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The Neotropical social wasp *Mischocyttarus 'alfkenii'* Ducke (Hymenoptera: Vespidae) is a pair of ethospecies

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Abstract. In Trinidad, West Indies, wasps matching the description of *Mischocyttarus alfkenii* build two readily distinguishable nest forms, differing both in architecture (excentric versus centric petiole) and colour (yellowish grey-brown versus reddish medium brown). Analysis of two mitochondrial genes (*16S* and cytochrome *c* oxidase subunit I, COI) in excentric- and centric-form *M. 'alfkenii'* consistently segregates individuals from the two nest forms, with genetic divergences comparable with those observed among other species in the genus. Geometric morphometric analysis of wing venation likewise recovers consistent differences between nest forms. Integrating behavioural, genetic and morphometric evidence corroborates the hypothesis that the two nest forms correspond to distinct species of recent common ancestry. Notes accompanying the description of *M. alfkenii* indicate that the name belongs to the species in which the nest has an excentric petiole and paler carton. The other species is described as *Mischocyttarus baconi* **sp.n.**

Introduction

It has been a working assumption of biological systematics during most of its history that physical differences provide a reliable indication of the boundaries among distinct breeding populations, so that biological species are congruent with morphospecies. It is now apparent that cryptic species pairs or complexes – in which individuals that appear to be a single species on physical grounds, in fact, represent genetically discrete populations – are not the rarity that they were once thought to be (Hebert *et al.*, 2004). Interest in cryptic species has grown exponentially in recent years (Pfenninger & Schwenk, 2007), and the growing task of accurately accounting for cryptic diversity has implications for the understanding of ecological and evolutionary processes (Molbo *et al.*, 2003), as well as biodiversity assessments and conservation planning (Bickford *et al.*, 2007).

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To address the challenges that cryptic species pose to taxonomy, an expanding array of new tools has been employed to help delimit them (Sites & Marshall, 2003; Wiens, 2007). Molecular data, including restriction fragment length polymorphisms (RFLPs; Murray *et al.* 2008), mitochondrial haplotypes (Malenke *et al.*, 2009) and standardized fragments of the mitochondrial gene cytochrome *c* oxidase subunit I (COI DNA barcodes; Hajibabei *et al.*, 2007), now frequently complement more traditional characters. In some cases putative species are separable by geometric morphometric analysis, which can yield subtle but reliable morphological differences to tie physical characters to genetic entities (Marsteller *et al.*, 2009). Classifications integrating diverse data, including morphological, behavioural, ecological and molecular data, are now becoming standard (Dayrat, 2005; DeSalle *et al.*, 2005; Damm *et al.*, 2010).

Ethospecies are a class of cryptic species in which members of a pair or complex can be distinguished by physical characters with difficulty, if at all, but are readily separable through behavioural characters. In some animals, the latter are part of sexual displays (e.g. wolf spiders; Uetz & Denterlein, 1979; Roberts & Uetz, 2004), whereas known examples in birds

(Hansell, 2000) and social insects (Emerson, 1956; Sakagami & Yoshikawa, 1968) mostly involve the structure of the nest.

Mischocyttarus Saussure, comprising approximately 25% of known species of polistine wasps (Hymenoptera: Vespidae; Polistinae), is probably the most speciose genus in the subfamily (Arévalo *et al.*, 2004; Silveira, 2008). Among its most salient features is a great diversity of nest structure within the general pattern of independent-founding polistine wasps: a single open comb of carton cells (Wenzel, 1991). As in the other large polistine genus, *Polistes*, the (usually single) nest petiole may be excentric (new cells mostly added on one side, with the comb descending from one side of the petiole) or centric (with new cells added approximately symmetrically on all sides, and usually with the comb face in a horizontal orientation).

