MORPHOLOGICAL AND DNA SEQUENCE DELINEATION OF TWO PROBLEMATIC SPECIES OF *CERATINA* (HYMENOPTERA: APIDAE) FROM EASTERN CANADA

S. M. REHAN[1], M. H. RICHARDS
Department of Biological Sciences, Brock University
St. Catharines, Ontario, Canada, L2S 3A1
email: sandra.rehan@gmail.com

Abstract

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Ceratina are small, twig-nesting carpenter bees of cosmopolitan distribution. In Eastern Canada, *C. calcarata* and *C. dupla* live in sympatry, and females are so similar morphologically that many previous species lists combined both taxa into one category. The problem is that the description of the traditional morphological character used to distinguish between them is easily misinterpreted, placing emphasis on puncture abundance rather than distribution. In this paper we propose a clearer description of this character and provide confirmation using scanning electron microscopy to confirm that puncture placement takes precedence over abundance. We tested the feasibility of the new character for distinguishing between these two species, by testing it on non-entomologists, who used it with 87% accuracy. Moreover, DNA sequencing of the cytochrome oxidase subunit 1 gene for the same specimens revealed 1.29% sequence divergence between the two morphs, providing additional support that *C. calcarata* and *C. dupla* are indeed distinct species that can be distinguished morphologically.

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Introduction

Ceratina are small, often abundant, carpenter bees that nest in pithy cores of dead broken stems and twigs (Michener 1985). Although they are found across the globe, they have rarely been studied in detail, and much remains unknown about their basic biology and behaviour (Michener 1979). The genus comprises 19 subgenera, of which three are found in North America (Michener 2000). In eastern Canada, from Ontario to the Maritimes, only three species are found, all members of the subgenus Zadontomerus: *Ceratina calcarata* Robertson, *C. dupla* Say, and *C. strenua* Smith (Daly 1973). These three species are also found throughout the eastern United States (Daly 1973). Although all three species often occur in sympatry in more southerly parts of their range, *C. strenua* is rarely found in Canada and southern Ontario is likely the northern edge of its range. However, in most of

[1] Author to whom all correspondence should be addressed.

eastern Canada, *Ceratina calcarata* and *C. dupla* are sympatric.

Morphologically, *C. strenua* is easily distinguishable from *C. dupla* and *C. calcarata*, most notably by its smaller size. However, separating *C. dupla* and *C. calcarata* is problematic: although there are recognizable differences among males, females are remarkably similar. As a result, many entomologists have given up trying to differentiate female *C. calcarata* and *C. dupla* in their samples and instead combine both taxa into one category for their species lists (Clinebell 2002; Mitchell 2002; Reed 1995). Although it is likely that both species occur in sympatry throughout much or all of their range, it is difficult to discern whether one species may predominate in an area as floral records and geographic distributions rely on males of each species (Daly 1973). The inability to differentiate females of these problematic species has also negatively affected studies on their life history. For example, Johnson's (1988) study on *Ceratina calcarata* sex ratios in Indiana, omitted all-female broods because he was only confident in identifying males of the species.

Currently, eastern North American *Ceratina* are identified using Mitchell (1962). In this key, females of *C. dupla* and *C. calcarata* are distinguished by comparison of the number of punctures in the area of the scutum between the notaulices: *C. dupla* have numerous punctures whereas *C. calcarata* have few, if any. The problem is that this character description is easily misinterpreted, and there is disagreement about whether the distinguishing character works. However, this taxonomic problem is difficult to resolve without independent evidence that the specimens being examined are indeed correctly identified members of the two species.

In this paper, we propose a simpler description of the traditional character used to distinguish female *C. calcarata* and *C. dupla*. Following thorough examination and description of the character based on scanning electron microscopy (SEM), we test this character's reliability by assessing non-entomologists' ability to use it to identify the two species. In addition, we provide genetic evidence that *C. calcarata* and *C. dupla* are indeed separate species based on comparisons of their DNA sequences for the cytochrome oxidase subunit 1 gene for the two species, allowing us to independently distinguish the species identities of specimens used to validate our proposed taxonomic character.

Methods

Sampling and Identification

Specimens were collected in pan traps during the summers of 2005 and 2006 from St. Catharines, Ontario. Pan traps used were blue, white and yellow plastic bowls (6 oz; SOLO PS6-0099) filled with soapy water and spaced ten metres apart in transects. Insects land on the water surface and drown, as the soap in the water acts as a surfactant. All specimens were subsequently preserved in 70% ethanol. *Ceratina* specimens were initially identified using Mitchell (1962). Following identification, most samples were returned to 70% ethanol for storage, but 24 specimens (12 females from each species) were dissected for SEM imaging and DNA extraction (see below). With legs and wings removed, the thoraces of these specimens were used for SEM imagery of the dorsal side, the heads and abdomens being reserved for DNA.

