many cases it is very difficult to characterise morphological variation, and the resolution of many species boundaries will inevitably require complementary information, especially on genetic variation (cf. SCHLICK-STEINER & al. 2010). However, the examples we have provided

represent clear morphological differentiation, and do not require the detailed morphometric analysis used to discriminate truly cryptic species (e.g., SEIFERT 2012). We are concerned that H&S (2011) note that extreme variability exists within what they consider species, but present no systematic analysis of it, and have ignored the extensive literature showing sympatric associations of morphologically differentiated taxa that are presented as being conspecific. It is particularly regrettable that many synonymies are proposed on the basis of unsubstantiated assertion, rather than being "credibly shown" (SEIFERT 2012).

We are particularly concerned that the revision of H&S (2011) gives a misleading picture of true diversity in such an ecologically dominant genus, with important implications for an understanding of the ecology and biogeography of a continent. A similar situation applies to a taxon presented as a "variable species" (*Monomorium fieldi* FOREL, 1910) in a revision of Australian *Monomorium* (see HETERICK 2001). This taxon actually includes eight species that remarkably co-occur in a single 10 m × 10 m plot in northern Australia, with all species clearly differentiated morphologically, genetically and behaviourally (ANDERSEN & al. in press). A lack of recognition of such clear species provides a totally misleading picture of speciation processes and patterns of species diversity and co-existence.

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