BARCODING ARTHROPODS

DNA barcoding a regional bee (Hymenoptera: Apoidea) fauna and its potential for ecological studies

CORY S. SHEFFIELD,* PAUL D. N. HEBERT,† PETER G. KEVAN‡ and LAURENCE PACKER*
*Department of Biology, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3, †Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, ‡Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Abstract

DNA barcoding has been evaluated for many animal taxa and is now advocated as a reliable and rapid means for species-level identification. The coming-to-light of this identification tool is timely as we are now facing perhaps the greatest rate of species loss in recent millennia. This study contributes to an ever-increasing number of published accounts of DNA barcoding successfully and accurately distinguishing animal taxa, in this instance, the bee fauna of Nova Scotia, Canada. Most members of this well-known fauna were resolved with particular clarity; the average intraspecific divergence was less than 0.5%, and COI sequences from over 75% of the province's species are now in the Barcodes of Life Data System. DNA barcoding also revealed some surprises within this fauna, including the possible recognition of two undescribed genetically unique species, one in the genus *Ceratina* (subgenus *Zadontomerus*), the second in the genus *Andrena* (subgenus *Larandrena*); both are presently receiving further taxonomic study. In addition, DNA barcoding has allowed sex-associations among two pairs of cleptoparasitic species. The resulting utility of DNA barcoding for ecological studies of bee communities is discussed.

Keywords: Apoidea, bees, cryptic species, DNA barcoding, Nova Scotia, sex associations

Received 15 October 2008; revision received 3 January 2009; accepted 25 January 2009

Bees (Hymenoptera: Apoidea) are among the most important groups of organisms due to their role as pollinators in both natural and managed terrestrial ecosystems (Michener 2007). This vital service provided by bees and other pollinating insects spans over 100 million years, their contributions having shaped modern terrestrial environments by facilitating plant reproduction (Grimaldi & Engel 2005; Novacek 2007). Bees, the most important pollinators (Kevan & Baker 1983), are a diverse taxa with estimates of 20 000–30 000 species worldwide (Michener 2007); the most recent account includes 19 261 valid names (Ascher *et al*.; http://www.discoverlife.org; accessed 19 September 2008). Bees are also behaviourally diverse, with several lifestyles (e.g. pollen vs. cleptoparasitic/social parasitic bees), nesting habits, and a range of sociality not seen in any other group of organisms (Wilson 1971; Michener 1974, 2007).

Bees have become the focus of much attention in recent years for various reasons, not the least of which is their importance in pollination and the recognition that this keystone process is under stress globally (Buchmann & Nabhan 1996; Matheson *et al*. 1996; Stubbs & Drummond 2001; Biesmeijer *et al*. 2006; Committee on the Status of Pollinators 2007). In addition, some attributes of bees have recently been recognized as making them even more extinction-prone; because of their haplodiploid sex-determining mechanism (reviewed by Heimpel & de Boer 2008), small populations, whether naturally limited or reduced through anthropogenic activities, may be particularly at risk due to diploid male production (Zayed & Packer 2005). The frailty of populations of several bee species is also linked to food plant specialization (Zayed *et al*. 2005; Zayed & Packer 2007), or for some cleptoparasitic species, specialized hosts (Sheffield *et al*. 2004). As such, bees may be among the most extinction-prone of all organisms, and bee communities may prove to be revealing indicators of environmental stress (Kevan *et al*. 1997; Kevan 1999).

Studies of bee communities within specific ecosystems have previously been recognized as having utility in environmental assessments; in Canada for instance, Kevan *et al*. (1997) studied species abundance patterns to assess the effects of environmental stress via insecticides. However,

Correspondence: Cory S. Sheffield, Fax: (613)759 1701;
E-mail: corys@yorku.ca

one of the main problems preventing bees (and many other insect taxa) from being used in diversity-based ecological studies is taxonomy-based (Weeks *et al*. 1999). To implement such studies, being able to accurately separate distinct taxonomic units (i.e. morphospecies) or identify the actual species involved is required (Gotelli 2004); but for bees, taxonomic knowledge for many groups is incomplete for various reasons. For instance, Sheffield & Westby (2007) reported that over 30% of the species of North American *Megachile* Latreille, one of the most important groups of pollinators for summer flowering plants, are known from one sex. In less-studied and/or more diverse faunas, the proportion is undoubtedly much higher.

These taxonomic impediments thus affect many community studies (Gotelli 2004; Bortolus 2008), and ecologists have often wished for ways to circumvent these issues as most lack the expertise to handle the required alpha-taxonomy reliably. Although there are taxonomists who specialize on specific groups of insects, many seldom have the time to identify what may be considered 'routine' material. As correctly pointed out by Will & Rubinoff (2004), taxonomy and systematics provide valuable information to many biological disciplines, but are not necessarily providers of 'identification' services. This only stresses the point — the inability to recognize individual species in a timely fashion is a large 'service' gap which has huge implications on basic community studies, especially if one hopes to use such data for inquiries of biodiversity, conservation, and ecosystem assessment.

In recognition of this problem, prototype semi-automated systems for specimen identification have been tested to help alleviate the difficulties faced by ecologists. Semi-automated systems are typically built around a database populated by material (i.e. linked to a species) identified by taxonomists, and it is through comparing unknown taxa to identified material within the database (normally with a probability of accuracy) by which identifications are made (see Ratnasingham & Hebert 2007). Two such systems for bee identification, the Digital Automated Identification System (DAISY) (Weeks *et al*. 1999) and Automated Bee Identification (Schröder *et al*. 2002) are based on image-analyses, the latter specifically utilizing wing venation patterns.

Genetic-based systems, primarily DNA barcoding [using cytochrome *c* oxidase I (*cox1* or COI) as per Hebert *et al*. 2003a, b] are now advocated as an accurate approach for identification of the world's biota (Waugh 2007). To facilitate DNA barcoding at this largest of scales, the Barcode of Life Data System (BOLD) has been developed to manage and provide analytical tools for large amounts of data (Ratnasingham & Hebert 2007). In addition to providing accurate species-level identification, DNA barcoding has also shown great promise in assessing and understanding the extent of diversity in groups that have proven difficult

by classical taxonomic techniques (Köhler 2007) through recognition of molecular operational taxonomic units (Floyd *et al*. 2002; Blaxter 2004; Smith *et al*. 2005). DNA barcoding has great implications for taxonomy, yet, perhaps one of the greatest promises is in studies of biological diversity within regional and poorly studied habitat-specific biotas (e.g. Smith *et al*. 2005).

The primary objectives of this study were to evaluate the performance of DNA barcoding to accurately distinguish bee species within a well-known fauna — that of Nova Scotia, Canada (Sheffield *et al*. 2003, 2004, 2008), and to demonstrate that DNA barcoding helps resolve difficult issues in bee taxonomy and therefore can be a great tool in ecological studies.

