Apidologie **Supplementary Materials**

Delimiting the species within the genus *Austroplebeia*, an Australian stingless bee, using multiple methodologies

Megan Therese Halcroft*, Anne Dollin, Tiago Mauricio Francoy, Jocelyn Ellen King, Markus Riegler, Anthony Mark Haigh, Robert Neil Spooner-Hart

*Corresponding author:

email: megan@beesbusiness.com.au

Addresses:

[1]*School of Science and Health, University of Western Sydney, Locked Bag 1797 Penrith NSW 2751, Australia*

[2]*Bees Business, PO Box 474, Lithgow NSW 2790, Australia*

Appendix I Abbreviated comparative descriptions (as written by the authors) of currently named *Austroplebeia* species, based on size and thoracic colour markings. Only the commonly used characteristics or comparative terms are listed here. Locations of holotype collection are shown in Figure 1.

Current species name	**Summary of the differentiating characteristics**	**Primary type location**
Austroplebeia cincta (Mocsáry 1898)	3.5 mm. Inner border of the eyes, antennal scapes, lateral edges of the dorsal thorax and lateral lobes of the scutellum yellow. Similar to *Trigona australis* but more shiny. (Translation - Dollin)	Madang, Papua New Guinea
Austroplebeia australis (Friese 1898)	4 mm. Scutellum on each side of tip broadly yellow-white edged, axillae entirely yellow-white.	Central Australia
Austroplebeia essingtoni (Cockerell 1905)	4 mm. Narrow stripe on each side of mesothorax, extending to axillae and all of scutellum. Easily known from *T. australis* by the entirely yellow scutellum.	Port Essington, Northern Territory
Austroplebeia cassiae (Cockerell 1910)	No size noted. Scutellum with broad transverse dusky-yellowish band. Very like *Trigona carbonaria.*	Mackay, Queensland
Austroplebeia percincta (Cockerell 1929)	ca. 5 mm. This name is proposed for the larger Australian race [of *A. cincta*]. (Previously *Trigona cincta percincta.*)	Hermannsburg, Finke River, Northern Territory
Austroplebeia cockerelli (Rayment 1930)	ca. 5 mm. Mesothorax black, a narrow creamy stripe laterally from the prothorax to the scutellum. Scutellum black, with a large cream dot laterally and a large marginate fulvous mark.	Borroloola, Northern Territory
Austroplebeia ornata (Rayment 1932)	ca. 4.5 mm. Mesothorax black, narrow dull-cream margins laterally. Apical half of scutellum with an interrupted yellow band (Rayment 1935). (Previously *Trigona cockerelli ornata.*)	Cape York, Queensland
Austroplebeia symei (Rayment 1932)	ca. 4.5 mm. Mesothorax black. Scutellum with two cream dots (Rayment 1935).	North Queensland
Austroplebeia websteri (Rayment 1932)	ca. 5 mm. Mesothorax black, with a fine narrow creamy line bordering the lateral margins. Scutellum creamy-yellow, except for a bracket shaped median dark mark.	Wyndham, Western Australia

Appendix II Male morphology - specimen preparation for imaging under SEM

Specimens were not critical-point dried, but were washed with 100% ethanol, air dried and mounted, in anatomical order, on a self-adhesive carbon-coated, 25 mm diam. × 5 mm high, with internal thread, lathe finished, anodised aluminium mount (ProSciTech, Australia, Thuringowa, Queensland). Specimens were sputter coated with gold under a 7 kpa vacuum and argon gas for 40 s (Polaron E5100, Quorum Technologies Ltd., UK). Images were captured using a JSM-6510LV scanning electron microscope (SEM) (JEOL, Japan). A high resolution of 3.0 nm was achieved at high vacuum mode 30 kV, GUI interface. Further investigations were conducted on the genital structures associated with S6, S7 and T7 and imaged using an Olympus BX60 compound light microscope and a Jenoptik ProgRes® C14 digital camera (SciTech Pty Ltd., Preston, Victoria). Differences detected in these images prompted the closer examination of stored specimens, to detect the presence of protruding genital structures. A Leica MZIZ stereomicroscope was used to study both dissected and *in-situ* genital structures.

Appendix III Geometric morphometry of wing venations – preparation and analysis techniques

The right forewing of five workers from each of 67 nests was mounted between two microscope slides. Digital images were captured using the Olympus BX60 compound light microscope and digital camera. Images were enhanced and compressed in Adobe® Photoshop® CS2 (Adobe Systems Inc.).

Ten homologous landmarks were plotted at the wing vein intersections, within the central part of the wing (Figure 2) using tpsDig2 V2.16 software (Rohlf 2005). Images were all scaled to the same size and superimposed, using the centroid of the landmarks distribution and rotated to the best fit of the landmarks. The aligned Cartesian coordinates of the landmarks were used to perform statistical analyses. In order to avoid distortions in the data, instead of individual measures, the mean configuration of bees from the same colony was used in the analysis. A principal component analysis (PCA) of the Cartesian coordinates of each landmark was conducted after alignment. A discriminant analysis was then conducted using forward stepwise analysis (tolerance 0.01; F to enter 1.00) using the same measures to determine the discriminant functions. A canonical analysis and a cross-validation test followed, to evaluate the accuracy of colony identification. Groups were labelled according to their morphological characteristics. The statistical analysis was carried out using Statistica 6.0 (StatSoft 2001). The Mahalanobis distances between the centroids of the groups were also calculated in the discriminant analysis and a dendogram of morphological similarity was constructed based on these data. The dendogram was constructed using neighbour-joining methodology (Saitou and Nei 1987) and MEGA version 4.1 (Tamura et al. 2007). The coefficients of cophenetic correlation, using TreeFit (Kalinowsi 2009), were calculated to determine if the dendogram gave a good representation of these data.