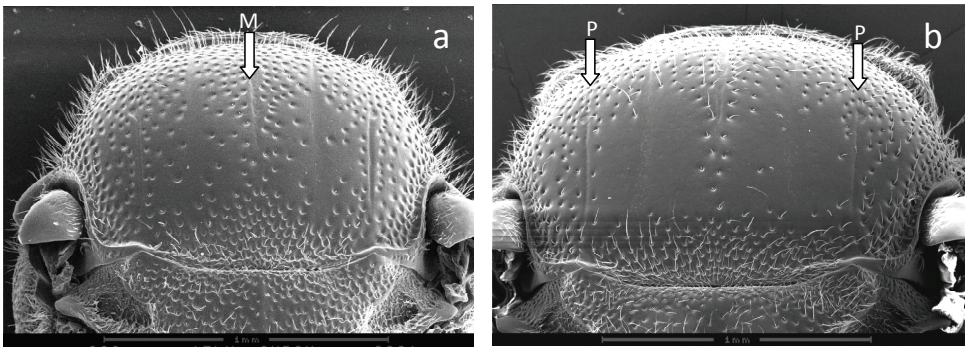

FIGURE 1. SEM images of female *Ceratina* thoraces. a. *C. dupla*, more punctate with two or more complete longitudinal rows of punctures inside the parapsidal lines and around the medial line. Arrow indicates the medial line (M). b. *C. calcarata*, less punctate with one to two incomplete longitudinal rows of punctures inside the parapsidal lines and around the medial line. Arrow indicates the parapsidal line (P).

SEM images were obtained using an AMRAY 1600 Turbo scanning electron microscope. Specimens were mounted onto a carbon adhesive tab and silver paint was applied to the specimen edges to aid in sample conductivity. Using secondary electron scintillation detector and 15kV accelerating voltage, images were processed using ORION Digital Image grabbing software (version 6.51). SEM images were used to confirm the validity and consistency of the revised morphological character developed to distinguish between female *C. calcarata* and *C. dupla*. SEM images and type specimens have been retained in the collection of M.H. Richards at Brock University.

Taxonomy Test for Non-Taxonomists

Since Mitchell's (1962) key is sufficient to identify male *C. calcarata* versus *C. dupla* and *C. strenua* of both sexes these characters were not altered from the original key descriptions. However, due to the difficulty distinguishing female *C. calcarata* versus *C. dupla* a new character was developed to differentiate females of the two species. Following scanning electron microscopy, SEM photographs of female *C. calcarata* and *C. dupla* were given to 24 naive non-entomologists along with 15 pinned specimens of *C. calcarata* and 13 pinned *C. dupla*. Bees were randomly labeled from 1 to 28 and placed in a box. Identifiers were given the box and a dissecting microscope and shown where on the bee the scutum was located (most volunteers had never seen a bee under a microscope before). Identifiers then assigned each specimen to either *C. calcarata* or *C. dupla* using figures 1 and 5, as well as by reference to a written description of the character (key to females, couplet 2). Identifiers were later graded against the original identifications by the senior author.

DNA Sequence Analysis

Total genomic DNA was isolated from the head and abdominal segments of the 24 SEM specimens, using the Sigma-Aldrich GenElute Mammalian Genomic DNA

Purification Kit. Manufacturer's instructions were followed with one additional step, in which an extra centrifugation was incorporated after proteinase K digestion to remove bee fragments that might clog the spin columns. Portions of the mitochondrial gene COI were amplified using primers mtd-8 and 12 (Simon et al. 1994; University of British Columbia Biotechnology Laboratory, Vancouver) in 20 µl reactions with 2.5 mM MgCl2, 200 µM dNTPs, 2.0 µM each primer, 0.25 U Sigma Jumpstart Taq polymerase, and 2 µl of Taq reaction buffer (supplied with the enzyme). PCR reactions were as follows: initial heating to 94°C for 5 min, followed by 30 repetitions of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5min. Amplification products were purified by ethanol precipitation and analyzed on 0.8% agarose gels containing 10mg/ml ethidium bromide. DNA sequencing was carried out at McGill University and Genome Quebec Innovation Centre in Montreal, using the same primers. Sequences were edited using BIOEDIT (Hall 1999) and aligned using CLUSTAL (Thompson et al. 1994) using default settings with the exception of gap open penalties increased to 50. All sequences have been deposited in GenBank under accession numbers EF534228-EF534247. We used Analysis of Molecular Variance (AMOVA) as implemented in Arlequin 3.11 (Excoffier et al. 2005) to compare genetic variation within and among specimens identified with the revised morphological character.