Mischocyttarus alfkenii (Ducke) is reported from widespread localities in South America, east of the Andes and north of the Amazon (Richards, 1978: 343–345). Although it has escaped explicit comment, remarks in the literature suggest that this species makes both excentric- and centric-petiole nests. Ducke (1905), in particular, illustrated two nests from the lower Amazon region, one of each form, associated with this species. Our own preliminary observations showed that wasps matching the description of *M. alfkenii* make nests of both forms (Fig. 1) sympatrically in Trinidad, West Indies. Furthermore, the nests differ sufficiently in colour that they are usually distinguishable at a glance: the carton of the excentric form is a yellow-tinged greyish brown, whereas that of the centric form is a reddish-tinged medium brown. Light-microscopic examination of both females and males (including male genitalia) by us and L.Y. Rusina & L. Firman (personal communication) failed, however, to reveal reliable physical differences between excentric- and centric-form adults. Here, we adopt an integrative approach to test the hypothesis that the two nest forms correspond to a pair of reproductively isolated ethospecies.

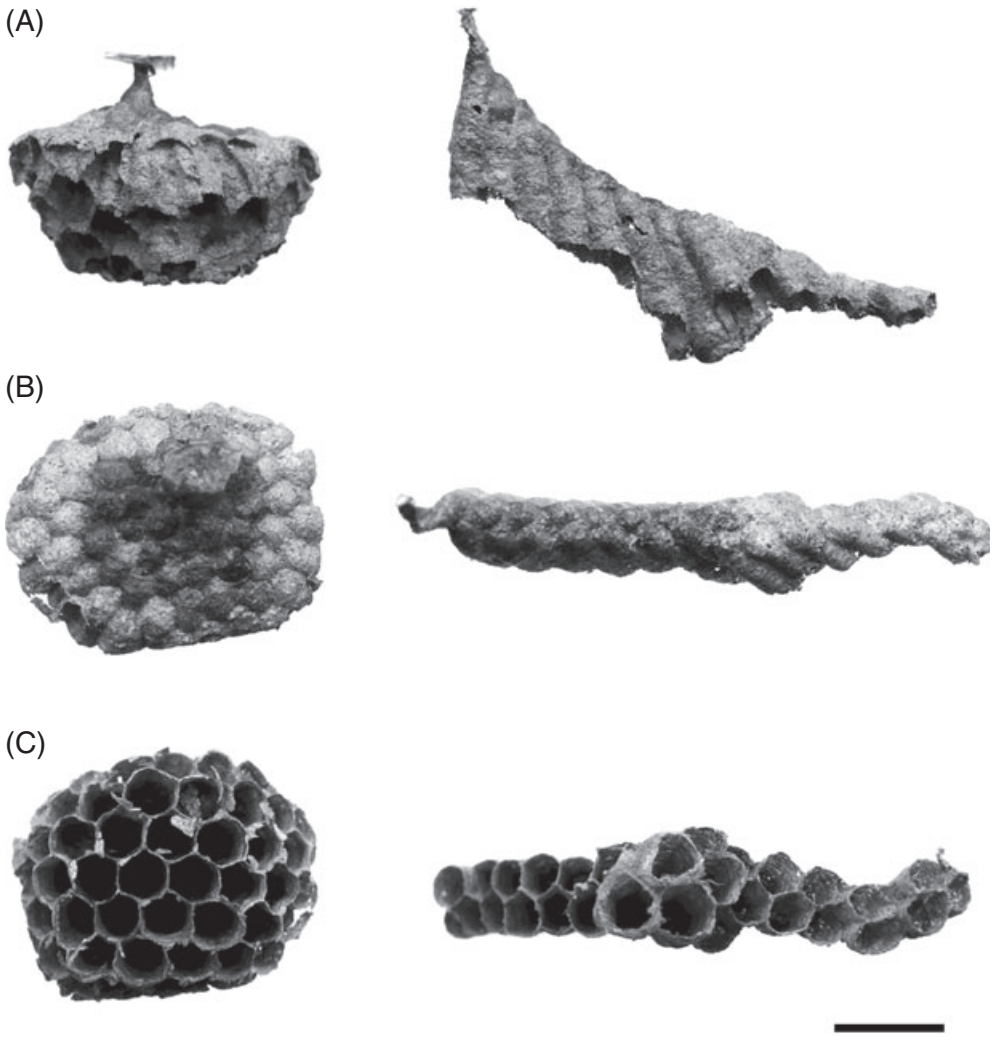

Fig. 1. Representative centric (left) and excentric (right) nest forms of *Mischocyttarus 'alfkenii'* from Trinidad in (A) lateral, (B) dorsal and (C) ventral views. Scale bar: 1 cm.

Material and methods

Between February 2005 and October 2007, we collected 36 females of *M. 'alfkenii'* from nests in north-western Trinidad, West Indies: 11 females from six excentric nests and 25 females from 11 centric nests (Table S1).

Fragments of two mitochondrial genes, *16S* ribosomal RNA (*16S*) and COI, were PCR amplified and sequenced for phylogenetic analysis. These genes share the advantage of rapid mutation rates (Knowlton & Weigt, 1998; Whitfield & Cameron, 1998) and maternal haploid inheritance, allowing for faster lineage sorting and greater phylogenetic resolution of recently diverged groups (Moore, 1995). Primers 16SWa and 16SWb (Dowton & Austin, 1994) amplified a ~520-bp fragment of *16S* with several indels, and COI primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) amplified a 658-bp fragment.

