Medical and Veterinary Entomology (2006) **20**, 413–424

Identifying Canadian mosquito species through DNA barcodes

A. CYWINSKA[1], F. F. HUNTER[1] and P. D. N. HEBERT[2]

[1]Department of Biological Sciences, Brock University, St. Catharines, Ontario, Canada and [2]Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada

Abstract. A short fragment of mt DNA from the cytochrome c oxidase 1 (CO1) region was used to provide the first CO1 barcodes for 37 species of Canadian mosquitoes (Diptera: Culicidae) from the provinces Ontario and New Brunswick. Sequence variation was analysed in a 617-bp fragment from the 5′ end of the CO1 region. Sequences of each mosquito species formed barcode clusters with tight cohesion that were usually clearly distinct from those of allied species. CO1 sequence divergences were, on average, nearly 20 times higher for congeneric species than for members of a species; divergences between congeneric species averaged 10.4% (range 0.2–17.2%), whereas those for conspecific individuals averaged 0.5% (range 0.0–3.9%).

Key words. Barcode of life, CO1–5′ region, evolution, mitochondrial DNA, molecular taxonomy, mosquitoes, sequence divergence.

Introduction

The increasing loss of biodiversity globally has led to numerous proposals to intensify efforts to produce a census of all biological diversity and to modernize taxonomy (Bisby *et al.*, 2002; Godfray, 2002; Besansky *et al.*, 2003; Lipscomb *et al.*, 2003; Mallet & Willmott, 2003; Seberg *et al.*, 2003; Tautz *et al.*, 2003). Traditional morphology-based taxonomic procedures are time-consuming and not always sufficient for identification to the species level, and therefore a multidisciplinary approach to taxonomy that includes morphological, molecular and distributional data is essential (Krzywinski & Besansky, 2003).

Hebert *et al.* (2003a, b) have shown that the analysis of short, standardized genomic regions (DNA barcodes) can discriminate morphologically recognized animal species. In particular, they suggest that the mitochondrial gene cytochrome c oxidase subunit 1 (CO1) can serve as a uniform target gene for a bioidentification system.

The ability of DNA barcodes to identify species reliably, quickly and cost-effectively has particular importance in medical entomology, where molecular approaches to species diagnoses are often of great benefit in the identification of all life stages, from eggs to adults. As Besansky *et al.* (2003) stated: 'Nowhere is the gap in taxonomic knowledge more urgent than for medically important pathogens and their invertebrate vectors'. For example, since the recent arrival of West Nile virus (WNv) in North America, mosquito identification and assessment of vector status has gained renewed significance on this continent. Successful longterm control of WNv will be aided by information on the epidemiological role of mosquitoes and the transmission biology of the virus.

Although biting insects have been studied more extensively than most other animal groups, our taxonomic knowledge of mosquitoes is far from complete. Since Edwards (1932) outlined the modern system of mosquito classification, the number of described mosquito species has more than doubled from 1400 to almost 3200 (Zavortink, 1990; Harbach & Kitching, 1998) and new species are still being identified.

Several genetic approaches have been applied to the identification of mosquito species, including protein electrophoresis (Green *et al.*, 1992; Foley *et al.*, 1995; Sukowati *et al.*, 1999; Van Bortel *et al.*, 1999), hybridization assays (Beebe *et al.*, 1996; Crampton & Hill, 1997; Cooper *et al.*, 2002) and polymerase chain reaction (PCR)-based sequence analysis. The latter has the advantage of requiring minute amounts of material for analysis. Methods based on PCR, such as satellite DNA (Krzywinski *et al.*, 2005), restriction fragment length analysis, single-strand conformation shifts, or heteroduplex analysis, have been applied to detect diagnostic differences among PCR products in mosquito species (Moriais & Severson, 2003; Santomalazza *et al.*, 2004; Weeto *et al.*, 2004; Garros *et al.*, 2005; Goswami *et al.*, 2005). Most PCR assays have examined sequence diversity in specific nuclear loci (Scott *et al.*,

Correspondence: Alina Cywinska, Department of Biological Sciences, Brock University, St. Catharines, Ontario L2S 3A1, Canada. Tel.: + 1 905 688 5550 (ext. 3879); Fax: + 1 905 688 1855; E-mail: alcywin@hotmail.com