Results

Morphological Differences

Using light microscopy and SEM images in combination with corresponding COI sequences revealed the subtle yet consistent dimorphism between these species. *Ceratina calcarata* are less punctate than *C. dupla*, just as Mitchell (1962) describes, but the placement of punctures around the parapsidal and medial lines is better at differentiation than their abundance (key to females, couplet 2). This difference was confirmed by an 87% correct identification rate among the non-entomologists tested.

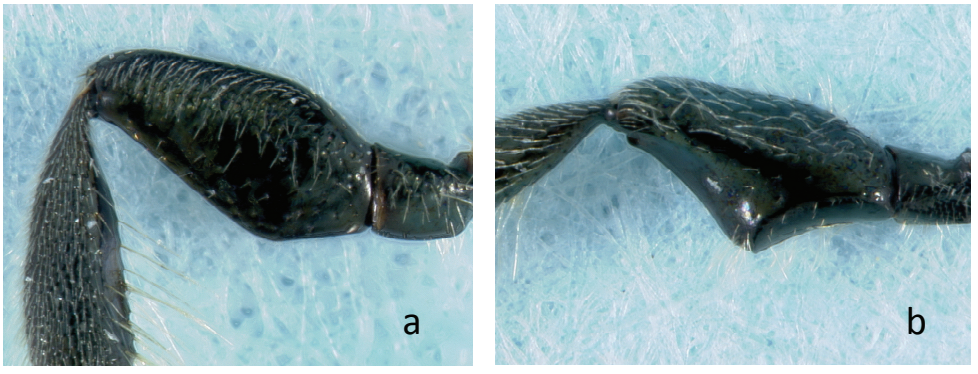

FIGURE 2. Hind femurs of male *Ceratina*. a. *C. dupla,* hind femur somewhat dilated toward base, but without a median projection. b. *C. calcarata*, hind femur with a median,

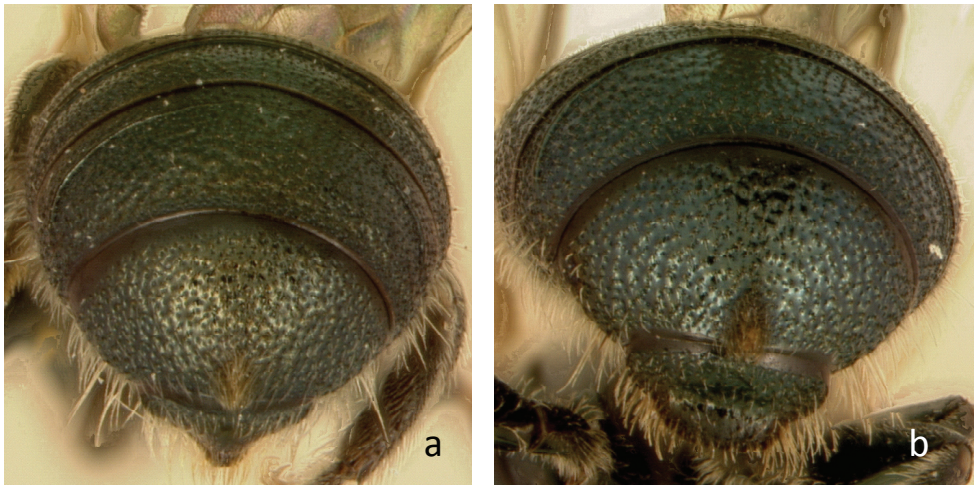

FIGURE 3. Tergum 7 of male *Ceratina*. a. *C. strenua,* carina of tergum 7 very narrow. b. *C. calcarata*, carina of tergum 7 broad.

FIGURE 4. Front tibia of female *Ceratina.* a. *C. strenua*, front tibia with a basal ivory stripe. b. *C. calcarata*, front tibiae with at most a basal ivory spot.

FIGURE 5. Thoracic puncture dimorphism of female *Ceratina*. a. *C. dupla*, more punctate with two or more complete longitudinal rows of punctures inside the parapsidal lines and around the medial line; central area between medial line and parapsidal lines always punctate in posterior half of mesoscutum. b. *C. calcarata*, less punctate with one to two incomplete longitudinal rows of punctures inside the parapsidal lines and around the medial line; areas between medial line and parapsidal lines usually impunctate with, at most, a couple of central punctures in posterior half of mesoscutum.