DNA was extracted from thoracic muscle tissue using the standard protocol of the DNeasy Tissue Kit (Qiagen, Valencia, CA). Gene fragments were amplified using Eppendorf HotMaster *Taq* and the following PCR protocol: 5-min initial denaturation at 94°C, 35 cycles of 1-min denaturation at 94°C, 1-min annealing at 48°C (*16S*) or 52°C (COI), and 1-min elongation at 68°C (*16S*) or 72°C (COI), followed by a final 5-min elongation at 68°C (*16S*) or 72°C (COI). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) with standard protocol. Sequencing reactions were performed for both forward and reverse strands using BigDye v3.1 (Applied Biosystems, Foster City, CA), and the resulting products were sequenced at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois. Forward- and reverse-strand sequences were assembled into contigs in BIOEDIT (Hall, 1999) and aligned using the default parameters of CLUSTAL W multiple alignment (Thompson *et al.*, 1994). Voucher specimens are retained in the collection of one of the authors (S.A.C.).

Initial trials were single-blind tests, in which one pair of individuals from each of three excentric and centric nests were sequenced and analysed (by T.K.O.) without knowledge of the nest forms. Blind analysis of these 12 specimens yielded two distinct groups corresponding to the two nest forms, which justified expanding our sampling but without maintaining blind testing.

To assess the relative magnitude of genetic distances among *Mischocyttarus* species, we analysed COI and *16S* sequences from eight additional species [*Mischocyttarus* nr. *collarellus* Richards, *Mischocyttarus immarginatus* Richards, *Mischocyttarus injucundus* (Saussure), *Mischocyttarus mastigophorus* Richards, *Mischocyttarus melanarius* Zikán, *Mischocyttarus mexicanus* (Saussure), *Mischocyttarus pallidipectus* (Smith) and *Mischocyttarus phthisicus* (Buysson)] and a population of *M. 'alfkeni'* from outside of Trinidad (Arévalo *et al.*, 2004; Hines *et al.*, 2007), representing five of the 11 currently recognized subgenera (Silveira, 2008) (Table S1).