Materials and methods

Study site and collection of specimens

The 629 specimens used in this study were collected by various means (i.e. netted, yellow pan-traps, Malaise traps, trap-nests) between 1999–2007 during surveys of the bee fauna throughout Nova Scotia (Sheffield *et al*. 2003, 2004, 2008), pinned and identified to species level using appropriate taxonomic literature (see Sheffield *et al*. 2003). All specimens, including the barcoded vouchers, are retained at the Packer Bee Collection, York University, ON, Canada. Collection information, photographs and other specimen details required (see Ratnasingham & Hebert 2007) are available in the project file 'Bees of Nova Scotia' in the Completed Projects section of the BOLD website (www.barcodinglife.org).

Tissue sampling and molecular protocols

Tissue samples (i.e. a single middle leg) from pinned specimens were removed and examined to ascertain the patterns of COI divergences among species. Whenever possible, we analysed multiple individuals of a species to quantify the extent of intraspecific sequence divergence; these specimens were obtained from several separated localities in Nova Scotia. Prior to 2005, DNA extracts were prepared using the GenElute DNA miniprep Kit (Sigma), following the manufacturer's protocols. DNA extracts were resuspended in 10 μL of H_2O, and the 5' region of the COI gene was amplified using the LepFl and LepRI primers whose sequences are 5'-ATTCAACCAATCAT-AAAGATAT-3', and 5'-TAAACTTCTGGATGTCCAAAAA-3', respectively. The 50 μL polymerase chain reaction (PCR) mixes included 40 μL of ultrapure water, 1U of *Taq* polymerase, 2.5 μL $MgCl_2$, 4.5 μL 10× PCR buffer, 0.5 μL of each primer (0.1 mM), 0.25 μL of each dNTP (0.05 mM), and 0.5–3.0 μL of DNA. Amplification was carried out using a thermal regime consisting of 1 min at 94 °C followed by

five cycles of 1 min at 94 °C, 1.5 min at 45 °C, 1.5 min at 72 °C, followed by 30 cycles of 1 min at 4 °C, 1.5 min at 51 °C, 1.5 min at 72 °C, and a final 5 min at 72 °C. PCR products were visualized in a 1.2% agarose gel. All PCRs that generated a single product were then cycle-sequenced, while gel purification was used to recover the target gene product in cases where more than one band was present. Sequencing reactions, which were carried out using BigDye version 3.1 and the LepF1 primer, were analysed on an ABI 377 sequencer. For variations in this protocol associated with fully automated procedures used post-2004, see Hajibabaei *et al.* (2005) and Smith *et al.* (2005). The electropherogram and sequence for each specimen are available in the 'Bees of Nova Scotia' project file of BOLD. In addition, all sequences obtained in this study have been deposited in GenBank (Accession nos FJ581961–FJ582507).

Data analyses

Regression analysis (using Minitab Release 13 software, Pennsylvania State College) was used to look at the relationship between both 'year of collection' and 'age of specimen' vs. the sequence length (bp). Sequence divergences among individuals with COI sequences > 400 bp were determined using the Kimura 2-parameter (K2P) distance model (Kimura 1980). Mean divergences were subsequently calculated for both conspecific individuals (when available) and for congeneric taxa. As well, a neighbour-joining (NJ) tree based on K2P distances was built using the 'Taxon ID tree' function of BOLD to provide a graphic representation of the patterning of divergences among species (Saitou & Nei 1987). Sequence data were then downloaded from BOLD for further analyses. MEGA version 4 (Tamura *et al.* 2007) was used for sequence alignment and then to build compressed trees using the neighbour-joining algorithm with the K2P model, with pairwise deletion of missing data, and the inclusion of all codon positions and substitution types (as used in the BOLD analytical module). Branch support was assessed by bootstrapping with 500 replicates.

In addition, the strength of cohesion at varied taxonomic levels was quantified using the 'Nearest-Neighbour module' on BOLD. This module examines the strength of association among lineages at each level in the taxonomic hierarchy based on COI sequence similarity. The key level of analysis in this study, that examining the strength of association among conspecific individuals, involved identification of the closest COI sequence match for each individual belonging to a species represented by more than one individual in the database. In addition, cohesion at the generic level was examined by identifying the nearest neighbour for each species belonging to a genus that was represented in the database by more than one species. The results at each level of the taxonomic hierarchy are reported as the percentage of cases in which expected associations based on current taxonomy were recovered (i.e. at a species level, the percentage of individuals whose closest sequence match was to another member of its species).

Results

PCR products were generated for 547 of 629 specimens, 414 of these with sequence lengths over 500 bp (i.e. the barcode standard minimum; Ratnasingham & Hebert 2007) (Fig. 1)
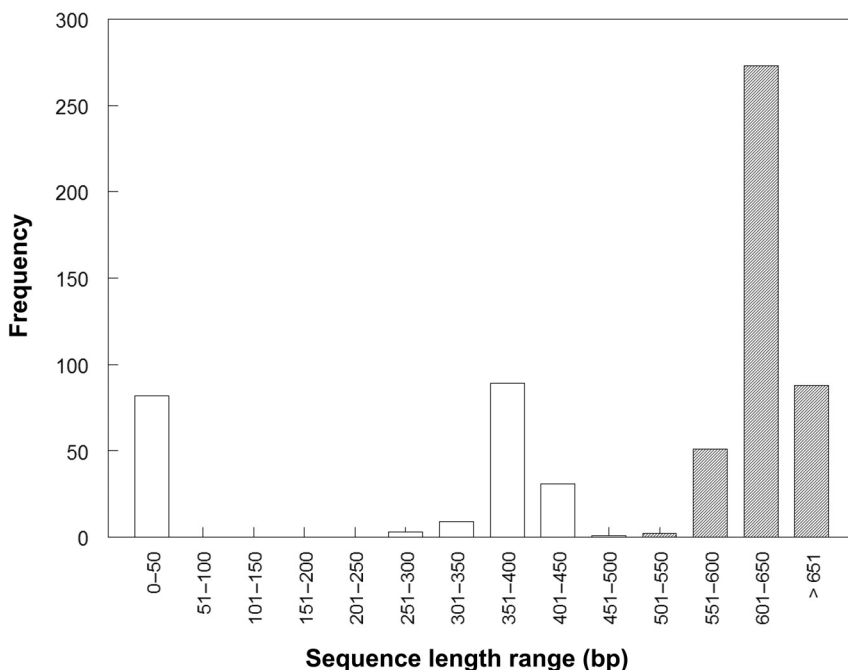