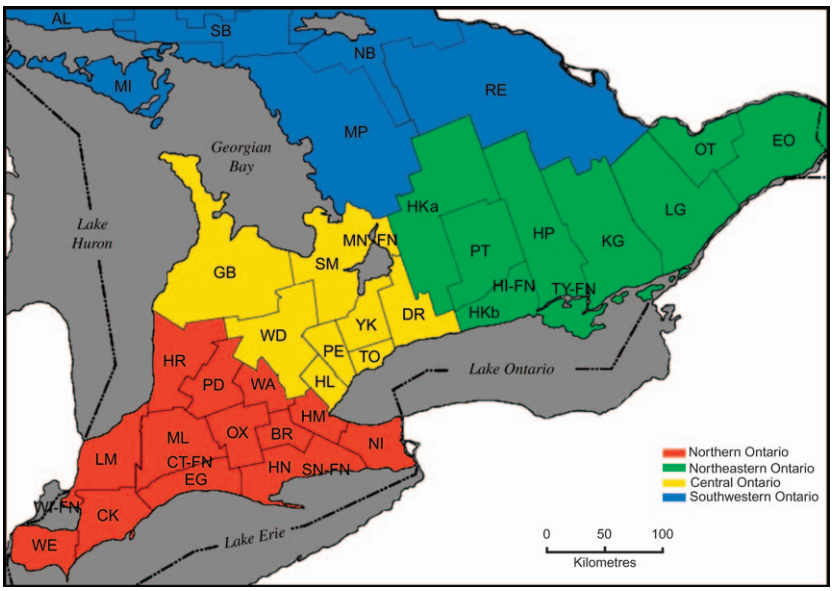

Fig. 1. Map of study area showing sampling sites within locations of the Health Units of the Ontario Ministry of Health and the First Nations territories. AL, Algoma; BR, Brant; GB, Bruce Grey Owen Sound; DR, Durham; EG, Elgin–St. Thomas; EO, Eastern Ontario; HK, Haliburton–Kawartha–Pine Ridge; HL, Halton; HM, Hamilton Wentworth; HN, Haldimand–Norfolk; HP, Hastings–Prince Edward; HR, Huron; CK, Kent–Chatham; KG, Kingston–Frontenac–Lennox–Addington; LM, Lambton; LG, Leeds–Grenville–Lanark; MI, Manitouli Island; ML, Middlesex–London; MP, Muskoka–Parry Sound; NB, North Bay and District; NI, Niagara; NW, North-western; OT, Ottawa–Carleton; OX, Oxford; PE, Peel; PC, Porcupine; PD, Perth District Health; PT, Peterborough County; RE, Renfrew County and District; SM, Simcoe; SB, Sudbury; TB, Thunder Bay; TK, Timiskaming; TO, Toronto; WA, Waterloo; WD, Wellington–Dufferin–Guelph; WE, Windsor–Essex County; YK, York. First Nations: CT-FN, Chippewas of the Thames; HI-FN, Hiavatha; MN-FN, Chippewas of Rama; NC-FN, Mississauga of New Credit; SN-FN, Six Nations of Grand River; TY-FN, Mohawks of the Bay of Quinte; WI-FN, Walpole Island.

1993; Beebe & Saul, 1995; Singh *et al.*, 1997; Koekemoer *et al.*, 1999; Proft *et al.*, 1999; Walton *et al.*, 1999; Hackett *et al.*, 2000; Favia *et al.*, 2001; Manonmani *et al.*, 2001; Cohuet *et al.*, 2003; Kent *et al.*, 2004; Smith & Fonseca, 2004; Kampen, 2005; Marrelli *et al.*, 2005). Other researchers have examined the taxonomic insights that can be gained by combining information from two or more genes (Nguyen *et al.*, 2000; Krzywinski *et al.*, 2001; Linton *et al.*, 2001; Mitchell *et al.*, 2002; Linton *et al.*, 2003; Dusfour *et al.*, 2004; Shaikevitch & Vinogradova, 2004; Cook *et al.*, 2005). Multiplex PCR assays that included both universal (conserved) and species-specific primers were performed by Phuc *et al.* (2003). By contrast with the many studies on nuclear genes, little taxonomic work has targeted haploid mitochondrial DNA sequences in mosquitoes and less yet has examined sequence diversity in the CO1 gene (Rey *et al.*, 2001; Fairley *et al.*, 2000, 2002; Sallum et al., 2002), despite its established potential for the diagnosis of biological diversity (Hebert *et al.*, 2003a, 2003b, 2004). The CO1 region is present in the hundreds of copies per cell, it generally lacks indels, and, in common with other protein-coding genes, its third position nucleotides show a high incidence of base substitutions. Changes in its amino acid sequence occur more slowly than those in any other mitochondrial gene, aiding resolution of deeper taxonomic affinities and primer design.