We also tested whether excentric- and centric-form wasps could be distinguished on the basis of differences in wing venation. Geometric morphometrics are a powerful method for detecting subtle shape differences among individuals, where shape is defined as the configuration of morphological landmarks (Adams *et al.*, 2004). The utility of this approach has previously been demonstrated in studies of closely related braconid species (Baylac *et al.*, 2003; Villemant *et al.*, 2007) and honey bee populations (Miguel *et al.*, 2010). Right forewings of the 36 '*M. alfkenii*' specimens used for genetic analyses were removed at the base, slide-mounted under glycerol, and photographed through a dissecting microscope. Seventeen homologous landmarks were selected at the intersections or distinct angles of wing veins (Fig. 2), and their coordinates recorded using TPSDIG (Rohlf, 2004). All further data manipulations were performed in IMP (Sheets, 2002). Raw coordinates were superimposed with a generalized Procrustes algorithm to eliminate all variation resulting from scaling, rotational and translational differences among individuals. The deviation of an individual's wing shape from the mean shape of their nest form (partial warp) was calculated for all individuals and a canonical variate analysis (CVA) was conducted upon partial warp scores. Bartlett's test ($P < 0.05$) identified the number of significant canonical axes, and a MANOVA was used to test for differences between nest forms. A classification algorithm using Mahalanobis distances based upon the approach of Cornuet *et al.* (1999) and implemented in CVAGEN 6 (Sheets, 2002) assigned individuals to either excentric or centric groups. Automated assignments were compared with known nest membership to assess the robustness of shape differences between nest forms.

Shape change implied by the first two canonical variates was visualized using a thin-plate spline. At each landmark, vectors indicated the degree and direction of difference between the mean shapes of each nest form, and an underlying grid illustrated how a virtual plane would deform in transition from the mean shape of one form to the other. We noted areas of greatest difference between groups and inspected these for potential diagnostic characters.

Results

The *16S* and COI divergences among excentric-form individuals (distance: *16S* = 0–0.19%; COI = 0–1.07%) were slightly greater than among centric-form individuals (distance: *16S* = 0–0.09%; COI = 0–0.53%), which were nearly invariant. Sequence divergences between the two nest forms

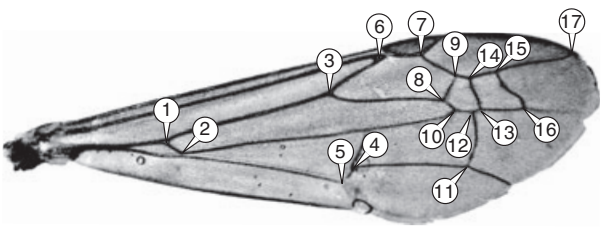

Fig. 2. Positions of 17 landmarks used in geometric morphometric analyses of the *Mischocyttarus 'alfkenii'* forewing.

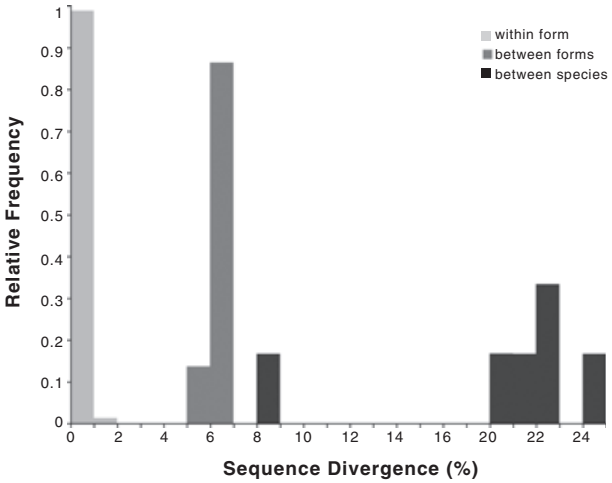

Fig. 3. Histogram of pairwise genetic distances in *16S* within nest forms of *Mischocyttarus 'alfkenii'*, between nest forms of *M. 'alfkenii'* and between *Mischocyttarus* species.

