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Scientific note

A scientific note on a rapid method for the molecular discrimination of *Apis andreniformis* and *A. florea**

Jessica S. Higgs, Marcus McHale, Benjamin P. Oldroyd

Behaviour and Genetics of Social Insects Lab, School of Biological Sciences A12, University of Sydney, NSW 2006, Australia

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***Apis florea* / *Apis andreniformis* / ribosomal RNA / identification**

Apis florea (Smith, 1858) and *A. andreniformis* (Fabricius, 1787) comprise the sole members of a subgenus of honey bee known as *Micrapis* (Maa, 1953) or dwarf honey bees (Oldroyd and Wongsiri, 2006). The species are superficially similar, but distinguishing characteristics include behaviour, the morphology of the crown of the nest, and body colour (Oldroyd and Wongsiri, 2006). However diagnosis is not always straightforward due to a rare yellow *A. andreniformis* morph that is very similar to *A. florea* (Higgs et al., 2009). Nor are the above characteristics diagnostic when only immature lifestages are available.

Here we report what we believe is an unequivocal test of species identity utilizing the mitochondrial large subunit ribosomal RNA gene (*rrnL*). The test is applicable to all life stages and castes, and will thus be useful for quarantine officials and studies of inter-specific reproductive parasitism.

POLYMERASE CHAIN REACTION PRIMER DESIGN

In March 2008 we retrieved all available *A. andreniformis* and *A. florea* mitochondrial sequences in GenBank in order to identify any sequences that showed significant differences between the two species. This search revealed a region of the *rrnL*, 13 484–13 706 bp relative to the *A. mellifera* mitochondrial genome (Crozier and Crozier, 1993), that contains an 11 base pair (bp) length polymorphism between *A. andreniformis* (250 bp) and *A. florea* (239 bp) (GenBank accession numbers *A. andreniformis* AY588425 (Raffiudin and Crozier, 2007), *A. florea* L22894 (Cameron, 1993)). From these sequences we designed primers for polymerase chain reaction (PCR) as follows: forward: 5'TGGGACGATAAGACCCTATAGA, reverse: 5'TCGAGGTCGCAATCATCTTT. To enable visualization of PCR product with a genetic analyser a second forward primer was designed with an additional unique 5' tag sequence: 5'CCTGGCGACTCCTGGAG complementary to an oligo fluorescently labeled with HEX (Genosys). Un-tagged PCR products were analysed by gel electrophoresis.

To verify that the polymorphism is a valid test of species identity we examined single workers from 15 *A. florea* and 17 *A. andreniformis* colonies collected from throughout Thailand. An additional 3 *A. florea* samples were examined from Kanataka, India. The species of each colony had been determined in the field by the colour of the first abdominal segment of the majority of workers and/or the morphology of the nest crown. DNA was extracted from a hind leg of each worker in 5% Chelex solution (Oldroyd et al., 1998).

Amplification from 0.75 μL of extracted DNA was conducted in a total volume of 25 μL containing 4 mM of each dNTP, 2 mM $MgCl_2$, 1 × reaction buffer (BIOLINE), 0.2 units BIOTAQ DNA polymerase (BIOLINE), 0.08 μM tagged forward primer, 0.4 μM reverse primer and 0.8 μM HEX labeled oligo (complementary to the tag sequence, Genosys). A thermal profile of 94 °C for 2 minutes, 30 × (94 °C, 57 °C, 72 °C for 30 seconds each), followed by 72 °C for 10 minutes was used.

Corresponding author: B.P. Oldroyd,
boldroyd@bio.usyd.edu.au
* Manuscript editor: Steve Sheppard

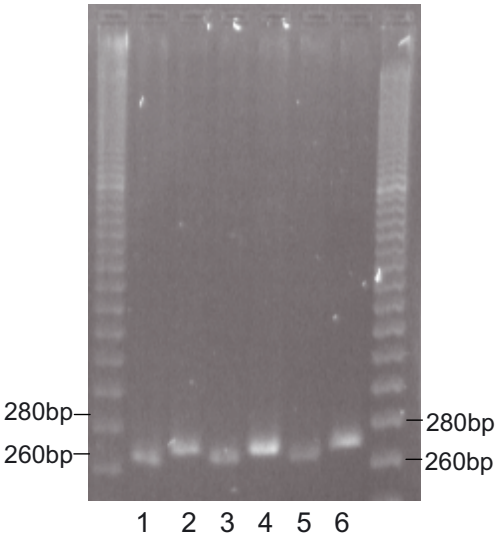

Figure 1. A fragment of *rrnL* from *A. florea* and *A. andreniformis* workers amplified and run on an EtBr stained, 3.5% agarose gel. Lanes 1, 3 and 5 are *A. florea* workers and lanes 2, 4 and 6 *A. andreniformis* workers. All workers originated from different colonies. Edge lanes are 20 bp molecular ladder (EZ Load, BIO-RAD).

GENETIC ANALYSIS

We sized the PCR products with a 3130 *xl* Genetic Analyser (AppliedBiosystems) before analysing the results with GeneMapper v 3.7. We found all 17 *A. andreniformis rrnL* fragments to be 250 bps, 15 *A. florea* to be 245 bps and 3 *A. florea*, those from India, to be 246 bps in length (lengths exclude the tag sequence). This is consistent with the mitochondrial divergence previously found between Thailand and India in *A. florea* (Smith, 1991).

As the *A. florea* PCR products were larger than expected, 5 samples of *A. florea* (accession number FJ348344), including 1 from India (accession number FJ348345), and *A. andreniformis* (accession number FJ348343) were sequenced in both directions at a commercial facility (Macrogen, Korea). The resulting sequences confirmed the fragment lengths obtained from the genetic analyser. Eight insertions and 1 (Indian sample) or 2 (Thailand samples) deletions in the sequences obtained explained the 6 bp difference from the published sequence (Cameron, 1993).

GEL ELECTROPHORESIS

PCR products obtained from *A. florea* and *A. andreniformis* can be resolved by agarose gel electrophoresis (Fig. 1). We diluted PCR products 1:5 with H_2O before running 4 μL with 1 μL of 5 × nucleic acid loading buffer (BIO-RAD) in a 3.5% low range ultra agarose gel (BIO-RAD), 0.5 μg/μL EtBr. Gels were run in 0.5 μg/μL EtBr, 1% TBE buffer for 7 hours at 70 V. Gels were visualised under UV light and products sized via the inclusion of a 20 bp molecular ladder (EZ load, BIO-RAD).

CONCLUSION

Our results demonstrate that the size polymorphism in this fragment of the *rrnL* gene reliably distinguishes *A. andreniformis* and *A. florea*. With some slight modifications to the DNA extraction protocol, we have successfully employed the above method to assign species to adult drones, a queen and drone brood of all stages.

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Note scientifique sur une méthode rapide de discrimination moléculaire d'*Apis andreniformis* et d'*A. florea*.

Eine wissenschaftliche Bemerkung über eine schnelle Methode der molekularen Unterscheidung zwischen *Apis andreniformis* und *A. florea*.

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