Fig. 1 Frequency plot of COI sequences of various length ranges (bp, base pairs). Shaded bars indicate sequences which are suitable for DNA barcode designation.
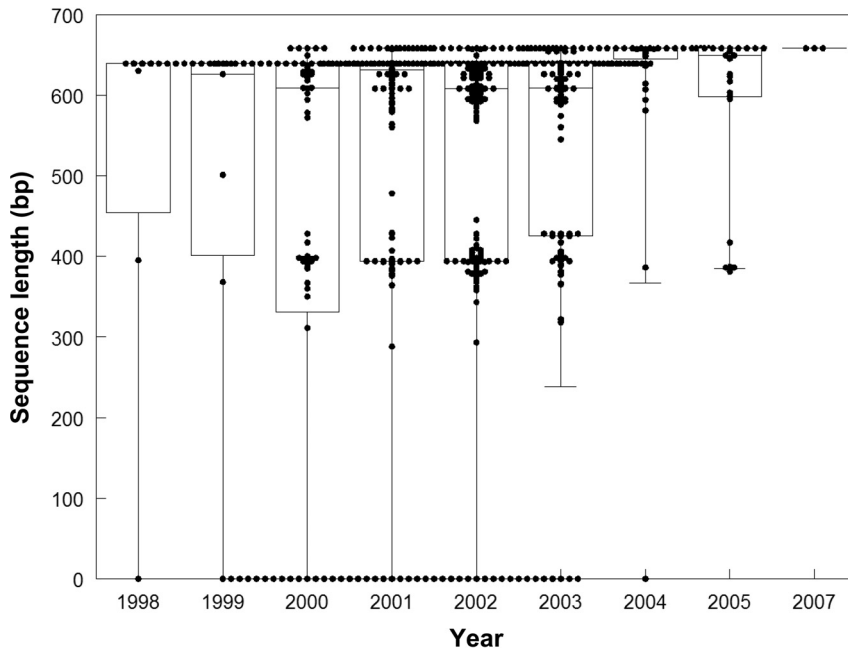
Fig. 2 Box-and-whisker plot of COI sequence length (bp, base pairs) data for the year of specimen collection. The relationship between sequence length and year was slight but significant, with a positive effect of R^2 = 3.5. A higher proportion of COI sequences from specimens collected after 2004 were longer than those collected prior to 2004 ($F_{1,604}$ = 22.21; P < 0.01).

and representing 144 species (approximately three-fourths of the known bee fauna of Nova Scotia; see Sheffield *et al*. 2003, 2004, 2008). The year of collection had a slight but significant positive effect (R^2 = 3.5) on COI sequence length ($F_{1,604}$ = 22.21; P < 0.01); a greater proportion of specimens collected prior to 2004 had variable sequence lengths compared to those collected beyond 2004, although median values for all years were above 600 bp (Fig. 2). The relationship between sequence length and age of specimen at the time of DNA extraction was not significant ($F_{1,604}$ < 0.1; P = 0.95). COI-5′ sequences were obtained for specimens from all 29 genera reported in the province (Sheffield *et al*. 2003, 2004, 2008), and were easily aligned as there were no insertion or deletion events. Translations confirmed that all of the sequences were free of nonsense and stop codons as would be expected if they were derived from the amplification of COI rather than a nuclear pseudogene (Bensasson *et al*. 2001).

Most of the species had a unique COI sequence; very few were shared between species; details of the exceptions are indicated below (and see Table 1). Each of the COI sequences exceeding 400 bp in the species represented by two or more individuals was either identical, or most closely related to a sequence from another individual of the same species (Table 1; Fig. 3). The strength of this association reflected the fact that COI sequence divergences were ordinarily far lower among individuals of a species than between closely related species; intraspecific distances averaged 0.48 ± 0.05% (SE) [max = 11.11%, within the *Hylaeus affinis* (Smith)/*H. modestus* Say complex discussed below], while species pairs in the same genus showed an

average sequence divergence of 13.32 ± 0.053% (SE), approximately 27 times higher. Species in different genera always showed marked differentiation with an average of 19.87 ± 0.026% (SE) divergence and a range from 11.52 to 32.51%.

Although this study primarily sought to evaluate the effectiveness of DNA barcoding for bee species recognition, an NJ tree based on sequence divergences recovered cohesive assemblages of genera and families (Fig. 3); this conclusion was supported by nearest-neighbour analysis that revealed cohesion across taxonomic strata; in the 18 genera with more than one species, 100% of the species paired most closely with another member of their genus. A similar pattern of association was apparent in families with more than one genus, as 99.3% of the genera paired most closely with another genus in their family, the exception being *Perdita octomaculata* (Say) (Andrenidae, Panurginae) which paired with the cleptoparasitic genus *Nomada* (Apidae) (Table 1).

Four species (i.e. two pairs) shared COI sequences, but each species was known from only the male or female sex. Thus, DNA barcoding associated two groups of previously unpublished conspecifics accurately. *Nomada inepta* Mitchell, described only from the female, showed very low divergence in COI sequences [mean = 0.042% ± 0.036% (SE); max 0.168%; *n* = 5] to *Nomada gracilis* Cresson, which is known only from the male (Table 1; Fig. 3). Similarly, *Sphecodes carolinus* Mitchell (female) and *S. coronus* Mitchell (male) showed low sequence divergence [mean = 0.266 ± 0.087% (SE); max 0.481%; *n* = 6] (Table 1; Fig. 3), confirming an unpublished sex association (M. Arduser, personal communication).

Table 1 COI sequence distance variability (%) among bee species in Nova Scotia, Canada, and the distance to nearest neighbour. Only data from specimens yielding COI sequence lengths > 400 base pairs were used. Species marked with (†) show either higher than expected mean distances or lower than expected distances to nearest neighbours (indicated in bold with *); see text for comments on these species. N/A (not applicable) indicates that distance calculations were not done due to a single representative of that species