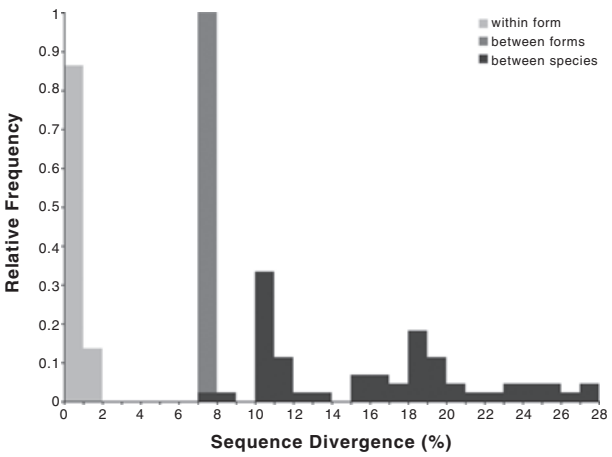

Fig. 4. Histogram of pairwise genetic distances in cytochrome *c* oxidase subunit I (COI) within nest forms of *Mischocyttarus 'alfkenii'*, between nest forms of *M. 'alfkenii'* and between *Mischocyttarus* species.

are comparable with those between the least different pairs of *Mischocyttarus* species used in this study (maximum between-form distance, *16S* = 3.23%, COI = 7.88%; minimum between-species distance, *16S* = 4.25%, COI = 7.01%) (Figs 3–4). The lengths of three out of 14 indels found in the *16S* alignment differed consistently between excentric and centric forms, whereas the other indels were invariant and identical in *M. 'alfkenii'* (Table 1). Sequences are available in GenBank under accession numbers HQ163801–HQ163868.

The CVA of partial warp scores identified a single canonical variate that effectively discriminated between groups (MANOVA: $\lambda = 0.0079$; $\chi^2 = 87.2123$; df = 30; $P > 0.00001$) (Fig. 5). Figure 6 displays shape differences between excentric and centric forms implied by variation along the first canonical

Table 1. Position and diagnostic sequences of indels in *16S* alignment.

Aligned position	Nest form	Sequence
150–159	Excentric	TCATTACATT
	Centric	TCATTAATT
455–464	Excentric	AAACTAATT
	Centric	AAATTTAATT
490–499	Excentric	TATAATTCAA
	Centric	TACAATCAA

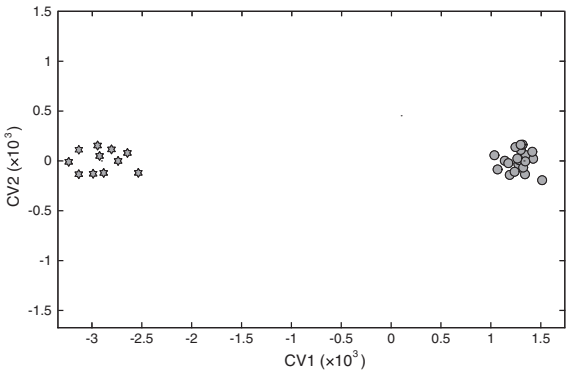

Fig. 5. Plot of partial warp scores on the first two canonical variates demonstrating two distinct clusters corresponding to excentric and centric forms of *Mischocyttarus 'alfkenii'*; stars, excentric; circles, centric.

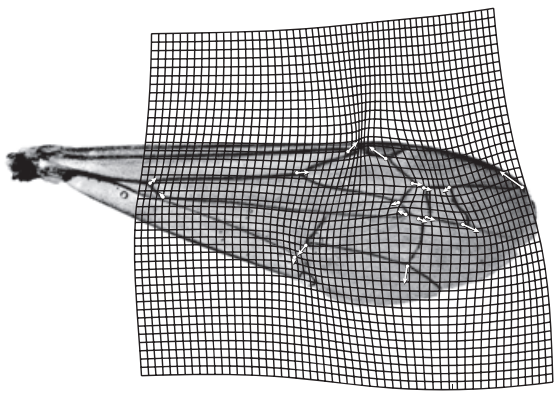

Fig. 6. Thin-plate spline overlain upon wing of *Mischocyttarus 'alfkenii'*, illustrating shape differences associated with the first canonical variate. Grid deformation and vectors illustrate a virtual transformation from mean shape of excentric-form wing to mean shape of centric-form wing. Note the particularly large shape differences (longer vectors) at landmarks near wing extremities.

