# **Article**

# Morphological and molecular delineation of a new species in the *Ceratina dupla* species-group (Hymenoptera: Apidae: Xylocopinae) of eastern North America[1]

SANDRA M. REHAN[2] & CORY S. SHEFFIELD[3]

[1]*This paper is contribution #11 from the Canadian Pollination Initiative*

[2]*Department of Biological Sciences, Brock University, 500 Glenridge Avenue, St. Catharines, Ontario, Canada L2S 3A1.*
*E-mail: sandra.rehan@gmail.com*

[3]*Department of Biology, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3.*
*E-mail: cory.silas.sheffield@gmail.com*

## Abstract

DNA barcoding is used to verify characters to morphologically differentiate genetically distinct species of eastern North American small carpenter bees, *Ceratina*. Here we reveal that the common eastern North American species, *Ceratina dupla s. l.*, is actually three separate species based on fixed differences in DNA barcode sequences and morphological characters. This study adds a new species, *C. mikmaqi* Rehan & Sheffield, to the *Ceratina dupla* species-group of eastern North America, and raises another form, *C. floridana* formerly *C. dupla floridana*, to full species. Temporal niche partitioning between *C. dupla* and *C. mikmaqi* and geographic isolation of *C. floridana* further support the division of the *C. dupla s. l.* group into three species. A diagnosis and description of the new species are provided, as is a key for eastern North American species of *Ceratina*.

**Key words:** small carpenter bee, DNA barcodes, cryptic species, *Ceratina (Zadontomerus), Ceratina floridana, Ceratina mikmaqi*

## Introduction

The small carpenter bees, *Ceratina* Latreille (Apidae: Xylocopinae), are a common and diverse group (Michener 2007). There are 21 described subgenera in this cosmopolitan genus, 16 of which are endemic to the Old World and five to the New World (Terzo 2000; Michener 2007). In America north of Mexico there are three subgenera containing 22 described species (Daly 1973). The subgenera *Ceratinula* Moure and *Zadontomerus* Ashmead are indigenous to North America, whereas *C. (Euceratina) dallatorreana* Friese was introduced to California from the Mediterranean region (Daly 1966). The subgenus *Ceratinula* is most diverse in Central and South America (Michener 1954; Moure 2007) but two species, *C. arizonensis* Cockerell and *C. cockerelli* H.S.Smith are found in the southern United States (Daly 1973). In contrast, the subgenus *Zadontomerus* is most diverse and widespread in North America, with 18 of the 25 described species occurring north of Mexico (Daly 1973; Michener 2007). Among the North American *Zadontomerus* species, there is a clear geographic division between morphological species groups, with 14 species found west and three species found east of the 104th meridian (Daly 1973).

In eastern North America the four recognized species are *Ceratina (Ceratinula) cockerelli* H. S. Smith, *C. (Zadontomerus) calcarata* Robertson, *C. (Z.) dupla* Say, and *C. (Z.) strenua* F. Smith (Daly 1973). *Ceratina cockerelli* is a small, black, mostly impunctate bee easily distinguished from the larger, metallic blue-green *Zadontomerus* species found in the east, and has a restricted distribution; found in Texas, Louisiana, Georgia and Florida. The three recognized eastern *Zadontomerus* species often occur in sympatry in more southern parts of their range, but *C. strenua* is rarely found in Canada, with southern Ontario likely the northern edge of its range. In contrast, both *C. calcarata* and *C. dupla* are largely sympatric and occur from Florida, north to Ontario and east to Nova Scotia (Sheffield *et al.* 2003). Of the three *Zadontomerus* species in the east, *C. strenua* is easiest to distinguish due to its tergal sculpturing and distinct maculations, and it is generally smaller than the other *Zadontomerus* species in

the east. Though males of *C. calcarata* and *C. dupla* are easily distinguished (Mitchell 1962; Daly 1973), differentiating females is difficult and often unattempted in faunistic surveys (Ginsberg 1984; Russell *et al.* 2005; Winfree *et al.* 2007; Tuell *et al.* 2008, 2009). Recently, morphological characters for separating females of these two species were clarified by integration of a molecular approach (Rehan & Richards 2008). Additionally, Mitchell (1962) described a deep blue, densely punctate subspecies of *C. dupla* from the southeast, *C. dupla floridana*, which has been recognized by some (Leuck & Hammons 1969; Deyrup *et al.* 2002), but regarded as an ecomorph rather than a genetically distinct variant by others (Daly 1973; Grissell 1976).

Traditional taxonomic methods used for bee identification rely on recognition and description of morphologically distinct characters, though for many species, sexual dimorphism often makes it difficult to associate sexes with confidence. For many cavity nesting bees, this is partially resolved by collection of nests with adult bees, which allows the association of males and females of each species (Sheffield & Westby 2007), but is difficult or not possible for other sampling approaches. The lack of distinguishing morphological characters is a common problem in many bee species (Packer *et al.* 2009), and this taxonomic impediment has negatively affected bee diversity surveys, floral associations and general biological studies of *Ceratina* when females cannot be associated with conspecific males (Johnson 1988; Reed 1995; Clinebell 2002; Mitchell 2002). Difficulty distinguishing sympatric females is common across this genus. Males present more overt and easily recognizable morphological variations while females have more subtle differences (Yasumatsu & Hirashima 1969; Rehan & Richards 2008).

Recent advances in molecular techniques have allowed independent assessment of insect species distinctions in the absence of obvious morphological characters. DNA sequencing of a standard gene region or "DNA barcoding" (Hebert *et al.* 2003a) can be helpful in species diagnosis. Sequence divergences are typically lower among individuals of a species than between closely related species (Moore 1995; Avise & Walker 1999; Hebert *et al.* 2003b; Gibbs 2009, 2010). DNA barcoding provides an independent data set to detect genetically discrete units and facilitates the discovery or verification of morphological differences among similar species (Packer *et al.* 2009). Studies on a wide array of taxonomic groups have resulted in the suggestion that there should be 2% average sequence divergence between closely related species (Hebert *et al.* 2003b). However, sequence divergences of less than this standard level have been found among morphologically discrete species (Gibbs 2009, 2010).

A recent DNA barcoding study of the bees of Nova Scotia, Canada revealed a putative new species of *Ceratina* ("*Ceratina* sp.") showing strong molecular and morphological affinities to both *C. dupla* and *C. calcarata*. All three species show less than 2% mean sequence divergence to nearest neighbours, but form discrete clusters nonetheless (Sheffield *et al.* 2009). Subsequently, we recognized distinct, albeit subtle, morphological differences among *C. dupla* specimens; these differences were later supported through DNA barcoding. In addition, a recent study of *Ceratina* in southern Ontario, found distinct behavioural and phenological patterns correlated to sequence differences (Vickruck *et al.* in press). The molecular data suggested the need for a formal re-evaluation of the taxonomic status of eastern *Zadontomerus* species. The objective of this study was to use DNA barcoding to verify morphological characters for an independent assessment of effective taxonomic units in eastern *Ceratina* species, and to provide morphology-based key to differentiate the genetically distinct species.


## Methods

*Ceratina* specimens from throughout eastern North America were used to obtain DNA for sequencing of the barcode region of cytochrome *c* oxidase subunit 1 (Herbert *et al.* 2003a,b), including both recognized subspecies of *C. dupla*. All specimen information is available on the barcode of life data system BOLD (www.barcodinglife.org), including collection locations (Fig. 1). DNA was extracted from a single leg using automated extraction protocols for 96-well plates (Ivanova *et al.* 2006). One set of primer pairs was used to amplify the DNA barcode region (LepF1 and LepR1; Hebert *et al.* 2004a). PCR and sequencing reactions followed standard Canadian Centre for DNA Barcoding (CCDB) protocols (Hajibabaei *et al.* 2005). Sequencing was performed at the CCDB at the Biodiversity Institute of Ontario at the University of Guelph using standard protocols available at URL: http://www.dna-barcoding.ca/pa/ge/research/protocols. Additional sequences from the "Bees of Nova Scotia" project on BOLD (Sheffield *et al.* 2009) were also used, including those of the putative new species in the *C. dupla* complex.