References

Rohlf, F.J. (2005) tpsDig2, version 2.04, Department of Ecology and Evolution, State University of New York, Stony Brook

Saitou, N., Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**, 406-425

Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007) MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24**, 1596 - 1599

StatSoft, I. (2001) STATISTICA (data analysis software system). Version 6. Available at [www.statsoft.com]. Accessed October 2010.

Appendix IV DNA amplification - preparation

DNA templates were amplified on a thermocycler (Thermomix comfort, Eppendorf Pty Ltd, North Ryde, NSW) performing 40 amplification cycles (94°C, 1 min; 42°C, 1.5 min; and 64°C, 1.5 min), followed by a final extension step at 72°C for 5 min (Costa et al. 2003).

Reaction components were provided in kit form (GoTaq® PCR core systems, Promega Corporation, www.promega.com) and initial reactions were carried out in a total volume of 20 µL. Ethidium bromide 0.5 µg / ml (AMRESCO 2011) was added to a 1% agarose gel and electrophoresis was carried out at 100 V for 1 h. Bands were visualised on a Gel Doc 2000 / ER (Bio-Rad Laboratories Ltd, www.bio-rad.com). PCR products were sent to Macrogen Inc. Korea for sequencing with BigDye™ terminator chemistry (Sanger sequencing).

For subsequent reactions, PCR amplifications were carried out in a total reaction volume of 40 µL, using 21.6 µL of PCR water, 8 µL of reaction buffer, 4 µL of $MgCl_2$ (25 mM), 0.8 µL of dNTP (10 mM), 1.6 µL of each primer (2) (10 mM), 0.4 µL of Taq polymerase and 2 µL of DNA template. Electrophoresis of the amplified fragments (40 µL) was carried out on a 2% agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA / pH 8.0), with ethidium bromide added 0.5 µg / mL, and run at 70 V for a minimum of 2.5 h, and up to 4 h, to obtain maximum resolution. Progress of gel bands and images were obtained with a Gel Doc 2000 / ER. Target bands were dissected under UV transilluminator (Cleaver Scientific Ltd, Warwickshire, UK). DNA extraction and purification of PCR products were carried out using Wizard® SV gel and PCR clean-up systems (Promega Corporation, www.promega.com). PCR products were sequenced as described above.

References

AMRESCO. (2011) Ethidium bromide, 0.625 mg/ml, Life Science Research Products and Biochemicals

Costa, M.A., Del Lama, M.A., Melo, G.A.R., Sheppard, W.S. (2003) Molecular phylogeny of the stingless bees (Apidae, Apinae, Meliponini) inferred from mitochondrial 16S rDNA sequences. Apidologie **34**, 73-84

Appendix V DNA accession numbers associated with nest specimens for the *Austroplebeia* groups

Group	Nest code	Collection locality	Collection date	GenBank accession number
'symei'	C36	Duaringa, QLD	Sep 1996	KM521445
	C42	Duaringa, QLD	Sep 1996	KM521446
	C43	Duaringa, QLD	Sep 1996	KM521447
	C58	Duaringa, QLD	Oct 1996	KM521434
	N23	Duaringa, QLD	Sep 1987	KM521444
	N1	Katherine, NT	Jul 1987	KM521453
	N2	Katherine, NT	Jul 1987	KM521454
	N12	Cobourg Peninsula, NT	Aug 1987	KM521455
	Roc	Rockhampton, QLD	Jul 2010	KM521443
'australis'	C35	Duaringa, QLD	Sep 1996	KM521431
	C39	Duaringa, QLD	Sep 1996	KM521432
	C56	Duaringa, QLD	Oct 1996	KM521433
	C70	Duaringa, QLD	Oct 1996	KM521435
	NSW15	Kempsey, NSW	1987	KM521436
	Tar	Tara, QLD	Jul 2010	KM521437
'intermediate'	S2	Kilcoy, QLD	Oct 1989	KM521438
	S3	Kilcoy, QLD	Oct 1989	KM521439
	S5	Kilcoy, QLD	Oct 1989	KM521440
	S9	Kilcoy, QLD	Oct 1989	KM521441
	S31	Blackbutt, QLD	Oct 1989	KM521442
'striped'	C47	Ti Tree, NT	Oct 1996	KM521452
	C49	Ti Tree, NT	Oct 1996	KM521450
	F53	Hughenden, QLD	Aug 1983	KM521448
	N21	Croydon, QLD	Aug 1987	KM521449
	W13	Timber Creek, NT	Sep 1985	KM521451
'curved'	N11	Cobourg Peninsula, NT	Aug 1987	KM521429
	N15	Cobourg Peninsula, NT	Aug 1987	KM521430
	W5	Home Valley, WA	Sep 1985	KM521427
	W8	Kununurra, WA	Sep 1985	KM521428
'cincta'	D5	Rossville, QLD	Jun 2010	KM521456
	D6	Rossville, QLD	Jun 2010	KM521457