In this study, sequence variation in the barcode region of CO1 was analysed to test its usefulness in the identification of mosquito species from eastern Canada.

Materials and methods

Mosquito collections

During 2002 and 2003, adults belonging to 37 mosquito species were collected across Ontario (Fig. 1, Table 1) as part of the West Nile Virus Surveillance Programme. Mosquitoes were sampled with CO_2-baited CDC (Center for Disease Control) miniature light traps (BioQuip, Rancho Dominguez, CA, U.S.A.) and were identified using standard taxonomic keys (Wood *et al.*, 1979; Darsie & Ward, 2005). In addition, individuals of five mosquito species from New Brunswick (Table 1) were sequenced to provide preliminary information about the degree of geographical variation in sequences within Canada and sequences from 19 species were chosen from GenBank (Table 1) to test for variation across a greater geographical area. These were the only mtDNA sequences for mosquitoes that matched our CO1 barcode region.

Sample preparation, DNA extraction, amplification and sequencing

Nearly half of the samples used for DNA extraction were obtained from 100 μL slurries of individual mosquitoes that had been homogenized earlier and pre-treated to test for WNv (Condotta *et al.*, 2004). DNA extractions for the remaining

Table 1. List of mosquito species, collection sites and number of sequences per species used in the study.

Species	Collection origin in Ontario	Collection origin outside Ontario	Number of specimens
Aedes abserratus	Central		2
	Northern		7
	South-western		1
Aedes aegypti		GenBank	5
Aedes atropalpus	South-western		5
		GenBank	1
Aedes aurifer	Central		1
Aedes canadensis	Central		7
	Eastern		4
	South-western		3
	Northern		2
		New Brunswick	4
Aedes cantator	South-western		2
		New Brunswick	5
Aedes cinereus	Central		2
	Eastern		1
	South-western		1
		New Brunswick	3
Aedes communis	Northern		5
Aedes dorsalis	Central		2
	South-western		1
	Northern		1
Aedes euedes	Central		3
	Eastern		4
Aedes excrucians	Central		5
	Eastern		2
Aedes fitchii	Central		1
	Eastern		4
Aedes grossbecki	Central		2
	South-western		2
Aedes implicatus	Central		1
	Eastern		6
	Northern		3
	South-western		1
		New Brunswick	2
Aedes intrudens	Eastern		1
	Northern		1
Aedes japonicus	Central		1
	Eastern		1
	South-western		4
Aedes provocans	Central		1
	Eastern		5
	South-western		1
Aedes riparius	Central		7
Aedes sollicitans	South-western		5
Aedes stictus	Northern		1
Aedes stimulans	Central		22
	South-western		4
Aedes triserratus	Central		3
	Eastern		4
	South-western		3
Aedes trivittatus	Central		6
	South-western		4
Aedes vexans	Central		8
	South-western		4
	Eastern		1
		New Brunswick	8
Anopheles funestus		GenBank	1
Anopheles earlei	Central		1
	Northern		3
		GenBank	1
Anopheles gambiae		GenBank	1
Anopheles maculipennis		GenBank	2
Anopheles messeae		GenBank	2
Anopheles punctipennis	Central		10
	South-western		5
Anopheles quadrimaculatus	Central		9
	South-western		2
		GenBank	1
Anopheles pullus		GenBank	2
Anopheles rivulorum		GenBank	1
Anopheles sacharovi		GenBank	2
Anopheles sinensis		GenBank	1
Anopheles stephensi		GenBank	1
Anopheles sundaicus		GenBank	2
Anopheles walkeri	Central		4
	Eastern		2
Coquillettidia perturbans	Central		5
	Northern		2
	Eastern		11
	South-western		1
Culex pipiens	Central		4
	South-western		7
Culex restuans	Central		5
	South-western		5
	Northern		1
Culex salinarius	Eastern		5
	South-western		1
Culex tarsalis		GenBank	1
Culex territans	Central		1
	Eastern		1
	Northern		1
Culiseta impatient		GenBank	1
Culiseta inornata	South-western		5
Culiseta minnesotae	Northern		1
	Eastern		1
Culiseta morsitans	Central		2
	Northern		3
	Eastern		1
Orthopodymia alba	Central		1
Sabethes cyaneus		GenBank	1
Toxorhynchites rutilus		GenBank	1
Toxorhynchites sp.		GenBank	1
Uranotaenia sapphirina	Central		3
	Eastern		1
	South-western		2