**FIGURE 1.** Species collection locations. *Ceratina floridana* indicated with black triangles, *C. dupla* in gray circles, and *C. mikmaqi* in black circles.

Sequences were downloaded from BOLD and edited using BIOEDIT (Hall 1999) and aligned using CLUSTAL (Thompson *et al*. 1994) using default settings. Sequences were all trimmed to 636 base pairs of the barcoding region of cytochrome oxidase subunit one with no gaps or missing bases. All sequences have been deposited in GenBank under accession numbers (FJ582163-FJ582182; GU707469-GU708169; HM386174; HM423191; HM905870; HM905873; JF271016-JF271101). Analysis of Molecular Variance (AMOVA) was used as implemented in Arlequin 3.11 (Excoffier *et al*. 2005) to compare genetic variation within and among putative *C. dupla* species. All three putative *C. dupla* species were compared in the full model followed by pair-wise compari-

sons of each possible pairing in subsequent AMOVA analyses. Sequences were analysed phylogenetically using the outgroup species *C. strenua*. Two tree-building methods were used. Neighbour-joining (NJ) analyses were conducted using ClustalX (Thompson *et al.* 1997) using the neighbour-joining algorithm with the Kimura 2-parameter (K2P) distance model (Kimura 1980). Branch support was assessed by bootstrapping with 500 replicates. Maximum parsimony (MP) analyses were conducted using PAUP* b4.10 (Swofford 2003). One hundred random sequence stepwise additions were used in the MP analysis, holding 10 trees at each step and with tree bisection and reconnection for searching tree space. Node support was estimated using 500 bootstrap pseudoreplicates, using the same methods as for the heuristic search, and retaining compatible groups with less than 50% bootstrap support. Morphological terminology follows Michener (2007). The following abbreviations are used: diameter (d), interspace (i), inside diameter (ID), outside diameter (OD), antennocular distance (AOD), interantennal distance (IAD), antennal flagellomere (F), metasomal tergum (T) and metasomal sternum (S).

## Results

In total, 210 DNA barcode sequences were obtained for the *C. dupla* complex across its known range, from which 27 haplotype sequences were identified. The specimens separated into three separate groups based on DNA barcode sequences (Figs. 2 and 3). Subsequently, subtle, yet distinct morphological differences were identified corresponding to the three species groups. Voucher specimens were compared to the *C. dupla s. l.* neotype (Université Laval, Quebec, as designated by Daly [1973]) and the holotype of *C. dupla halophila* Cockerell (University of Colorado Museum of Natural History) for proper designation of species names.

Pair-wise sequence divergence between putative species was low ranging from 1.11–1.75% between *C. dupla s. l.* group comparisons (Table 1). Variable sites among the three putative species showed a strong bias for the 3rd codon position (34/36 variable sites). From 2 to 7 fixed nucleotide differences were found among species (Table 1). Intraspecific sequence variation was relatively low (0.47% and 0.79%) in *C. floridana* and *C. mikmaqi* new species respectively, but high (1.43%) in *C. dupla* (Table 1). Pair-wise comparisons among all individuals revealed significantly greater sequence divergence between species than within species (full model AMOVA: $F_{ST} = 0.77029$, df = 2,103, p<0.0001; pair-wise tests: *mikmaqi-floridana* $F_{ST} = 0.86174$, df = 1, 52, p<0.0001; *floridana-dupla* $F_{ST} = 0.76364$, df = 1, 66, p<0.0001; *dupla-mikmaqi* $F_{ST} = 0.73710$, df = 1, 87, p<0.0001).

**TABLE 1.** Pair-wise sequence divergence of DNA barcodes from the *C. dupla* species group. The diagonal (bold) gives the maximum within species variation. Above the diagonal indicates the number of fixed pair wise nucleotide differences followed by the total number of variable sites in parentheses. Below the diagonal gives the minimum percent sequence divergence.

|  | *C. calcarata* | *C. mikmaqi* | *C. floridana* | *C. dupla* |
|---|---|---|---|---|
| *C. calcarata* | **1.75** | 3 (37) | 8 (28) | 5 (37) |
| *C. mikmaqi* | 1.11 | **0.79** | 5 (16) | 3 (23) |
| *C. floridana* | 1.75 | 0.95 | **0.47** | 6 (20) |
| *C. dupla* | 1.43 | 1.11 | 1.44 | **1.43** |

Phylogenetic analyses of the *C. dupla* species group also revealed three distinct, well supported clusters. The MP and NJ results were largely congruent (Figs. 2 and 3). Neighbour joining and maximum parsimony analyses strongly support (bootstrap support %) the monophyly of *C. calcarata* (68% NJ, Fig. 2; 55% MP, Fig. 3), *C. dupla* (88% NJ, Fig. 2; 78% MP, Fig. 3), *C. mikmaqi* (61% NJ, Fig. 2; 62% MP, Fig. 3) and *C. floridana* (96% NJ, Fig. 2; 95% MP, Fig. 3). *Ceratina dupla* and *C. floridana* were sister species while *C. mikmaqi* was resolved as sister to the *C. dupla* + *C. floridana* clade.

*Ceratina dupla* and *C. mikmaqi* were analyzed from locations in broadly overlapping geographic regions (Fig. 1). However, *C. dupla* had almost twice the intraspecific genetic variation of *C. mikmaqi*. Both species are found in Nova Scotia, New York, Ontario and Wisconsin. To ensure that species distinction was not attributable to genetic variation across the species' range sequence divergence of these species were compared in sympatry. If sequence divergence between *C. dupla* and *C. mikamqi* are equally distinct in sympatry as found across their range this would support the distinction of two species rather than a single species presenting two ecomorphs across its range.

Comparison of sequence divergence of specimens collected in the same region (Table 2) revealed that species were consistently genetically distinct, with interspecific sequence divergence ranging from 1.27 to 2.24%. In Ontario sample sizes were large enough to allow statistical analysis. Pair-wise comparisons among all individuals revealed significantly greater sequence divergence between species than within species (AMOVA: $F_{ST}$ = 0.91927, df = 1,156, p<0.0001). These data further support that *C. dupla* and *C. mikmaqi* are genetically distinct across their range (Table 1) as well as in sympatry (Table 2).

The molecular data support the division of the *Ceratina dupla s. l.* group into three distinct clades. Moreover, these three putative species can be distinguished from each other (see species description and key below modified from Mitchell [1962] and Rehan & Richards [2008]). In combination, the molecular and morphological data support separation of *C. dupla s. l.* into three species. Two species (*C. dupla* and *C. mikmaqi*) are known to be sympatric in some geographical areas, while the third (*C. floridana*) is allopatric, distinctly separated from the others (Fig. 1).

**TABLE 2.** Comparison of sequence divergence between *C. dupla* and *C. mikmaqi* collected in the same regions.

|  | Sample size | | Interspecific sequence divergence (%) | | |
| --- | --- | --- | --- | --- | --- |
| Collection location | *C. dupla* | *C. mikmaqi* | Minimum | Mean | Maximum |
| Nova Scotia | 3 | 1 | 1.59 | 1.96 | 2.24 |
| New York | 3 | 1 | 2.07 | 2.07 | 2.07 |
| Ontario | 41 | 117 | 1.27 | 1.40 | 1.91 |
| Wisconsin | 3 | 4 | 1.27 | 1.38 | 1.59 |

**Taxonomy**

***Ceratina (Zadontomerus) mikmaqi* Rehan & Sheffield, new species**

**Diagnosis.** Males of *C. mikmaqi* can be recognized by the combination of the metallic greenish blue colour, T3 with punctures separated by > 1 puncture diameter, and the hind femur which is only slightly dilated toward base, the lower margin carinate only in the apical half. They are very similar to *C. floridana* and *C. dupla*. Males of *C. floridana* have T3 densely punctate, with interspaces ≤ 1 puncture diameter, and the carina of T7 is more truncate. Males of *C. dupla* have a complete carina running the length of the hind femur.