Species	No. of specimens	Mean distance within species (%)	Maximum distance within species (%)	Distance to nearest neighbour	Nearest neighbour
Andrena algida	3	0	0	9.39	*Andrena tridens*
Andrena alleghaniensis	3	0	0	14.09	*Andrena rugosa*
Andrena barbilabris	3	0	0	12.76	*Andrena robertsonii*
Andrena canadensis†	4	0.24	0.48	**0.81***	*Andrena nubecula*
Andrena carolina	5	0.07	0.18	6.03	*Andrena milwaukeensis*
Andrena ceanothi	1	N/A	N/A	4.37	*Andrena hippotes*
Andrena dunningi	9	0.23	0.8	9.14	*Andrena regularis*
Andrena erigeniae	2	0	0	8.45	*Andrena tridens*
Andrena forbesii	5	0	0	4.71	*Andrena sigmundi*
Andrena frigida	2	0.78	0.78	6.41	*Andrena milwaukeensis*
Andrena hippotes	3	0.44	0.66	4.24	*Andrena sigmundi*
Andrena hirticincta	2	0.17	0.17	8.8	*Andrena canadensis*
Andrena kalmiae	2	0.16	0.16	16.65	*Andrena nivalis*
Andrena mandibularis	2	0.17	0.17	6.69	*Andrena rufosignata*
Andrena melanochroa	1	N/A	N/A	15.25	*Andrena vicina*
Andrena milwaukeensis	3	0.22	0.33	5.63	*Andrena rufosignata*
Andrena miserabilis†	4	**5.91***	8.9	12.67	*Andrena nivalis*
Andrena nivalis	3	0.94	1	2.3	*Andrena vicina*
Andrena nubecula†	3	0.12	0.19	**0.81***	*Andrena canadensis*
Andrena regularis	3	0	0	9.14	*Andrena dunningi*
Andrena robertsonii	1	N/A	N/A	12.76	*Andrena barbilabris*
Andrena rufosignata	5	0.06	0.15	5.63	*Andrena milwaukeensis*
Andrena rugosa	2	0.16	0.16	4.91	*Andrena ceanothi*
Andrena sigmundi	3	0.1	0.15	4.24	*Andrena hippotes*
Andrena tridens	1	N/A	N/A	8.45	*Andrena erigeniae*
Andrena vicina	3	0.78	0.84	2.3	*Andrena nivalis*
Andrena wheeleri	5	0.32	0.72	14.86	*Andrena robertsonii*
Andrena wilkella	3	0.17	0.17	12.13	*Andrena sigmundi*
Andrena wscripta	6	0.1	0.34	11.84	*Andrena vicina*
Calliopsis andreniformis	3	0.37	0.64	21.16	*Andrena vicina*
Perdita octomaculata	3	0.1	0.16	16.15	*Nomada lehighensis*
Pseudopanurgus nebrascensis	4	0.31	0.48	9.4	*Pseudopanurgus andrenoides*
Pseudopanurgus andrenoides	2	0	0	9.4	*Pseudopanurgus nebrascensis*
Anthophora bomboides	2	0	0	11.97	*Anthophora furcata*
Anthophora furcata	2	1.5	1.5	11.97	*Anthophora bomboides*
Apis mellifera	5	0.21	0.34	16.32	*Nomada sayi*
Bombus ashtoni	3	0	0	11.2	*Bombus citrinus*
Bombus borealis	4	0.16	0.32	9.1	*Bombus fervidus*
Bombus citrinus	5	0.48	0.8	5.96	*Bombus insularis*
Bombus fernaldae	3	0.1	0.16	11.96	*Bombus ashtoni*
Bombus fervidus	6	0.2	0.51	9.1	*Bombus borealis*
Bombus impatiens†	3	**2.41***	3.62	5	*Bombus ternarius*
Bombus insularis	1	N/A	N/A	5.96	*Bombus citrinus*
Bombus perplexus	3	0.42	0.64	6.74	*Bombus vagans*
Bombus rufocinctus	3	1.47	2.33	7.62	*Bombus vagans*
Bombus sandersoni	3	0.52	0.74	6.58	*Bombus vagans*
Bombus ternarius†	6	**2.24***	4.59	5	*Bombus impatiens*
Bombus terricola	3	0.21	0.32	9.21	*Bombus sandersoni*
Bombus vagans	5	0	0	6.58	*Bombus sandersoni*
Ceratina calcarata†	6	0	0	**1.55***	*Ceratina* sp.
Ceratina dupla†	6	0.11	0.32	**1.4***	*Ceratina* sp.
Ceratina sp.†	8	0.24	0.62	**1.4***	*Ceratina dupla*
Epeoloides pilosula	1	N/A	N/A	13.39	*Nomada sayi*

Table 1 *Continued*

| Species	No. of specimens	Mean distance within species (%)	Maximum distance within species (%)	Distance to nearest neighbour	Nearest neighbour
Epeolus autumnalis	3	0.59	0.88	10.93	*Epeolus scutellaris*
Epeolus scutellaris	1	N/A	N/A	10.93	*Epeolus autumnalis*
Holcopasites calliopsidis	2	0.31	0.31	14.78	*Nomada inepta*
Melissodes desponsa	2	0.33	0.33	3.74	*Melissodes druriella*
Melissodes druriella	1	N/A	N/A	3.74	*Melissodes desponsa*
Nomada articulata	2	0.17	0.17	6.35	*Nomada australis*
Nomada australis	3	1.46	2.19	6.35	*Nomada articulata*
Nomada bella	3	0.32	0.32	4.29	*Nomada* sp.
Nomada cressonii†	3	2.15	3.23	**1.03***	*Nomada pygmaea*
Nomada dentaria	3	0.5	0.79	3.46	*Nomada inepta*
Nomada gracilis†	3	0.84	1.3	**0***	*Nomada inepta*
Nomada illinoiensis	3	0.44	0.67	**0***	*Nomada lehighensis*
Nomada imbricata	3	0.16	0.32	4.93	*Nomada subrutila*
Nomada inepta†	3	0	0	**0***	*Nomada gracilis*
Nomada lehighensis†	6	0.86	1.91	**0***	*Nomada illinoiensis*
Nomada lepida	5	0.59	1.04	2.88	*Nomada* sp.
Nomada maculata	8	1.38	4.19	5.43	*Nomada ovata*
Nomada ovata	1	N/A	N/A	4.77	*Nomada* sp.
Nomada pygmaea†	2	2.44	2.44	**1.03***	*Nomada cressonii*
Nomada sayi†	1	N/A	N/A	**1.03***	*Nomada pygmaea*
Nomada sp.	1	N/A	N/A	2.88	*Nomada lepida*
Nomada subrutila	4	0	0	4.93	*Nomada imbricata*
Nomada vicina	2	0	0	4.42	*Nomada illinoiensis*
Triepeolus brittaini	4	0	0	14.81	*Nomada subrutila*
Colletes compactus	3	0.53	0.79	9.93	*Colletes simulans*
Colletes inaequalis	3	0.42	0.63	8.31	*Colletes simulans*
Colletes simulans	2	0.95	0.95	8.31	*Colletes inaequalis*
Hylaeus affinis/modestus	4	**7.65***	11.11	8.99	*Hylaeus basalis*
Hylaeus annulatus	5	0.38	0.67	10.69	*Hylaeus verticallis*
Hylaeus basalis	3	1.02	1.53	8.99	*Hylaeus affinis/modestus*
Hylaeus mesillae	2	0.17	0.17	8.9	*Hylaeus verticallis*
Hylaeus verticallis	2	0	0	8.9	*Hylaeus mesillae*
Agapostemon virescens	3	0	0	18.84	*Lasioglossum divergens*
Augochlorella aurata†	5	2.89	7.36	18.61	*Lasioglossum divergens*
Dufourea novaangliae	6	0.43	0.79	18.24	*Lasioglossum divergens*
Halictus confusus	5	0	0	14.6	*Halictus rubicundus*
Halictus ligatus	2	0	0	12.15	*Halictus rubicundus*
Halictus rubicundus	3	1.31	1.82	12.15	*Halictus ligatus*
Lasioglossum acuminatum	1	N/A	N/A	2.9	*Lasioglossum coriaceum*
Lasioglossum cinctipes	1	N/A	N/A	17.09	*Lasioglossum divergens*
Lasioglossum coriaceum	2	0.66	0.66	2.9	*Lasioglossum acuminatum*
Lasioglossum cressonii	2	0.17	0.17	11.11	*Lasioglossum pectorale*
Lasioglossum divergens	6	0.15	0.34	7.91	*Lasioglossum pectorale*
Lasioglossum foxii	1	N/A	N/A	12.61	*Lasioglossum quebecense*
Lasioglossum leucozonium	6	0.18	0.48	12.7	*Lasioglossum zonulum*
Lasioglossum pectorale	3	0	0	7.91	*Lasioglossum divergens*
Lasioglossum quebecense	2	0.17	0.17	12.61	*Lasioglossum foxii*
Lasioglossum zonulum	3	0.11	0.16	12.7	*Lasioglossum leucozonium*
Sphecodes autumnalis	2	0	0	9.59	*Sphecodes cressonii*
Sphecodes carolinus†	4	0.23	0.48	**0***	*Sphecodes coronus*
Sphecodes clematidis	3	0.11	0.17	3.44	*Sphecodes dichrous*
Sphecodes confertus	3	0	0	10.5	*Sphecodes autumnalis*
Sphecodes coronus†	2	0.46	0.46	**0***	*Sphecodes carolinus*
Sphecodes cressonii†	4	0.08	0.17	**0***	*Sphecodes galerus*
Sphecodes dichrous	4	0.17	0.34	2.44	*Sphecodes minor*
Sphecodes galerus†	1	N/A	N/A	**0***	*Sphecodes cressonii*