DNA Sequence Divergence Between *Ceratina calcarata* and *Ceratina dupla*

We compared 774 base pairs of cytochrome oxidase one (COI) for 11 individuals of *C. calcarata* and 9 of *C. dupla* that had been previously identified using the morphological character described above. Sequences fell cleanly into two groups, distinguished by 7 fixed differences between the two species, providing evidence that *C. calcarata* and *C. dupla* are indeed genetically isolated in sympatry. Pair-wise comparisons among all individuals revealed significantly greater sequence divergence between species (mean pairwise difference, 1.29%) than within species (*C. calcarata*, 0.46 %; *C. dupla*, 0.029 %; AMOVA: $F_{ST} = 0.80993$, df= 1,18, $p<0.00001$).

Discussion

The biological species concept defines species as groups of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups (Mayr 1942). Previous studies have provided some evidence that *C. calcarata* and *C. dupla* are separate species due to a lack of heterozygotes at multiple isozyme loci in natural populations (Hung & Norden 1987). Our study confirms that the two species are indeed

genetically as well as morphologically distinct. Although hybridization is thought to be possible in artificial environments such as greenhouses and flight cages (Hung & Norden 1987), the fact that these sympatric species are genetically distinct suggests differences in life history and behaviour are possible.

Limited research has been done on the life history and behaviour of natural populations of *Ceratina* in North America. Grothaus (1962) found that *C. calcarata* prefer to nest in open areas, usually in older, drier stems, while *C. dupla* prefer shaded areas and recently dead stems. This life history difference between sister species parallels a remarkably similar finding in two Asian species of the subgenus *Ceratinidia, Ceratina flavipes* and *C. japonica*, which are sympatric throughout Japan. Females of *C. japonica* prefer to nest in bushes or sparsely wooded areas, remaining shaded and humid, whereas *C. flavipes* nest in comparatively open, dry areas (Sakagami & Maeta 1977). As with *C. calcarata* and *C. dupla*, males of the two Japanese species are quite distinct, but females are very difficult to distinguish (Shiokawa 1963; Yasumatsu & Hirashima 1969).

Previous studies have shown COI to be a useful indicator gene to differentiate even morphologically indistinguishable sister species. *Halictus ligatus* and *H. poeyi* are sympatric, eusocial species with slightly different phenologies (Dunn et al. 1998). They exhibit approximately 0.4% COI sequence variation within species and 4 to 5% between species (Danforth et al. 1998). The degree of divergence between these two *Halictus*, is comparable to that between the aforementioned *C. flavipes* (Genbank accession no. AY250190) and *C. japonica* (AY250192) (Cronin 2004); comparison between these two sequences reveals about 4.1% divergence. Both these species pairs are considerably more divergent than *C. calcarata* and *C. dupla* appear to be.

Morphologically similar females are found in various Old World subgenera of *Ceratina*, including *Ceratinidia* (Shiokawa 1963; Yasumatsu & Hirashima 1969), *Ceratina sensu stricto* and *Neoceratina* (Hirashima 1971), and numerous New World *Zadontomerus* (Daly 1973). Although males of this genus have proven simple to distinguish based on femoral tooth projections, tergal processes, elaborate genitalia and sternal suture modifications, it seems that females across the genus *Ceratina* have more understated distinctions. Eventually, closer examination for subtle differences among sympatric species, perhaps aided by molecular systematics approaches, may reveal consistent female key characters across the genus.

Key to species

Males

1. Hind femur somewhat dilated toward base, but without a median projection, the greatest width near the base no more than a third its length (Figure 2a)..... .....***C. dupla*** Say
– Hind femur with a median, triangular projection, femoral width at this point about equal to half its length (Figure 2b) ……...…….…..…...2
2. Carina of tergum 7 very narrow, fully as long as broad, and not over a fourth as broad as the tergum (Figure 3a)……***C. strenua*** Smith
– Carina of tergum 7 at least twice as broad as long, fully half as broad as width of the tergum (Figure 3b) ……..…….....…***C. calcarata*** Robertson

Females

1. Front tibia with a basal ivory stripe; small (body length 5-6 mm) (Figure 4a)..... ……..……***C. strenua*** Smith
– Front tibia with at most a basal ivory spot; usually larger (body length 7 mm or more) (Figure 4b) ...……...…….…..2
2. One to two complete and up to four incomplete longitudinal rows of punctures inside parapsidal lines and around medial line; area between medial line and parapsidal lines always punctate in posterior half of mesoscutum (Figures 1a and 5a)…….....
...……...…… ***C. dupla*** Say
– One to two incomplete longitudinal rows of punctures inside the parapsidal lines and on either side of the medial line; areas between medial line and parapsidal lines usually impunctate with, at most, a couple of central punctures in posterior half of mesoscutum (Figures 1b, 5b)....……..……***C. calcarata*** Robertson

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