Females of *C. mikmaqi* are recognized by the combination of the metallic greenish colour, the small basal ivory spot on the front tibia, the largely impunctate surface in the posterior half of the mesoscutum, and the clypeus which has an elongate, median maculation, and sinuate lateral edges. They are very similar to *C. dupla* and *C. calcarata*. Females of *C. dupla* have distinct rows of punctures medially on the posterior half of the mesoscutum. Females of *C. calcarata* have the lateral edges of the clypeus more angulate, and often have the clypeal maculation greatly reduced or absent.

**Description.** FEMALE. Length 6–8 mm; head length 1.67–2.22 mm; head width 1.75–2.04 mm; forewing length 4.63–5.3 mm.

*Colouration.* Body mostly bluish green. Mesoscutum often black with violet reflections centrally. T1 largely black. Apical half of clypeus, malar area, basal half of mandible, lower paraocular area, and genal area adjacent to eye margin black. Antenna dark brown to black, flagellum with ventral surface reddish brown. Tegula reddish brown. Wing membrane subhyaline, venation and pterostigma dark brown. Legs bluish green, tibia and tarsi dark brown to black, distitarsus and tarsal claw reddish, all spurs yellowish brown. Clypeus with a pale, vertically elongate, median maculation. Posterior half of pronotal lobe with pale maculation. All tibia with a small, pale basal maculation.

*Pubescence.* Dull white. Very sparse and mostly short (≤1 OD). More elongate (1.5–2 OD) on vertex, frons, labrum, metanotum and mesopleuron, on legs, and basal sterna, with a few very long (>3 OD) hairs on the apical sternum.
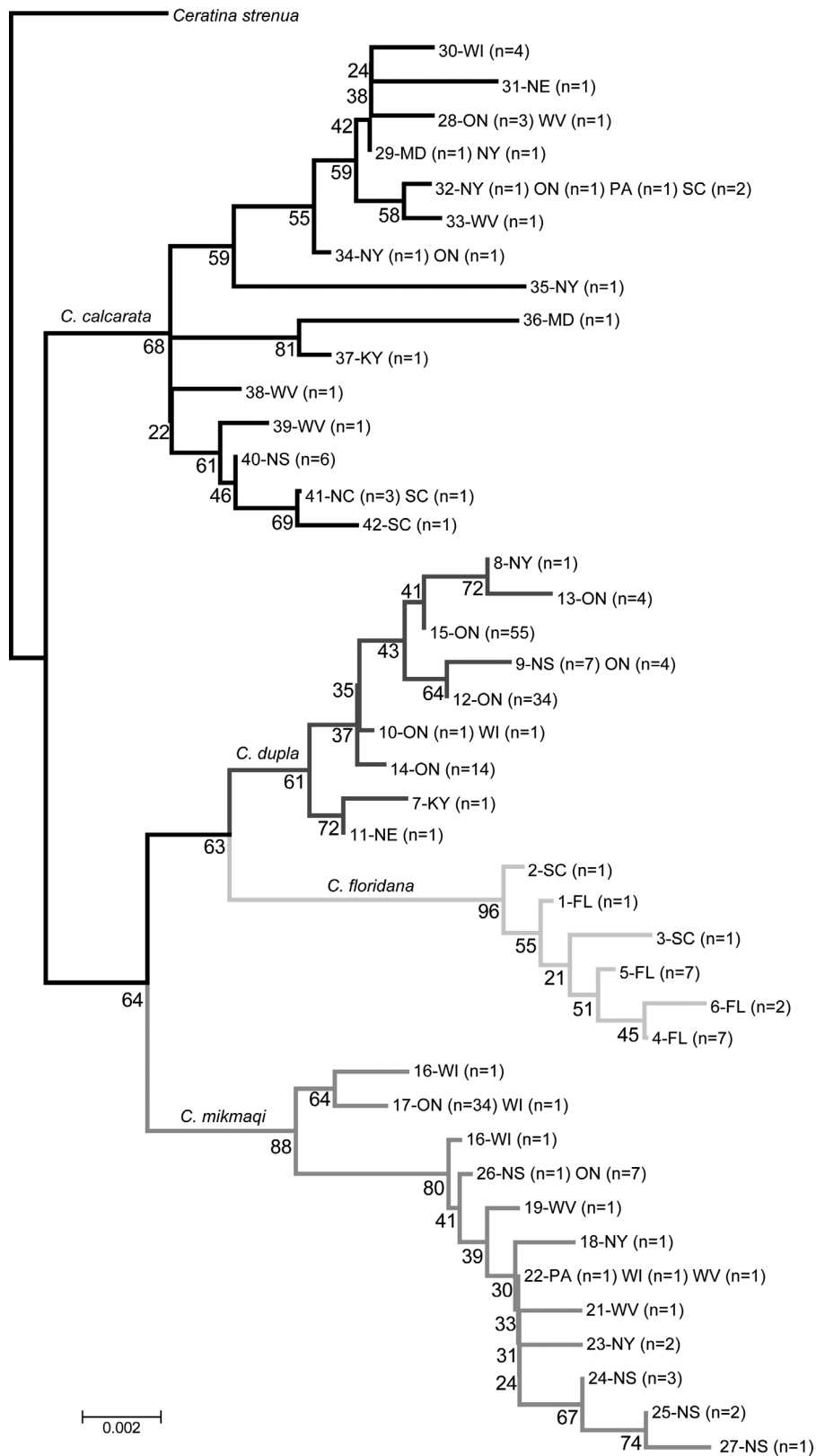
**FIGURE 2.** Neighbour-joining phylogram of three clades within the *Ceratina dupla* species-group. Ingroup variants of the *C. dupla* species-group are indicated in gray and outgroup species in black. Bootstrap support is indicated for each node. Each of the 27 terminal ingroup taxa represents a unique haplotype of the 210 specimens sequenced and names are replaced with collection state/province (sample size) for each haplotype.
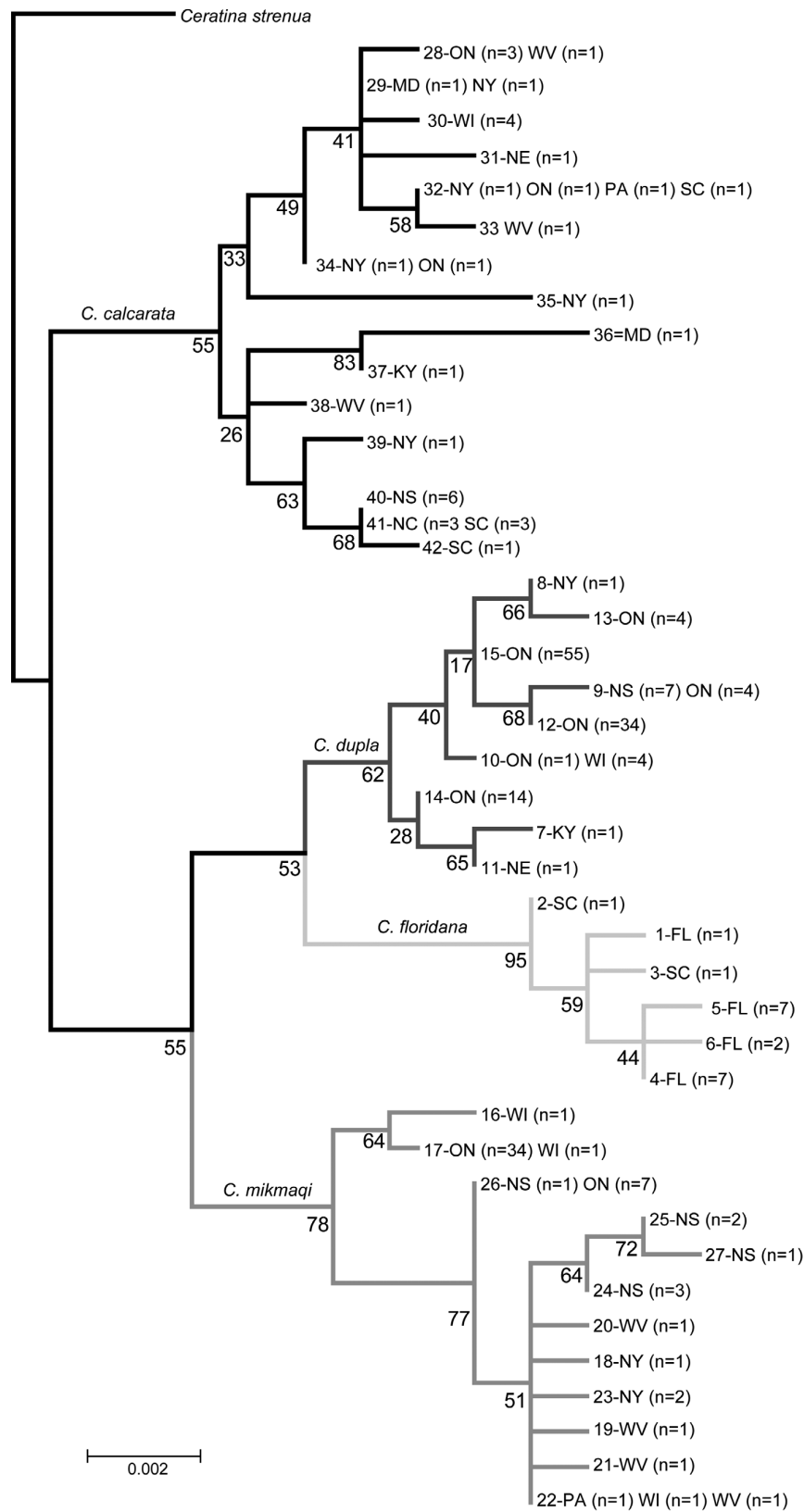
**FIGURE 3.** Maximum parsimony consensus phylogram of three clades within the *Ceratina dupla* species-group. Ingroup variants of the *C. dupla* species-group are indicated in gray and outgroup species in black. Bootstrap support is indicated for each node. Each of the 27 terminal ingroup taxa represents a unique haplotype of the 210 specimens sequenced and names are replaced with collection state/province (sample size) for each haplotype.