Table 1 *Continued*

Species	No. of specimens	Mean distance within species (%)	Maximum distance within species (%)	Distance to nearest neighbour	Nearest neighbour
Sphecodes minor	2	0.17	0.17	2.44	*Sphecodes dichrous*
Sphecodes prosphorus	2	1.23	1.23	8.12	*Sphecodes clematidis*
Sphecodes ranunculi	5	0	0	14.64	*Sphecodes cressonii*
Sphecodes solonis	1	N/A	N/A	14.5	*Sphecodes clematidis*
Sphecodes sp.†	1	N/A	N/A	**1.71***	*Sphecodes carolinus*
Sphecodes stygius	2	0	0	8.61	*Sphecodes carolinus*
Coelioxys funeraria	5	0.76	1.43	9.55	*Coelioxys moesta*
Coelioxys moesta	4	0.39	0.47	8.13	*Coelioxys rufitarsis*
Coelioxys rufitarsis	3	0	0	8.13	*Coelioxys moesta*
Coelioxys sodalis	3	0.21	0.32	10.99	*Coelioxys moesta*
Heriades carinata	3	0.53	0.79	12.87	*Hoplitis spoliata*
Hoplitis pilosifrons	1	N/A	N/A	5.21	*Hoplitis producta*
Hoplitis producta	4	0.71	1.08	5.21	*Hoplitis pilosifrons*
Hoplitis spoliata	2	0.34	0.34	9.96	*Hoplitis pilosifrons*
Megachile centuncularis	1	N/A	N/A	9.9	*Megachile gemula*
Megachile frigida	4	0.16	0.32	6.57	*Megachile gemula*
Megachile gemula	2	0	0	5.89	*Megachile melanophea*
Megachile inermis	3	0	0	11.17	*Megachile gemula*
Megachile latimanus	2	0	0	6.74	*Megachile gemula*
Megachile melanophaea	2	0	0	5.89	*Megachile gemula*
Megachile montivaga	1	N/A	N/A	9.21	*Megachile frigida*
Megachile pugnata	3	0	0	9.72	*Megachile gemula*
Megachile rotundata	3	0	0	9.9	*Megachile gemula*
Osmia atriventris	3	0.33	0.5	4.7	*Osmia proxima*
Osmia bucephala	3	0	0	4.71	*Osmia atriventris*
Osmia coerulescens	3	0	0	8.66	*Osmia simillima*
Osmia inermis	2	0	0	5.77	*Osmia proxima*
Osmia lignaria	2	0	0	9.23	*Osmia simillima*
Osmia proxima	5	0.24	0.69	4.7	*Osmia atriventris*
Osmia simillima	6	0.19	0.33	4.94	*Osmia virga*
Osmia tersula	7	0.24	0.51	6.78	*Osmia inermis*
Osmia virga	2	0.32	0.32	4.94	*Osmia simillima*
Stelis foederalis	2	0.76	0.76	9.79	*Stelis subemarginata*
Stelis subemarginata	1	N/A	N/A	9.79	*Stelis foederalis*
Macropis nuda	3	0.32	0.47	18.77	*Colletes inaequalis*

DNA barcoding indicated the possible presence of a third (undescribed) species of *Ceratina* (Apidae: Xylocopinae) in Nova Scotia (Fig. 3, as 'Ceratina sp.') which would key out to *C. dupla* Say in Mitchell (1962), Daly (1973) and Rehan & Richards (2008); these species showed low levels of divergence (Table 1; Fig. 3). Similarly, *Andrena miserabilis* Cresson, the sole Nearctic member of the subgenus *Larandrena* LaBerge (Ribble 1967; Michener 2007), appears to have a morphologically similar (i.e. indistinguishable) and closely related (mean = 5.907 ± 1.68% (SE); max 8.903%; *n* = 4) sister species (Fig. 3).

Among the bumble bees, *Bombus ternarius* Say showed particularly high levels of sequence divergence (mean = 2.237 ± 0.515% (SE); max 4.592%; *n* = 6), separating into two distinct clusters. *Bombus impatiens* Cresson also showed divergence, [mean = 2.406 ± 0.982% (SE); max 3.618%; *n* = 3], albeit entirely on the account of one individual.

Discussion

We are presently in the midst of a major biodiversity crisis, and loss of species continues to happen at an unprecedented rate (Wilson 1992; Novacek 2007; Chivian & Bernstein 2008). Recognizing the need to conserve the Earth's biodiversity (in terms of both species and functional diversity, as per Maclaurin & Sterelny 2008), and actually knowing how many species there are remains the major challenge to these efforts. Our estimates of the Earth's biota differ by at least one degree of magnitude, ranging from 10 million to upwards of 100 million species (Chivian & Bernstein 2008). DNA barcoding offers great hope to understanding the extent of biodiversity, allowing rapid identification of previously studied species (Hebert *et al.* 2003a, b), cryptic species (Janzen *et al.* 2005; Waugh 2007), and assisting in the recognition of new 'genetically distinct'
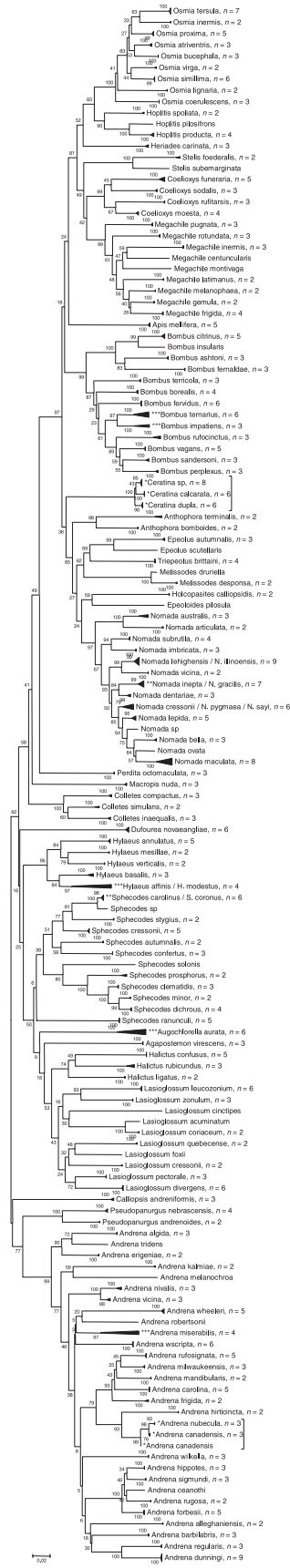
taxa (reviewed in Hajibabaei *et al.* 2007), as was also demonstrated in the present study. DNA barcoding also provides a simple and accurate way to associate sexes in insects, many of which are dimorphic and may presently be described as separate species, as demonstrated in this study and elsewhere (Janzen *et al.* 2005). Mound (1998) suggested that even in well-studied insect groups, at least 25% of all recognized species names are subsequently placed into synonymy upon revisionary work. Accurate sex association certainly would contribute to this effort, which ultimately assists in generating accurate regional and global species lists.