variate. Shape differences between the two nest forms were distributed throughout the wing, with notable differences in the positions of landmarks at the wing extremities. Although we were unable to develop diagnostic characters for subjective discrimination of the two nest forms based on shape, automated CVA classification tests correctly assigned all 36 specimens to their respective nest forms with >95% confidence.

Discussion

Analyses of *16S* and COI fragments, diagnostic indels, relatively large sequence divergences, and geometric morphometric analysis of wing characters corroborate the hypothesis that the excentric and centric forms of *M. 'alfkenii'* in Trinidad are distinct species, and are consistent with relatively recent speciation.

It is not known whether wasps matching the description of *M. 'alfkenii'* in the rest of its broad geographic range represent these same two species. The report of both excentric and centric nests near the southern end of the range (Ducke, 1905) is consistent with the simple scenario of a division into two species, followed by the spread of each resulting in sympatry over much of the combined range. However, present evidence does not exclude the possibility of a larger species complex.

The gross nest structure distinction between the two forms is unequivocal, but differences in fine structure remain to be elucidated. In particular, the cause of the distinct colour difference in their carton is not known. Preliminary observations of the at-nest behavioural repertoire of adult females have revealed no differences (Scobie, 2003; C.K. Starr, unpublished data).

Our results provide convincing evidence for the species-level nature of both sympatric forms of *M. 'alfkenii'* in Trinidad. They do not, in themselves, resolve the problem of which of the two forms should retain the name *M. alfkenii*. However, as part of his description, Ducke (1904: 362) remarked (in translation) that, 'The nest is the same as that of the preceding species, although a little larger'. The preceding species in his paper was *Mischocyttarus surinamensis* (Saussure), the nest of which consists of a single comb of yellow-tinged light-brown carton with a distinctly excentric petiole (Ducke, 1904; C.K. Starr, personal observation); in other words, it closely resembles that of the excentric form of *M. 'alfkenii'*, the cells of which are, indeed, somewhat larger than those of *M. surinamensis*. Furthermore, the two described subspecies – *M. alfkenii excrucians* Richards and *M. alfkenii trinitatis* Richards – both have excentric nests (Richards, 1945: figs 130–131). Accordingly, regardless of whether these and the typical form are conspecific, we conclude that the centric form of *M. 'alfkenii'* is not conspecific with any of these, and is as yet undescribed. A description follows in the Appendix.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-3113.2011.00575.x

Table S1. Specimens used in this study. Asterisks indicate specimens in the initial tests, in which the experimenter was blinded to the nest form.

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Appendix

Mischocyttarus baconi sp.n. Starr

(Figs 1, 7)

Diagnosis. The new species shares the characters of subgenus *Monocyttarus*: small wasps, neither exceptionally robust nor exceptionally slim for the genus; pronotum with a distinct fovea; pronotal keel very low or interrupted centrally; male mandible with four teeth; ocelli forming an almost equilateral triangle (Fig. 7A). Adults are almost indistinguishable from *Mischocyttarus alfkenii* Ducke, at least in Trinidad, where the two are sympatric. The only distinguishing physical characters of adults known to us are minor morphometric features of the forewings (Fig. 6), although the species can be reliably separated through genetic characters. On the other hand, the two make distinct nests (Fig. 1). That of *M. alfkenii* has a yellowish grey-brown carton and an excentric petiole, whereas that of *M. baconi* has a reddish brown carton and a centric petiole.