*Surface sculpture*. Body in large part polished and shiny. Clypeus polished, with large, well spaced (i=1–3d), elongate punctures in apical half, punctures becoming rounded and smaller basally. Supraclypeal area with large, well spaced (i=1–2d) punctures apically, becoming much finer and dense (i≤d) between antennal sockets. Lower paraocular and antennocular area punctation rather dense (i=1–1.5d). Upper paraocular and ocellocular areas with punctation sparser (i=2–3d), becoming closer (i=1–1.5d) on vertex. Frons with swellings largely impunctate, punctures fine and close (i≤d) adjacent to median ocellus. Gena above with punctation close (i=1–1.5d), punctures elongate, becoming sparse (i>3d) to virtually impunctate below and adjacent to eye. Mesoscutum polished, punctation coarse, moderately dense (i=d) anteriorly and along lateral and posterior margins, largely impunctate medially, with irregularly spaced punctures along medial line and parapsidal lines. Mesoscutellum similar to mesoscutum, submedial punctation moderately sparse (i=1–3d). Axilla and metanotum with punctation fine and close (i=d). Tegula largely impunctate, with very fine, close (i<d) punctures on inside margin. Pronotum with punctation fine and close (i=d), with small impunctate area anterior to pronotal lobe. Pleural punctation course and close (i=d) throughout, punctures becoming slightly sparser (i=1.5d) below. Metapostnotum largely tessellate apically, with strong, well spaced striae basally. Propodeum finely and densely punctate throughout (i≤d). T1 largely shiny and impunctate, often with a few fine, sparse (i=2–3d) punctures at apicolateral edge, and with with a triangular area of coarser, closer (i=d) punctures apicomedially, T2–T4 punctation more coarse, quite deep and distinct, close laterally (i≤ 1d), becoming well separated (i=2–3d) medially, punctures on apical edge of T2 and T3 becoming finer (i≤ 1.5d), T4 becoming irregularly roughened in apical half, though with punctures visable to apical edge, T5 rugose, without distinct punctures except on apical edge, T6 entirely rugose; all sterna coarsely and closely punctate (i<d), punctures becoming slightly separated (i≤d) laterally on S2–S4.

*Structure*. Head width varying (length/width ratio = 0.95–1.09). Eyes with inner margins subparallel to slightly converging below. Clypeus inverted T-shaped, 1/3 below suborbital tangent, apicolateral margins broadly rounded. Antennal sockets evenly spaced (IAD=AOD). Gena narrower than eye (0.54–0.8). Hypostomal carinae parallel, reflexed distally. Mandibles 3-dentate, with median tooth larger and longer than 1st and inner teeth. F1 longer than broad (1.5:1), nearly as long as F2+F3, F2–F5 broader than long, F6 quadrate, remaining flagellomeres slightly longer than broad. T6 apical margin coming to a fine triangular point.

MALE. Similar to female except for the usual secondary sexual characters and as follows. Length. 5–7 mm; head length 1.3–1.48 mm; head width 1.38–1.57 mm; forewing length 3.7–4.1 mm.

*Colouration*. As in female except tibia typically dark blue; labrum with a large, pale central maculation; clypeal maculation inverted T-shaped, large, filling most of surface.

*Pubescence*. Generally as in female, except long hairs (2 OD) present laterally on T4–T7, on apical margin of S2–S5. T6 with apex of process with a tuft of dense, short, yellowish pubescence. Carina of T7 laterally with elongate (2 OD) yellowish hairs.

*Surface sculpture*. Generally as in female. T6 entirely rugose; T7 more distinctly punctate basally, surface of carina smooth and sparsely punctate; all sterna coarsely and closely punctate.

*Structure*. Head round to slightly elongate (length/width ratio = 0.94). Eyes strongly convergent below (UOD/LOD ratio = 1.2–1.3). Clypeus inverted T-shaped, 1/3 below suborbital tangent, apicolateral margins broadly rounded. Antennal sockets slightly separated (IAD/OAD=1.2). Gena narrower than eye (2:3) in smaller specimens, to wider than eye (4:3) in larger ones. Hypostomal carinae parallel, reflexed distally. Mandibles 2-dentate, with upper tooth larger and longer than 1st tooth. F1 quadrate to very slightly longer than broad, F2–F5 broader than long, F6 quadrate, remaining flagellomeres slightly longer than broad. Hind femur somewhat dilated toward base but not angulate, the length more than twice the width, the lower margin carinate only in the apical half. T6 with a prominant rounded medial process overhanging the apical margin of T7. T7 with a wide, broadly rounded to subtruncate carina. Apical margin of S6 with a deep median cleft, lateral margins bent ventrally. S7 and genital armature as in *C. dupla* and *C. calcarata* (see Mitchell 1962).

**Etymology.** *Ceratina mikmaqi* is named in honour of the Mi'kmaq, the First Nations People of Nova Scotia where this species was first discovered with DNA barcoding (Sheffield *et al.* 2009).

**Type material.** The male holotype of *C. mikmaqi* was collected in Middleton, N44.9665, W65.5755, Annapolis Co., Nova Scotia, Canada on 20.vi.2002, col. Cory Sheffield [DNA barcode sample ID "02-NS-1619"]; the specimen is in good condition, but missing the right antenna and middle leg. The female allotype was collected in Forest Home, N44.9117, W64.5288, Kings Co., Nova Scotia, Canada on 2.vii.2003, cols. C. Sheffield, S. Rigby, and K. Jansen [DNA barcode sample ID "sheffT-58"]; the specimen is in excellent condition, but missing the right hind leg. Both holotype and allotype are in the Packer Collection at York University (PCYU).