Considering that most insect surveys in terrestrial systems, including those of bees, use sampling methods which capture adults, DNA barcoding facilitates linking studies of alpha diversity to those which investigate functional diversity (as per Tilman & Lehman 2001; Maclaurin & Sterelny 2008), thus facilitating broader ecological knowledge (Mound 1998). For instance, DNA barcoding allows immature life stages to be matched to adult specimens (Janzen *et al.* 2005; Köhler 2007). In the present context (i.e. Apoidea), DNA barcoding could facilitate associations of cleptoparasitic bees (and/or other nest associates) to respective host taxa within nests, which ultimately would provide valuable information for further studies of bee ecology (e.g. Wcislo 1981; Wcislo & Cane 1996) and phylogeny (e.g. Alexander 1991, 1994).

This study establishes two facts that jointly indicate the feasibility of assembling a comprehensive COI library for bees for species-level identification, the primary goal of the Bee-BOL campaign (http://www.bee-bol.org). First, this study confirms for the Apoidea that COI sequences can be recovered from a wide range of bee species through PCR amplification with a uniform primer set. Second, it reveals that sequences can be obtained from many dried bee specimens at least 8 years old, and curated museum collections can contribute material for analysis and/or building a COI library (L. Packer *et al.*, in preparation). The proportion of specimens that did not yield PCR material (Fig. 1) is undoubtedly a reflection of collection methods used; much of the material samples collected prior to 2004 (Fig. 2) were killed using ethyl acetate which is known to negatively affect DNA (Prendini *et al.* 2002). Subsequent (post-2004) killing methods used for bees (e.g. pan-trapping in salt water solution, cyanide, ethanol, propylene glycol) greatly enhanced successful amplification (Fig. 2) and are recommended (Dick *et al.* 1993; Dillon *et al.* 1996).

Fig. 3 Neighbour-joining tree for bees in Nova Scotia, Canada. Groups of species separated by small (< 2%) genetic divergence are indicated with a bracket and *; those which are believed to be associated males and females are indicated with **; species indicated with *** show high (> 2.2%) intraspecific genetic divergence and may contain undescribed taxa (see text for details). See Materials and methods for details on the tree-building algorithm.

Aside from demonstrating the recovery of COI sequences from diverse bee taxa, our results make clear that the resultant sequences are taxonomically informative. Species boundaries signalled by deep COI divergences are generally congruent with those established through classical taxonomic work within this regional fauna. Our results also establish that individuals of a species possess little variation with COI divergences averaging just 0.49%, a value slightly higher similar to those reported for lepidopterans (0.25%; Hebert *et al*. 2003a) and birds (0.27%; Hebert *et al*. 2004). Because of their elevated rates of mitochondrial evolution (Crozier *et al*. 1989), we had expected higher intraspecific divergences in bees than in other insect groups. However, the COI divergences reported are probably higher than the actual values; our results were skewed primarily by the *Hylaeus affinis* / *H. modestus* complex, with 11.11% divergence among the members; in Nova Scotia, at least three species comprise this difficult complex (Fig. 3). Additional morphological/molecular studies of eastern North American *Hylaeus* are currently ongoing (S. Droege, US Geological Survey, personal communication).

Higher-than-expected sequence divergence was also observed among *Bombus ternarius* specimens (Table 1; Fig. 3). Despite this species being among the easiest to identify in eastern Canada (Laverty & Harder 1988), two distinct clusters were observed in Nova Scotia, a trend which was supported with the addition of material from across Canada (C.S. Sheffield, unpublished). Additional divergences occurred among species within the genera *Nomada* and *Sphecodes* (Table 1; Fig. 3) which are notoriously difficult to identify accurately as many species are described from one sex (Mitchell 1960, 1962) and show variable morphological characters (i.e. colour patterns, scutellum shape, punctation). These genera accounted for almost all of the remaining intraspecific sequence divergences; this is primarily a reflection of not being able to assign a species epithet accurately. Clearly, DNA barcoding has a tremendous amount to offer taxonomic studies of these common, yet poorly known cleptoparasites.

In addition to the success with the Nova Scotia bee fauna, prior studies of COI diversity in bees reinforce our conclusion that conspecific individuals normally show little variation, while congeneric species pairs typically show marked divergence; Pederson (1996) found no intraspecific variation in his study of COI diversity in 11 European species of *Bombus.* Similarly, a detailed study on *Halictus rubicundus* revealed less than 2.5% sequence divergence among individuals from widely separated locales in both North America and Europe (Soucy & Danforth 2002). Although bee assemblages in some arid settings have several magnitudes more species than those in our study area (Michener 1979, 2007), we expect similar identification success for two reasons. First, DNA barcoding has proven very effective in distinguishing species in several hyper-diverse tropical insect assemblages (Smith *et al*. 2005; Hajibibaei *et al*. 2006). Second, DNA barcodes diagnosed most of the bee species in our study area with particular clarity. Levels of sequence divergence between congeneric pairs were 27 times higher than intraspecific divergences (13.3% vs. 0.5%). Once again, we attribute this result to the high rates of mitochondrial DNA evolution in hymenopterans (Crozier *et al*. 1989; Hebert *et al*. 2003a), suggesting that this rate acceleration derives from selective sweeps that lead to both the rapid accumulation of differences between species and to the erosion of variation within species. Because of its rapid sequence evolution, mitochondrial DNA has long been valued for its ability to resolve recent evolutionary events (reviewed in Avise 2000). As such, the ability of COI to discriminate closely allied species comes as little surprise.