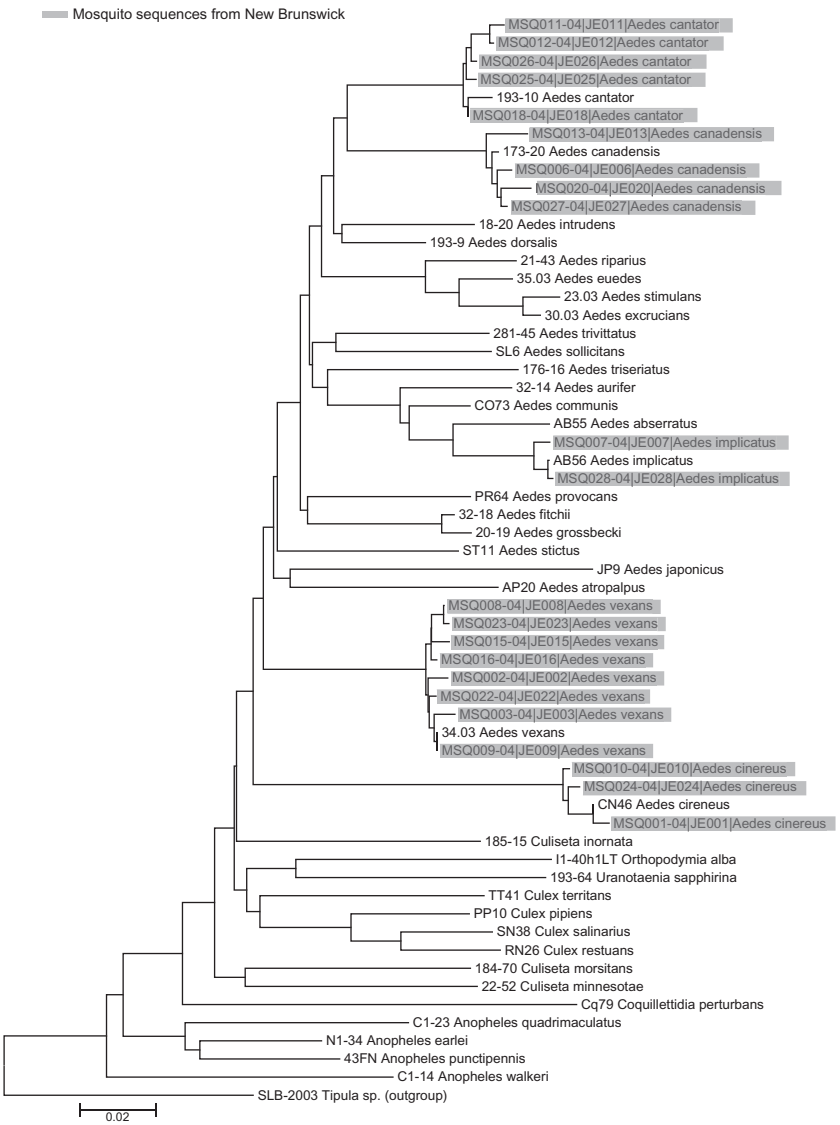

Fig. 2. Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of CO1 mosquito sequences from Ontario and New Brunswick. Labels indicate mosquitoes collected in New Brunswick.

samples were obtained from small amounts of tissue (two to three legs) from frozen pinned mosquitoes.

For each mosquito, 30 μL of total DNA was extracted using the GeneElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich Co., St. Louis, MO, U.S.A.). The primer pairs LCO1490 and HCO2198 (Folmer *et al.*, 1994) or LepF (5′-ATT CAACCAATCATAAAGATATTGG-3′) and HCO2198 were subsequently used to amplify ~ 650 bp fragments of CO1 which were trimmed later to 617 bp in the barcode region of CO1. Each PCR cocktail contained 2.5 μL 10 X PCR buffer, pH 8.3 (10 mM Tris-CH1, pH 8.3 and 50 mM KC1, 0.01% NP-40), 1.5 mM $MgCl_2$, 200 μM of each NTP, 1 unit Taq polymerase, 0.3 μM of each primer, 1–5 μL of DNA template and the remaining volume of ddH_2O up to 25 μL. The PCR thermal regime consisted of one cycle of 1 min at 95 °C, 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C, and a final cycle of 7 min at 72 °C. All PCR products were subjected to dye terminator cycle sequencing reactions (30 cycles, 55 °C annealing), and sequenced on ABI 377 or 3730 automated sequencers, using Big Dye vs. 3.1 and LCO1490 primer.