Paratypes were designated from the following locations: **CANADA: NS**; **Avonport**, N45.119, W64.273, Kings Co., 21.vi.2001 [♂; "01-NS-1622"], col. Cory Sheffield; **East Torbrook**, N44.927, W64.93, Kings Co., 20.vi.2002 [♂ ; "02-

NS-1618"], col. Cory Sheffield; **Somerset**, N45.0836, W64.7322, Kings Co., 22.vi.2001 [♂; "sheffT-62"], col. Cory Sheffield; **West Black Rock**, N45.13, W64.74, Kings Co., 5.vi.2002 [♂, "02-NS-1621"], col. Cory Sheffield; **ON**; **Cambridge**, Shade`s Mills Conservation Area, N43.38, W80.284, 12.vii.2007 [♀, "G5S-20070712-002"], 30.viii.2007 [♀, "G5B-20070830-001"], 23.vii.2007 [♂, "O1Y-20070723-006"], col. M. Horn; **Waterloo**, Chesapeake Drive, N43.508, W80.505, 01.vii.2008 [♂, "N3Y-20080701-005"; ♀, "N3Y-20080701-006"]; **St. Catharines**, Brock University Campus, N43.119, W79.249, 1.viii.2008 [♀, "08-ON-2151"]; 3.viii.2008 [♀, "08-ON-2146"]; 14.viii.2008 [2♀, "08-ON-2150", "08-ON-2147"], col. J. Vickruck; **UNITED STATES: MD;** N38.909, W76.683, Pr. George's Co., 18.vi.2009 [♀, "CCDB-01570 D03'], col. S.W. Droege; **NE;** N41.2774, W95.9116, Douglas Co., 10.v.2007 [♂, "CCDB-01570 F11"], col. S.W. Droege; **NY;** N41.029, W72.138, Suffolk Co., 07.ix.2005 [♀, "CCDB-01570 E03"], col. S.W. Droege; **KY;** N36.924, W84.872, Wayne Co., 27.vii.2007 [♀, "CCDB-01570 B06"], col. S.W. Droege; **WI;** N43.338, W89.949, Sauk Co., 5.ix.2007 [4♀, "CCDB-01570 D08 – D11"], col. M. Mossman. Paratypes are in the Packer Collection at York University (PCYU).

    **Distribution.** *Ceratina mikmaqi* ranges in Canada from Nova Scotia to southern Ontario, and south into the northeastern United States, as far west as Nebraska, and south to Kentucky. Its range overlaps that of *C. dupla* and *C. calcarata* (see Mitchell [1962] and Daly [1973]).

## Key to *Ceratina* species of Eastern North America
(modified from Mitchell [1962] and Rehan & Richards [2008])

### Males
| | |
|---|---|
| 1 | Body black; apex of T7 with two widely separated, acute tubercles (Fig. M1). . . . . . . . . . . . . . . . . . *C. cockerelli* H. S. Smith |
| - | Body metallic blue-green; apex of T7 with a single, median, lobe-like carina (Fig. M2) . . . . . . . . . . . . . . . . . . . . . . . . . . . .2 |
| 2 | Hind femur somewhat dilated toward base, but without a median process, the greatest width hardly more than a third its length (Fig. M3) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 3 |
| - | Hind femur with a median, triangular process, greatest width about half the length (Fig. M4) . . . . . . . . . . . . . . . . . . . . . 5 |
| 3 | Colour deep metallic blue; T3 more closely punctate, interspaces no greater than diameter of punctures (Fig. M5); carina of T7 broadly rounded, subtruncate medially (Fig. M7) . . . . . . . . . . . . . . . . . . . . . . . . . *C. floridana* Mitchell (new combination) |
| - | Colour usually more greenish; T3 less closely punctate, interspaces considerably greater than diameter of punctures (Fig. M6); carina of T7 more evenly rounded (Fig. M8) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 4 |
| 4 | Hind femur with ventral edge separating inner and outer surfaces rounded in basal half, becoming more carinate in apical half, basal lateral edge more rounded than carinate (Fig. M9) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .*C. mikmaqi* new species |
| - | Hind femur with ventral edge separating inner and outer surfaces sharply carinate for entire length, basal lateral edge more carinate (Fig. M10) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . *C. dupla* Say |
| 5 | Carina of T7 very narrow, fully as long as broad, and not over a fourth as broad as the tergum (Fig. M11) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .*C. strenua* F. Smith |
| - | Carina of T7 at least twice as broad as long, fully half as broad as width of the tergum (Fig. M12) . . .*C. calcarata* Robertson |

### Females
| | |
|---|---|
| 1 | Body black; head and mesoscutum largely impunctate (Fig. F1) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . *C. cockerelli* H. S. Smith |
| - | Body metallic blue-green; head and mesoscutum with coarse deep and distinct punctures (Fig. F2) . . . . . . . . . . . . . . . . . . . . .2 |
| 2 | Front tibia with a basal ivory stripe (Fig. F3). . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 3 |
| - | Front tibia not maculated (Fig. F4) or with a basal ivory spot (Fig. F5) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 4 |
| 3 | Body small (length 5–6 mm.); scutellum somewhat swollen, the punctures separated by >1 puncture diameter; mesoscutum between medial line and notaulus sparsely punctate, punctures >1 puncture diameter apart, impunctate in large part in posterior half (Fig. F6); colour usually more greenish . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . *C. strenua* F. Smith |
| - | Usually larger (length 5-7 mm); scutellum quite flat and closely punctate, separated by < 1 puncture diameter; mesoscutum between medial line and notaulus and posteriorly more densely punctate, punctures separated by ≤ 1 puncture diameter (Fig. F7); colour more bluish. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . *C. floridana* Mitchell (new combination) |
| 4 | Mesoscutum more regularly punctate in posterior half, with one to two complete and up to four incomplete longitudinal rows of punctures near medial line (Fig. F8) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .*C. dupla* Say |
| - | Mesoscutum largely impunctate in posterior half, with only a few scattered punctures, with one to two incomplete longitudinal rows of punctures on either side of the medial line (Fig F9). . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 5 |
| 5 | Clypeus with inner lateral margins more angulate [clypeus OFTEN with maculation reduced to an apical spot or absent[1] (Fig. F10) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .*C. calcarata* Robertson |
| - | Clypeus with lateral margins more sinuate [clypeus with maculation present and elongate] (Fig. F11) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .*C. mikmaqi* new species |