Female. Forewing length from base of costal vein 9.5–10.5 mm. Pronotal fovea very small but distinct, with only a slight prominence in front of it; pronotal keel very low in centre, sharper at sides, with distinct 'shoulders'; claws of mid and hind tarsi asymmetrical, inner (or hind) claw thicker and longer; pronotal furrow shallow with no more than a weak

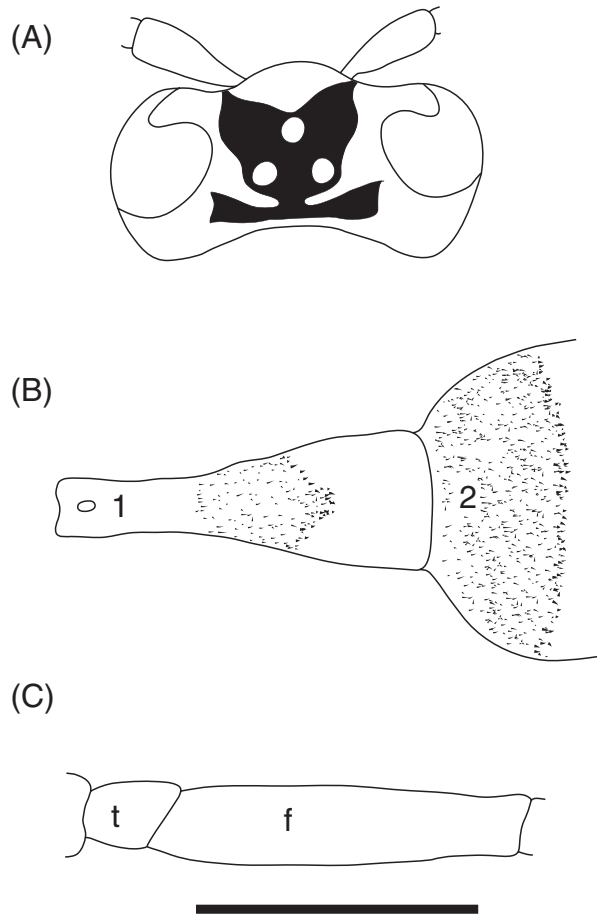

Fig. 7. Features of *Mischocyttarus baconi* female, all in top view. (A) Head, to show dark markings and ocellar triangle. (B) Gastral terga 1–2, to show shape of petiole and dark markings; 1, tergum 1; 2, tergum 2. (C) Hind femur, for length comparison with tergum 1; f, femur; t, trochanter. Scale bar: 2 mm.

central keel in lower half; first half of gastral tergum 1 (to spiracles) forming an almost parallel-sided petiole about half as wide as tergum at apex (Fig. 7B); tergum 1 not noticeably shorter than hind femur (Fig. 7B, C). Ground colour yellow. Orange–brown: antennae, humeral stripes. Brown–black: area around ocelli, extending as narrowing stripes down to or close to antennal sockets; stripes along vertex inward from each eye to meet or almost meet behind ocelli (Fig. 7A); central and two lateral longitudinal stripes on mesoscutum; sometimes narrow central line in lower part of propodeum furrow; indistinct mark on gastral tergum above bases of terga 2–5 (Fig. 7B).

Male. Very like female, brown–black bases of gastral terga 2–6.

Holotype. ♂, TRINIDAD, W.I. St Augustine 10°38′N, 61°24′W, 12.ii.2011, C.K. Starr, Nest series no. 2211. Deposited in the American Museum of Natural History (AMNH), New York.

Paratypes. Four ♂ and ten ♀ from the same colony (nest series no. 2211) as the holotype. Deposited in the AMNH, Natural History Museum (London), Museu Paulista (São Paulo) and Land Arthropod Collection of the University of the West Indies. The nest and several larvae from nest series no. 2211 are likewise deposited in the AMNH.

Etymology. The species is named in honour of Peter R. Bacon (1938–2003), late Professor of Zoology at the University of the West Indies and an outstanding all-around naturalist.