Data analysis

Electropherograms for the CO1 gene were edited and aligned with Sequencher™ Version 4.5 (Gene Codes Corp., Ann Arbor, MI, U.S.A.). Pairwise nucleotide sequence divergences were calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980), and neighbour-joining (NJ) analysis (Saitou & Nei, 1987) in MEGA 2.1 was used to examine relationships among taxa. All sequences obtained in this study have been deposited in GenBank. Collection localities and other specimen information, such as the GenBank submission numbers, will be available in the 'Mosquitoes of Canada' file in the Completed Project section of the Barcode of Life website (http://www.barcodinglife.org).

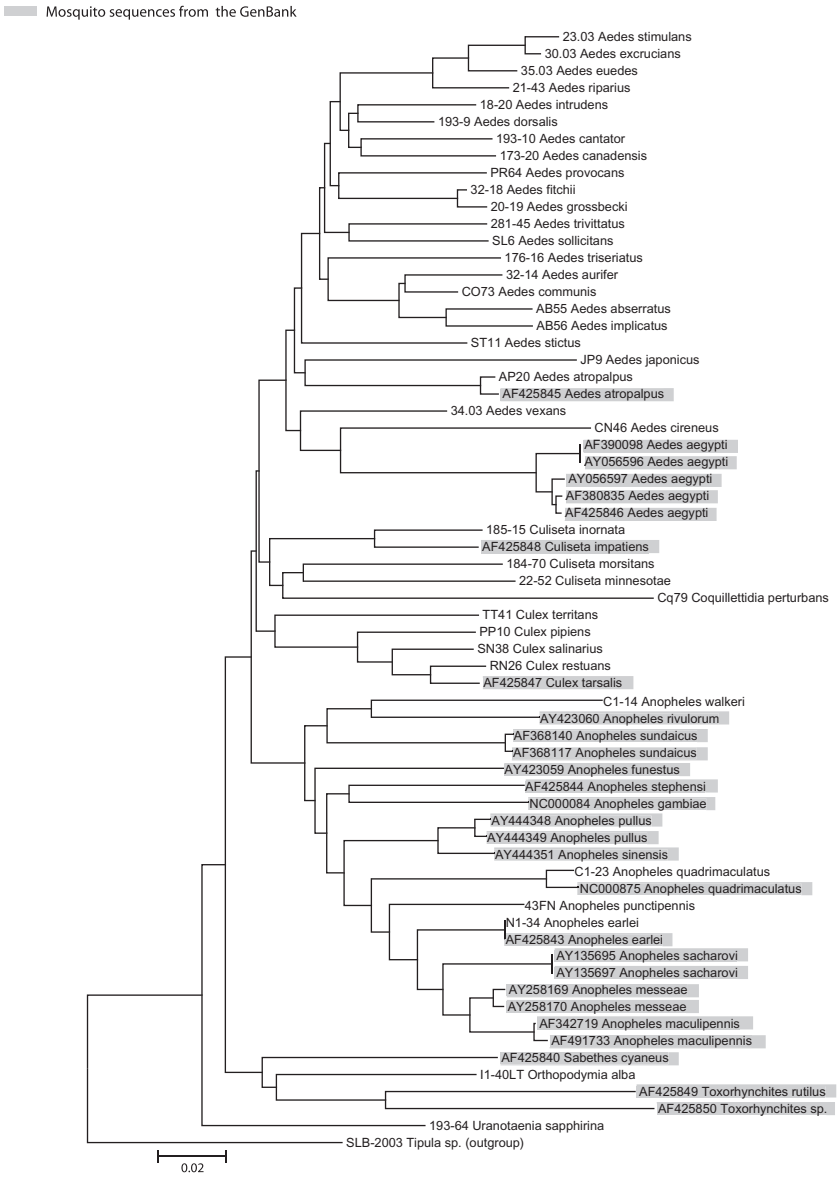

Fig. 3. Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of CO1 mosquito sequences from Ontario and from GenBank. Labels indicate sequences obtained from GenBank.