---

1.   This character is based on DNA barcoded specimens, but may not hold true throughout the range of these species.
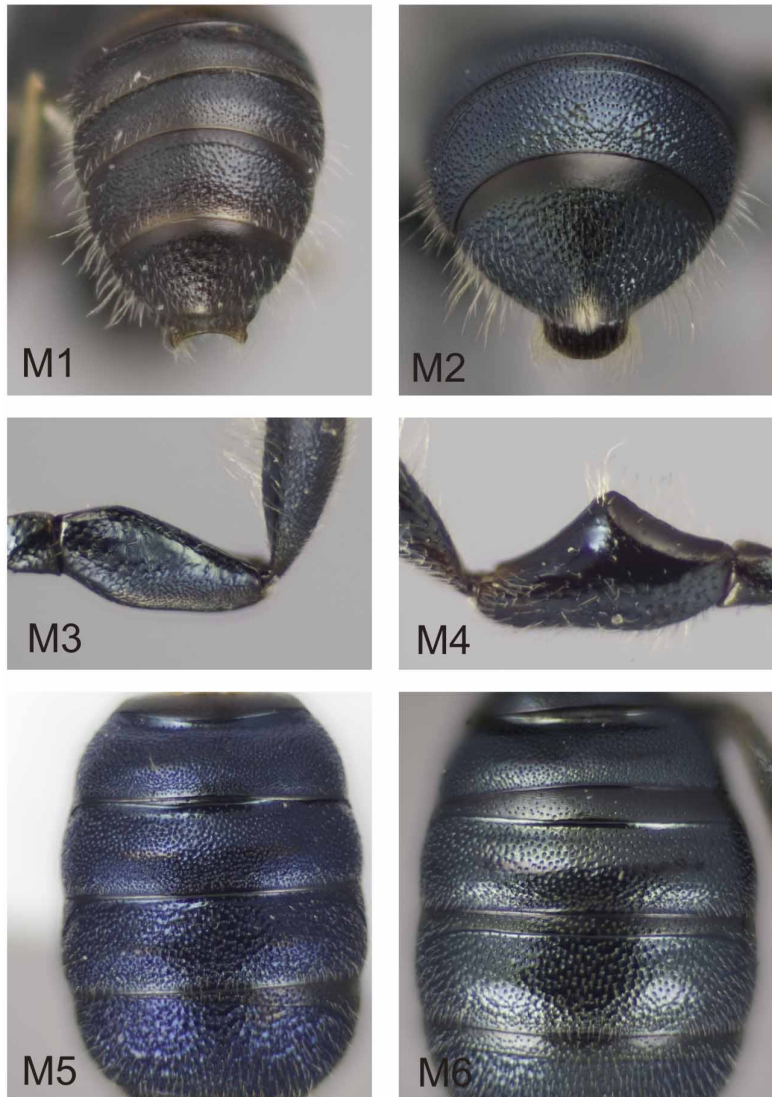
## Discussion

DNA barcoding was developed for species identification under the proposition that sequence divergences are typically less within than between species (Hebert *et al.* 2003a). Studies on broad taxonomic groups suggested 2–3% sequence divergence as the advised average sequence divergence between closely related species (Hebert *et al.* 2003b). However, more detailed research has shown that this value may range from 0.32 to 7.93% (Hebert *et al.* 2004a, b). In this study the average minimum pair-wise genetic difference between species was 1.38%, ranging from 1.11 to 1.75%, while the average maximum pair-wise genetic difference within species was 0.90%, ranging from 0.47 to 1.43%. On average there was greater variation between than within species, however the range of intraspecific variability indicates that thresholds such as the percentage of genetic divergence should be regarded as guidelines rather than rules for species recognition as comparable rules for delimiting species with morphological characters do not exist (Packer *et al.* 2009).
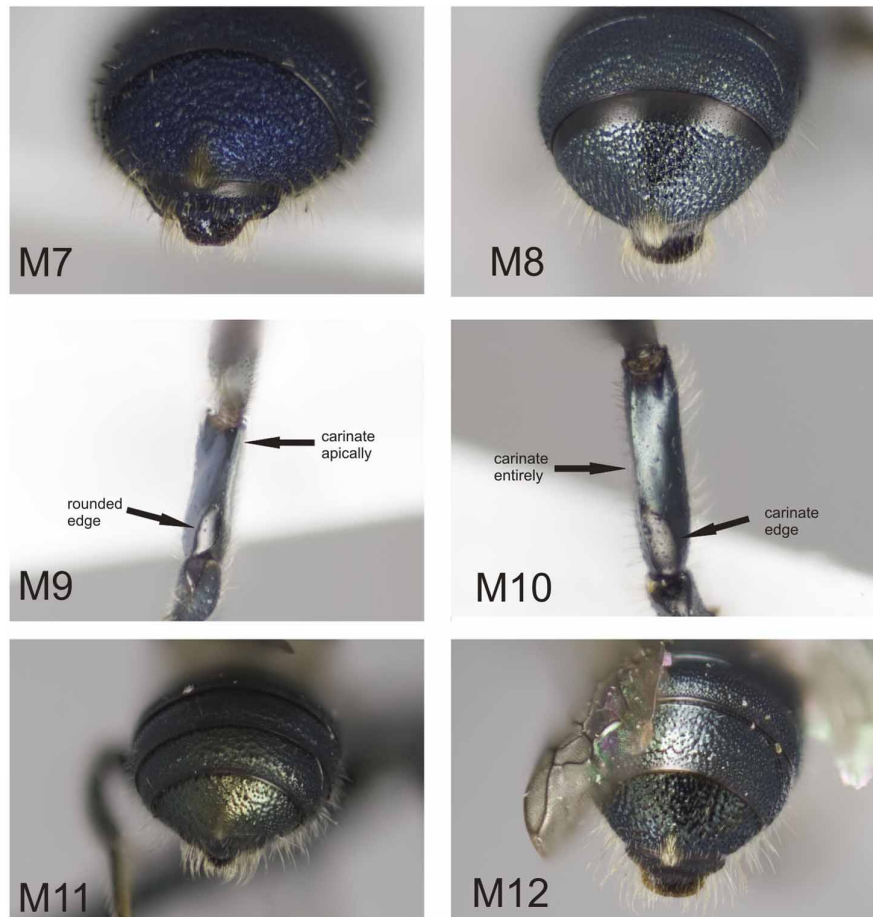
The phylogenetic species concept defines a species as an irreducible group whose members are descended from a common ancestor and who all possess a combination of certain defining, or derived, traits (Eldridge & Cracraft 1980; Nelson & Platnick 1981). Hence, this concept defines a species as a group having a shared and unique evolutionary history. In this study we differentiated three genetic variants within what would have formerly been identified as *C. dupla* (Mitchell 1962). The three species of the *C. dupla s. l.* group are not only genetically distinct, but also morphologically distinct, albeit subtly. *Ceratina floridana* can be easily distinguished from *C. dupla* and *C. mikmaqi* by its deep-blue colouration, distinct geographic range (Florida to North Carolina [Mitchell 1962]), and four fixed base pair differences in comparison to the other two species. *Ceratina mikmaqi* is more subtly distinguished but has consistent morphological distinction. *Ceratina mikmaqi* is sympatric with *C. dupla* as specimens of both species were found in the same collection localities in New York, Nova Scotia, Ontario and Wisconsin. These species are also genetically distinct throughout their range with three fixed base pair differences in the barcoding region.

The biological species concept defines a species as a group of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups (Mayr 1942). Since the inception of this study ongoing research on the nesting biology and life history of the *C. dupla s. l.* group in southern Ontario has revealed temporal niche partitioning between the two sympatric species. *Ceratina dupla* and *C. mikmaqi* are found nesting in the same substrate (teasel) yet *C. dupla* nests earlier in the season and is possibly bivoltine while *C. mikmaqi* nests later in the season and is univoltine (Vickruck *et al*. in press). The nesting behaviour of *C. floridana* remains unknown but would be an interesting study to improve our understanding of the variation in behaviour, morphology, and DNA barcodes across the *Ceratina* of eastern North America.
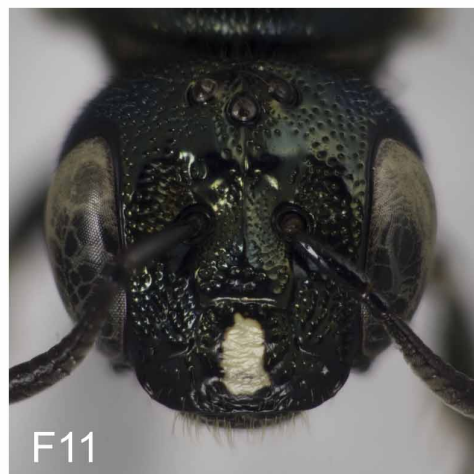
Direct comparisons of morphology, genetic divergence and ecology among sympatric sister species in bees are few, but the following three studies further support the utility of an integrated taxonomic approach. Two sympatric species of *Halictus* in the Piedmont region (Packer 1999) are morphologically indistinguishable but have marked genetic and ecological differences. Specifically, *H. ligatus* Say and *H. poeyi* Lepeletier are cryptic species with different phenologies (Dunn *et al*. 1998) and COI divergence of 4 to 5% between species (Danforth *et al*. 1998). Two species of *C. (Ceratinidia)* from Japan have fixed morphological, genetic and ecological differences. *Ceratina flavipes* Smith and *C. japonica* Cockerell are morphologically distinct (Shiokawa 1963) with 4.1% COI divergence between species (Cronin 2004; Rehan & Richards 2008). These sister species are further differentiated by spatial niche partitioning; *C. flavipes* nest in open areas and *C. japonica* nest in shaded wood margins (Sakagami & Maeta 1977). Finally, two species of *Zadontomerus* from Canada have marked ecological differences (Vickruck *et al*. in press) and fixed yet more subtle morphological and molecular divergence. *Ceratina calcarata* and *C. dupla* have low (1.29%) COI divergence, yet fixed morphological divergence in males, and subtle variation in females (Rehan