The ability of COI-based systems to provide a tentative taxonomic placement for cryptic (Carman & Packer 1996; Danforth *et al*. 1998) or newly encountered (i.e. genetically distinct) species is important because it aids their referral to the appropriate taxonomic specialist (Hajibabaei *et al*. 2007). In the present study, DNA barcoding revealed an undescribed species of *Ceratina* within Nova Scotia. Only five species of *Ceratina* are known from eastern North America (Mitchell 1962; Daly 1973), and the females of the two most common (*C. dupla* and *C. calcarata*) are thought to be indistinguishable using traditional morphology (Daly 1973), although recent combined molecular/morphological studies of these two species have clarified this (Rehan & Richards 2008). Using molecular techniques (i.e. DNA barcoding) as a guide, consistent morphological characters have now been found to separate males of all the species in eastern North America (S. Rehan and C. Sheffield, in preparation).

This study contributes to a growing body of work that demonstrates the effectiveness of DNA barcodes in species identifications for members of the phylum Arthropoda. The approach has now gained preliminary validation in all major lineages of arthropods including spiders (Barrett & Hebert 2005), crustaceans (Witt *et al*. 2006), collembolans (Hogg & Hebert 2004) and other insects (Janzen *et al*. 2005). The current results extend prior barcoding investigations on insects by establishing its effectiveness in a group with an unusually high rate of mitochondrial evolution. Moreover, because of the limited intraspecific variation (even among individuals from widely separated locales), an effective identification system can be created by analysing just a few specimens of each species. Collectively, these results establish that identification systems based on COI will regularly provide species-level resolution. However, Schaefer & Renner (2008) suggest caution against a potential problem with pseudogenes in COI in bees (observed in their study of Ctenoplectrini), and comment on the importance of screening COI when used for barcoding purposes.

While the potential constraints of DNA-based identification systems have been discussed in detail, there has been less consideration of the new opportunities that will arise from their development. In the case of bees, the ability to identify all life stages and to associate individuals of different sex represents an important extension of existing identification capabilities. The assembly of a barcode library for the bees of the world will not only provide insights into the origins and extent of bee diversity, but it will create a new tool for both routine and challenging identifications.

Acknowledgements

Conflict of interest statement

The authors have no conflict of interest to declare and note that the funders of this research had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Alexander BA (1991) *Nomada* phylogeny reconsidered. *Journal of Natural History*, **25**, 315–330.

Alexander BA (1994) Species-groups and cladistic analysis of the cleptoparasitic bee genus *Nomada*. *University of Kansas Science Bulletin*, **55**, 175–238.

Ascher JS, Eardley C, Griswold T, Melo G, Polaszek A, Tadauchi O, Walker K, Warrit N, Williams P (2008) Discover Life Apoidea species guide. http://www.discoverlife.org

Avise JC (2000) *Phylogeography. The History and Formation of Species*. Harvard University Press, Cambridge, Massachusetts.

Barrett R, Hebert PDN (2005) Identifying spiders through DNA barcodes. *Canadian Journal of Zoology*, **83**, 481–491.

Bensasson D, Zhang D, Hartl DL, Hewitt GM (2001) Mitochondrial pseudogenes: evolution's misplaced witnesses. *Trends in Ecology & Evolution*, **16**, 314–321.

Biesmeijer JC, Roberts SPM, Reemer M *et al.* (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science*, **313**, 351–354 (doi: 10.1126/science.1127863.)

Blaxter ML (2004) The promise of a DNA taxonomy. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **359**, 669–679.

Bortolus A (2008) Error cascades in the biological sciences: the unwanted consequences of using bad taxonomy in ecology. *Ambio*, **37**, 114–118.

Buchmann SL, Nabhan GP (1996) *The Forgotten Pollinators*. Island Press, Washington D. C.

Carman GM, Packer L (1996) A cryptic species allied to *Halictus ligatus* Say (Hymenoptera: Halictidae) detected by allozyme electrophoresis. *Journal of the Kansas Entomological Society*, **69**, 168–176.

Chivian E, Bernstein A (2008) *Sustaining Life: How Human Health Depends on Biodiversity*. Oxford University Press, New York.

Committee on the Status of Pollinators in North America (2007) *Status of Pollinators in North America*. National Academic Press, Washington, DC.

Crozier RH, Crozier YC, Mackinley AG (1989) The CO-I and CO-II region of the honeybee mitochondrial DNA: evidence of variation in insect mitochondrial evolutionary rates. *Molecular Biology and Evolution*, **6**, 399–411.

Daly HV (1973) Bees of the genus *Ceratina* in America north of Mexico. *University of California Publications in Entomology*, **74**, 1–114.

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

Dick M, Bridge DM, Wheeler WC, DeSalle R (1993) Collection and storage of invertebrate samples. *Methods in Enzymology*, **224**, 51–65.

Dillon N, Austin AD, Bartowsky E (1996) Comparison of preservation techniques for DNA extraction from hymenopterous insects. *Insect Molecular Biology*, **5**, 21–24.

Floyd R, Eyualem A, Papert A, Blaxter M (2002) Molecular barcodes for soil nematode identification. *Molecular Ecology*, **11**, 839–850 (doi: 10.1046/j.1365-294X.2002.01485.x).

Gotelli NJ (2004) A taxonomic wish-list for community ecology. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **359**, 585–597.

Grimaldi D, Engel MS (2005) *Evolution of the Insects*. Cambridge University Press, New York.

Hajibabaei M, deWaard JR, Ivanova NV *et al.* (2005) Critical factors for assembling a high volume of DNA barcodes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **360**, 1959–1967.

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN (2006) DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences, USA*, **103**, 968–971.

Hajibabaei M, Singer GAC, Hebert PDN, Hickey DA (2007) DNA barcoding: how it compliments taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics*, **23**, 167–172.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 313–322.

Hebert PDN, Ratnasingham S, deWaard JR (2003b) Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society B: Biological Sciences*, **270**, S96–S99.

Hebert PD, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. *Public Library of Science, Biology*, **2**, 1657–1663.

Heimpel GE, de Boer JG (2008) Sex determination in the Hymenoptera. *Annual Review of Entomology*, **53**, 209–230.

Hogg ID, Hebert PDN (2004) Biological identifications of springtails (Hexapoda: Collembola) from the Canadian arctic, using mitochondrial barcodes. *Canadian Journal of Zoology*, **82**, 749–754.

Janzen DH, Hajibabaei M, Burns JM, Hallwachs W, Remigio E, Hebert PDN (2005) Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **360**, 1835–1845.

Kevan PG (1999) Pollinators as bioindicators of the state of the environment; species, activity and diversity. *Agriculture, Ecosystems and Environment*, **74**, 373–393.

Kevan PG, Baker HG (1983) Insects as flower visitors and pollinators. *Annual Review of Entomology*, **28**, 407–453.

Kevan PG, Greco CF, Belaoussoff S (1997) Log-normality of biodiversity and abundance in diagnosis and measuring of ecosystem health: pesticide stress on pollinators on blueberry heaths. *Journal of Applied Ecology*, **34**, 1122–1136.