& Richards 2008). These sympatric species are also segregated by spatial niche partitioning; *C. calcarata* found nesting in shaded areas and *C. dupla* nest in open wood lots (Grothaus 1962; Vickruck *et al*. in press). Here we reveal that *C. dupla s. l*. are actually three separate species based on fixed differences in DNA barcodes and morphological distinctions. Temporal niche partitioning between *C. dupla* and *C. mikmaqi* and geographic isolation of *C. floridana* further support the division of the *C. dupla s. l*. group into three species.
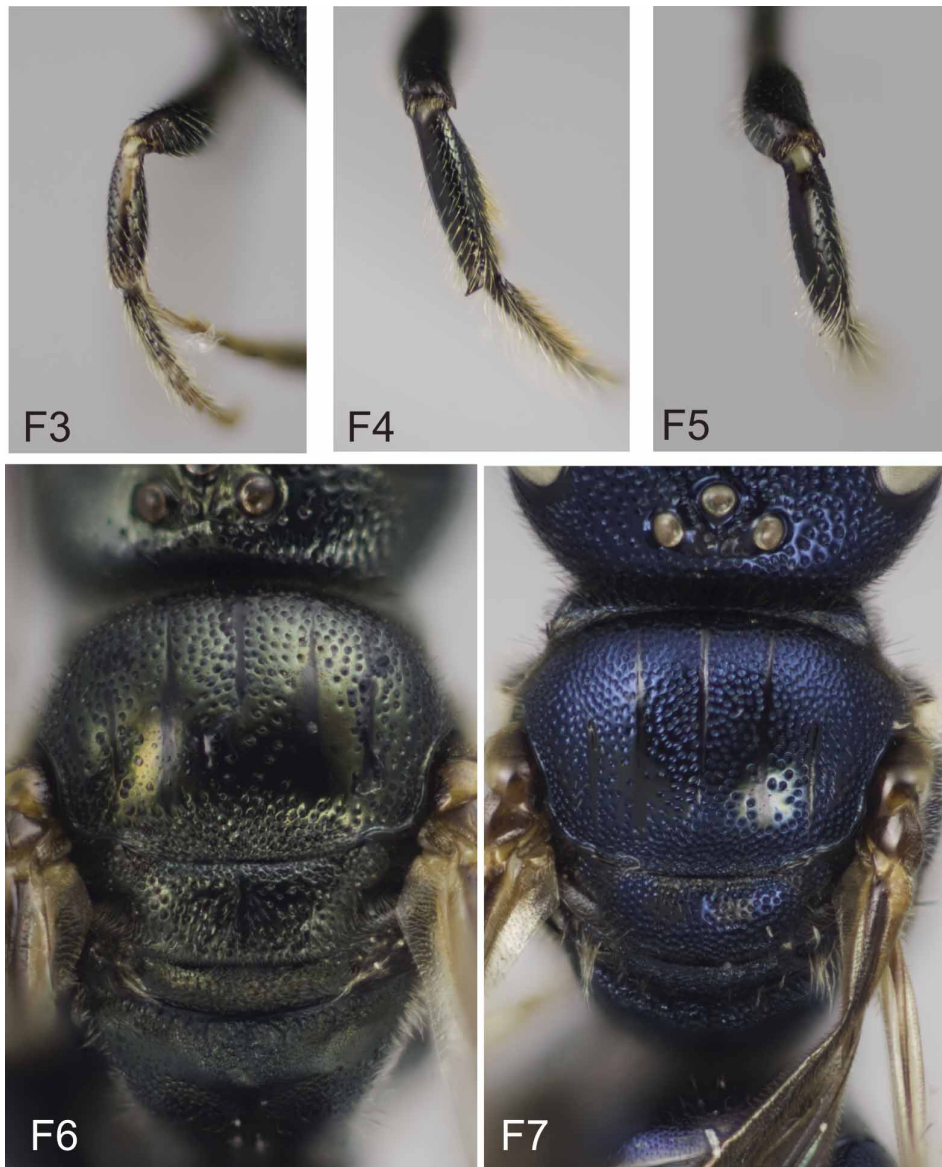


One additional characteristic of eastern *Ceratina*, and perhaps many members of the subgenus *Zadontomerus*, is the occurance of allometric variation. This variability has no doubt lead to much of the difficulty in distiguishing females; as mentioned in the description above, compound eye to gena ratios for *C. mikmaqi* range from greater than one in smaller individuals, to less than one in larger ones. The effect of allometric variation on other morphological features, such as surface sculpture (puncture size and density) may be great. Further studies on allometric variation in *Ceratina*, facilitated with DNA barcoding, may help to further clarify the taxonomy of these bees.

F8



F9



F10



F11

## Acknowledgements

# Literature cited

Avise, J.C. & Walker, D. (1999) Species realities and numbers in sexual vertebrates: perspectives from an asexually transmitted genome. *Proceedings of the National Academy of Science USA*, 96, 992–995.

Clinebell, R.R. (2002) Foraging ecology in selected prairie wildflowers (*Echinacea, Liatris, Monarda,* and *Veronicastrum*) in Missouri prairie remnants and restorations. *Proceedings of the 18th North America Prairie Conference*. Truman State University, Kirksville, Missouri, pp. 194–212.

Cronin, A.L. (2004) A molecular phylogeny and social behaviour of Japanese *Ceratina* (Hymenoptera, Apidae, Xylocopinae). *Insect Systematics and Evolution*, 35, 137–146.

Daly, H.V. (1966) Biological studies on *Ceratina dallatorreana*, an alien bee in California which reproduces by parthenogenesis (Hymenoptera: Apoidea). *Annals of the Entomological Society of America*, 59, 1138–1154.

Daly, H.V. (1973) *Bees of the Genus* Ceratina *in America North of Mexico*. University of California Press, Berkley, California, 114 pp.

Danforth, B.N., Mitchell, P.L. & Packer, L. (1998) Mitochondrial DNA differentiation between two cryptic *Halictus* (Hymenoptera: Halictidae) species. *Annals of the Entomological Society of America*, 91, 387–391.

Deyrup, M., Edirisinghe, J. & Norden, B. (2002) The diversity and floral hosts of bees at the Archbold Biological Station, Florida (Hymenoptera: Apoidea). *Insecta Mundi*, 16, 87–120.

Dunn, M., Mitchell, P.L. & Packer, L. (1998) Phenology and social biology of two sibling species of *Halictus* in an area of sympatry. *Canadian Journal of Zoology*, 76, 2207–2213.

Excoffier, L., Laval, G. & Schneider, S. (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, 1, 47–50.

Eldridge, N. & Cracraft, J. (1980) *Phylogenetic Patterns and the Evolutionary Process*. Columbia University Press, New York.

Gibbs, J. (2009) An integrative taxonomic approach reveals new (and old) species in the *Lasioglossum (Dialictus) tegulare* (Robertson) species group (Hymenoptera, Halictidae). *Zootaxa*, 2032, 1–38.

Gibbs, J. (2010) Revision of the metallic species of *Lasioglossum* (*Dialictus*) of Canada (Hymenoptera, Halictidae, Halictini). *Zootaxa, 2591*, 1–382.