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

Köhler F (2007) From DNA taxonomy to barcoding — how a vague idea evolved into a biosystematic tool. *Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe*, **83**, 44–51 (doi: 10.1002/mmnz.200600025.)

Laverty TM, Harder LD (1988) The bumble bees of eastern Canada. *Canadian Entomologist*, **120**, 965–987.

Maclaurin J, Sterelny K (2008) *What is Biodiversity?* University of Chicago Press, Chicago.

Matheson A, Buchmann SL, O'Toole C, Westrich P, Williams IH (1996) *The Conservation of Bees*. Academic Press, London.

Michener CD (1974) *The Social Behavior of the Bees: A Comparative Study*. Belknap Press of Harvard University Press, Cambridge, Massachusetts.

Michener CD (1979) Biogeography of the bees. *Annals of the Missouri Botanical Garden*, **66**, 277–347.

Michener CD (2007) *The Bees of the World*, 2nd edn. Johns Hopkins University Press, Baltimore, Maryland.

Mitchell TB (1960) Bees of the eastern United States. Volume I. *North Carolina Agricultural Experiment Station Technical Bulletin*, **141**, 1–538.

Mitchell TB (1962) Bees of the eastern United States. Volume II. *North Carolina Agricultural Experiment Station Technical Bulletin*, **152**, 1–557.

Mound LA (1998) Insect taxonomy in species-rich countries — the way forward? *Anais da Sociedade Entomológica do Brasil*, **27**, 1–8.

Novacek M (2007) *Terra: Our 100-Million-Year-Old Ecosystem — and the Threats that Now Put it at Risk*. Farrar, Straus and Giroux, New York.

Pederson BV (1996) A phylogenetic analysis of cuckoo bumblebees (*Psithyrus*, Lepeletier) and bumblebees (*Bombus*, Latreille) inferred from sequences on the mitochondrial gene cytochrome oxidase I. *Molecular Phylogenetics and Evolution*, **5**, 289–297.

Prendini L, Hanner R, DeSalle R (2002) Obtaining, storing and archiving specimens and tissue samples for use in molecular studies. In: *Techniques in Molecular Evolution and Systematics* (eds DeSalle R, Giribet G, Wheeler WC), pp. 176–248. Birkhäuser Verlag AG, Basel, Switzerland.

Ratnasingham S, Hebert PDN (2007) BOLD: the Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes*, **7**, 355–364.

Rehan SM, Richards MH (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.

Ribble DW (1967) The monotypic North American subgenus *Larandrena* of *Andrena*. *Bulletin of the Nebraska State Museum*, **6**, 27–42.

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406–425.

Schaefer H, Renner SS (2008) A phylogeny of the oil bee tribe Ctenoplectrini (Hymenoptera: Anthophila) based on mitochondrial and nuclear data: evidence for Early Eocene divergence and repeated out-of-Africa dispersal. *Molecular Phylogenetics and Evolution*, **47**, 799–811.

Schröder S, Wittmann D, Drescher W, Roth V, Steinhage V, Cremers AB (2002) The new key to bees: automated identification by image analysis of wings. In: *Pollination Bees: The Conservation Link Between Agriculture and Nature* (eds Kevan PG, Imperatriz-Fonseca VL), pp. 209–216. Ministry of Environment, Secretariat for Biodiversity and Forests, Brasília-DF, Brazil.

Sheffield CS, Westby SM (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.

Sheffield CS, Kevan PG, Smith RF, Rigby SM, Rogers REL (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 CS, Rigby SM, Smith RF, Kevan PG (2004) The rare cleptoparasitic bee *Epeoloides pilosula* (Hymenoptera: Apoidea: Apidae) discovered in Nova Scotia, Canada, with distributional notes. *Journal of the Kansas Entomological Society*, **77**, 161–164.

Sheffield CS, Kevan PG, Westby SM, Smith RF (2008) Diversity of cavity-nesting bees (Hymenoptera: Apoidea) within apple orchards and wild habitats in the Annapolis Valley, Nova Scotia, Canada. *Canadian Entomologist*, **140**, 235–249.

Smith MA, Fisher BL, Hebert PDN (2005) DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **360**, 1825–1834.

Soucy SL, Danforth BN (2002) Phylogeography of the socially polymorphic sweat bee *Halictus rubicundus* (Hymenoptera: Halictidae). *Evolution*, **56**, 330–341 (doi: 10.1111/j.0014-3820.2002.tb01343.x).

Stubbs CS, Drummond FA (2001) *Proceedings: Bees and Crop Pollination — Crisis, Crossroads, Conservation*. Thomas Say Publications in Entomology. Entomological Society of America, Lanham, Maryland.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**, 1596–1599.

Tilman D, Lehman C (2001) Biodiversity, composition, and ecosystem processes: theory and concepts. In: *The Functional Consequences of Biodiversity: Empirical Progress and Theoretical Extensions* (eds Kinzig AP, Pacala SW, Tilman D), pp. 9–41. Princeton University Press, Princeton, New Jersey.

Waugh J (2007) DNA barcoding in animal species: progress, potential and pitfalls. *Bioessays*, **29**, 188–197 (doi: 10.1002/bies.20529.)

Wcislo WT (1981) The roles of seasonality, host synchrony, and behaviour in the evolutions and distributions of nest parasites in Hymenoptera (Insecta), with special reference to bees (Apoidea). *Biological Reviews*, **62**, 515–543.

Wcislo WT, Cane JH (1996) Floral resource utilization by solitary bees (Hymenoptera: Apoidea) and exploitation of their stored foods by natural enemies. *Annual Review of Entomology*, **41**, 257–286.

Weeks PJD, O'Neill MA, Gaston KJ, Gauld ID (1999) Automating insect identifications: exploring the limitations of a prototype system. *Journal of Applied Entomology*, **123**, 1–8.

Will KW, Rubinoff D (2004) Myth of the molecule: DNA barcodes for species cannot replace morphology for identifications and classification. *Cladistics*, **20**, 47–55.

Wilson EO (1971) *The Insect Societies*. Harvard University Press, Cambridge, Massachusetts.

Wilson EO (1992) *The Diversity of Life*. W.W. Norton & Company, New York.

Witt JDS, Threloff DL, Hebert PDN (2006) DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. *Molecular Ecology*, **15**, 3071–3082 (doi: 10.1111/j.1365-294X.2006.02999.x).

Zayed A, Packer L (2005) Complementary sex determination substantially increases extinction proneness of haplodiploid populations. *Proceedings of the National Academy of Sciences, USA*, **102**, 10742–10746.

Zayed A, Packer L (2007) The population genetics of a solitary oligolectic sweat bee, *Lasioglossum (Sphecodogastra) oenotherae* (Hymenoptera: Halictidae). *Heredity*, **99**, 397–405.

Zayed A, Packer L, Grixti JC, Ruz L, Owen RE, Toro H (2005) Increased genetic differentiation in a specialist versus a generalist bee: implications for conservation. *Conservation Genetics*, **6**, 1017–1026.