Ginsberg, H.S. (1984) Foraging behaviour of the bees *Halictus ligatus* (Hymenoptera: Halictidae) and *Ceratina calcarata* (Hymenoptera: Anthophoridae) foraging speed on early-summer composite flowers. *New York Entomological Society*, 92, 162–168.

Grissell, E.E. (1976) The carpenter bees of Florida (Hymenoptera: Apidae: Xylocopinae). *Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Entomology Circular*, No. 167.

Grothaus, R.H. (1962) *The biology of the species of* Ceratina *(Hymenoptera, Xylocopidae) in Indiana*. Unpublished Master's thesis, Purdue University, Lafayette, Indiana, 201 pp.

Hajibabaei, M., deWaard, J.R., Ivanova, N.V., Ratnasingham, S., Dooh, R.T., Kirk, S.L., Mackie, P. & Hebert, P.D.N. (2005) Critical factors for assembling a high volume of DNA barcodes. *Philosophical Transactions of the Royal Society of London (Series B)*, 360, 1959–1967.

Hall, T.A. (1999) Bioedit: a user friendly biological sequence alignment editor and alignment program for Windows 95/98 NT. *Nucleic Acid Symposium Series*, 41, 95–98.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003a) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B—Biological Sciences*, 270, 313–321.

Hebert, P.D.N., Penton, E.H., Burns, J.M., Janzen, D.H. & Hallwachs, W. (2004a) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 14812–14817.

Hebert, P.D.N., Ratnasingham, S. & deWaard, J.R. (2003b) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London Series B—Biological Science*, 270, S96–S99.

Hebert, P.D.N., Stoeckle, M.Y., Zemlak, T.S. & Francis, C.M. (2004b) Identification of birds through DNA barcodes. *PLoS Biology,* 2, e312.

Ivanova, N., deWaard, J. & Hebert, P. (2006) An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes*, 6, 998–1002.

Johnson, M.D. (1988) The relationship of provision weight to adult weight and sex ratio in the solitary bee, *Ceratina calcarata*. *Ecological Entomology,* 13, 165–170.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16, 111–120.

Leuck, D.B. & Hammons, R.O. (1969) Occurrence of atypical flowers and some associated bees (Apoidea) in the peanut, *Arachis hypogaea* L. *Agronomy Journal*, 61, 958–960.

Mayr, E. (1942) *Systematics and the Origin of Species*. Harvard University Press, New York, 372 pp.

Michener, C.D. (1954) The bees of Panama. *Bulletin of the American Museum of Natural History*, 104, 1–176.

Michener, C.D. (2007) *The Bees of the World*, second ed. John Hopkins University Press, Baltimore, xvi+[i]+953 pp.

Mitchell, R.S. (2002) New York Flora Association Newsletter—*New York State Museum Associates, New York State Museum*, Vol. 13, No. 4 (September).

Mitchell, T. (1962) *Bees of the Eastern United States*, Volume 2. North Carolina Agricultural Experiment Station Technical

Bulletin Number 152. North Carolina, USA. 557 pp.

Moore, W.S. (1995) Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear-gene trees. *Evolution*, 49, 718–726.

Moure, J.S. (2007) Xylocopini Latreille, 1802. In: Moure, J. S., Urban, D. & Melo, G. A. R. (Eds), Catalogue of Bees (Hymenoptera, Apoidea) in the Neotropical Region. Sociedade Brasileira de Entomologia, Curitiba, pp. 637–673.

Nelson, G. & Platnick, N. (1981) *Systematics and Biogeography: Cladistics and Vicariance*. Columbia University Press, New York. 567 pp.

Packer, L. (1999) The distribution of *Halictus ligatus* Say and *H. poeyi* Lep. (Hymenoptera: Halictidae) in North America. *In:* Byers, C. W., R. H. Hagen, and R. W. Brooks (Eds.), *Entomological Contributions in Memory of Byron A. Alexander. University of Kansas Natural History Museum Special Publication* 24, pp. 81–84.

Packer, L., Gibbs, J., Sheffield, C. & Hanner, R. (2009) DNA barcoding and the mediocrity of morphology. *Molecular Ecology Resources*. 9 (Suppl. 1), 42–50.

Reed, C.C. (1995) Species richness of insects on prairie flowers in southeastern Minnesota. *In:* Hartnett, D. C. (Ed.) *Fourteenth North American Prairie Conference: Prairie Biodiversity.* Kansas State University, Manhattan, Kansas, pp. 103–115.

Rehan, S.M. & Richards, M.H. (2008) Morphological and DNA sequence delineation of two problematic species of *Ceratina* (Hymenoptera: Apidae) from Eastern Canada. *Journal of the Entomological Society of Ontario*, 139, 59–67.

Russell, K.N., Ikerd, H. & Droege, S. (2005) The potential conservation value of unmowed powerline strips for native bees. *Biological Conservation*, 124, 133–148.

Sakagami, S.F. & Maeta, Y. (1977) Some presumably presocial habits of Japanese *Ceratina* bees, with notes on various social types in Hymenoptera. *Insectes Sociaux*, 24, 319–343.

Sheffield, C.S., Kevan, P.G., Smith, R.F., Rigby, S.M. & Rogers, R.E.L. (2003) Bee species of Nova Scotia, Canada, with new records and notes on bionomics and floral relations (Hymenoptera: Apoidea). *Journal of the Kansas Entomological Society*, 76, 357–384.

Sheffield, C.S., Hebert, P.N., Kevan, P.G. & Packer, L. (2009) DNA barcoding a regional bee (Hymenoptera: Apoidea) fauna and its potential for ecological studies. *Molecular Ecology Resources*, 9, 196–207.

Sheffield, C.S. & Westby, S.M. (2007) The male of *Megachile nivalis* Friese, with an updated key to members of the subgenus *Megachile s. str.* (Hymenoptera: Megachilidae) in North America. *Journal of Hymenoptera Research*, 16, 178–191.

Shiokawa, M. (1963) Redescriptions of *Ceratina flavipes* Smith and *C. japonica* Cockerell (Hymenoptera, Apidae). *Kontyu*, 31, 276–280.

Swofford, D.L. (2003) *PAUP*. Phylogenetic analysis using parsimony (*and other methods)*. Version b4.10. Sinauer Associates, Sunderland, Massachusetts.

Terzo, M. (2000) *Classification phylogénétique des Cératines du monde et monographie des espèces de la région ouest-paléarctique et de l'Asie Centrale (Hymenoptera, Apoidea, Xylocopinae: Ceratina Latreille)*. Ph.D. dissertation, Universite de Mons-Hainaut, 293 pp.

Thompson, J.D., Higgins, D.G. & Gibons, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Research*, 22, 4673–4680.

Thompson, J.D., Gibson, T.J., Plewniak, F, Jeanmougin, F. & Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25, 4876–4882.

Tuell, J.K., Ascher, J.S. & Isaacs, R. (2009) Wild Bees (Hymenoptera: Apoidea: Anthophila) of the Michigan Highbush Blueberry Agroecosystem. *Annals of the Entomological Society of America*, 102, 275–287.

Tuell, J.K., Fiedler, A.K., Landis, D. & Isaacs, R. (2008) Visitation by wild and managed bees (Hymenoptera: Apoidea) to eastern U.S. native plants for use in conservation programs. *Environmental Entomology*, 37, 707–718.

Vickruck, J.L., Rehan, S.M., Sheffield, C.S. & Richards, M.H. (in press) Nesting Biology and barcode analysis of two cryptic species within *Ceratina dupla*, with comparisons to *C. calcarata* (Hymenoptera: Apidae: Xylocopinae). *Canadian Entomologist*.

Winfree, R., Williams, N.M., Dushoff, J. & Kremen, C. (2007) Native bees provide insurance against ongoing honey bee losses. *Ecology Letters*, 10, 1105–1113.

Yasumatsu, K. & Hirashima, Y. (1969) Synopsis of the small carpenter bee genus *Ceratina* of Japan. *Kontyu*, 37, 61–70.