Results

Sequences for 37 mosquito species from Ontario were compared with those for five species from New Brunswick (Fig. 2) and 19 species from GenBank (Fig. 3), producing sequence records for 53 mosquito species belonging to nine genera and three subfamilies (Anophelinae, Culicinae and Toxorhynchitinae). Individual species were represented by one to 26 individuals, for a total of 302 CO1 sequences. Because these sequences contained no indels, alignments were straightforward. The CO1 sequences had a strong A + T bias (average 67% for all codons), especially, at third codon positions (91%) (Table 2). The pattern was generally consistent across genera, with the A + T content ranging from 64% (*Coquillettidia* sp.) to 70% (*Uranotaenia* sp.).

All but one of the sequences lacked nonsense or stop codons, supporting their origin from the mitochondrial gene. One probable pseudogene, a CO1 sequence from an individual of *Culex restuans* (Theobald), contained one stop codon and 15 amino acid substitutions in comparison with the consensus sequence for other mosquitoes. In addition, it showed substantial sequence divergence (3.9−4.4% nt) from the other representatives of its species.

Individuals of a single species always grouped closely together, regardless of where they were collected (Figs 2, 3 and 4). All species also possessed a distinctive set of CO1 sequences, most of which showed low intraspecific divergences. Conspecific K2P divergence averaged 0.5% (range 0–3.9%), whereas sequence divergences between congeneric species averaged 10.4% (range 0.2–17.2%) (Fig. 5a, b). Sequence divergences were even higher among species in different genera, averaging 16.0% (range 7.2–26.3%; Fig. 5c). Most conspecific sequences (98%) showed $< 2\%$ divergence, including those between two

Table 2. Sequence divergence and nucleotide composition for the mosquito genera from Ontario, Canada. The frequencies of nucleotides in sequences are presented as the total average values for all codon positions and for each codon position separately with the accuracy to tenths of a percent.

		Total					First codon position					Second codon position					Third codon position				
Genus	Sequence divergence %	% A	% T	% C	% G	% A + T	% A	% T	% C	% G	% A + T	% A	% T	% C	% G	% A + T	% A	% T	% C	% G	% A + T
Aedes	17.0–23.0	30.0	37.6	16.3	16.2	67.6	29.1	27.5	15.1	28.4	56.6	13.5	43.3	26.3	16.9	56.8	47.4	41.8	7.5	3.3	89.2
Anopheles	15.8–19.8	29.2	37.5	16.2	17.1	66.7	27.8	26.2	15.1	31.0	54.0	13.8	42.2	27.2	16.8	56.0	46.0	44.1	6.5	3.4	90.1
Coquillettidia	22.3–22.6	26.6	36.9	18.9	17.5	63.5	27.7	23.5	18.7	30.0	51.2	13.1	42.2	27.2	17.4	55.3	39.1	45.1	10.8	5.0	84.2
Culex	18.0–21.4	28.9	40.1	15.4	15.7	69.0	29.1	24.6	17.2	29.1	53.7	13.6	42.7	26.8	16.9	56.3	43.9	53.0	2.2	0.9	96.9
Culiseta	19.5–20.8	29.2	38.5	16.2	16.0	67.7	28.7	26.9	15.7	28.6	55.6	13.7	42.7	26.7	16.9	56.4	45.4	45.9	6.3	2.5	91.3
Orthopodymia	20.5	32.1	37.4	14.9	15.7	69.5	29.1	25.8	16.1	28.6	54.9	13.6	42.3	26.3	17.8	55.9	53.5	44.1	1.9	0.5	97.6
Sabethes	19.9	28.8	40.2	16.1	14.9	69.0	30.5	26.3	16.9	26.3	56.8	14.1	42.7	26.8	16.4	56.8	41.8	51.6	4.7	1.9	93.4
Toxorhynchites	25.5	30.7	38.7	16.5	14.1	69.4	31.9	24.6	18.1	25.4	56.5	15.0	43.4	25.4	16.2	58.4	45.1	48.1	6.1	0.7	93.2
Uranotaenia	17.3	31.5	38.2	14.8	15.5	69.7	30.0	26.8	15.5	27.7	56.8	13.6	42.7	26.8	16.9	56.3	50.7	45.2	2.2	1.9	95.9
Average nt content		29.4	38.0	16.4	16.2	67.4	29.3	26.0	16.4	28.3	55.2	13.8	42.7	26.6	16.9	56.5	45.7	46.6	5.5	2.3	92.3

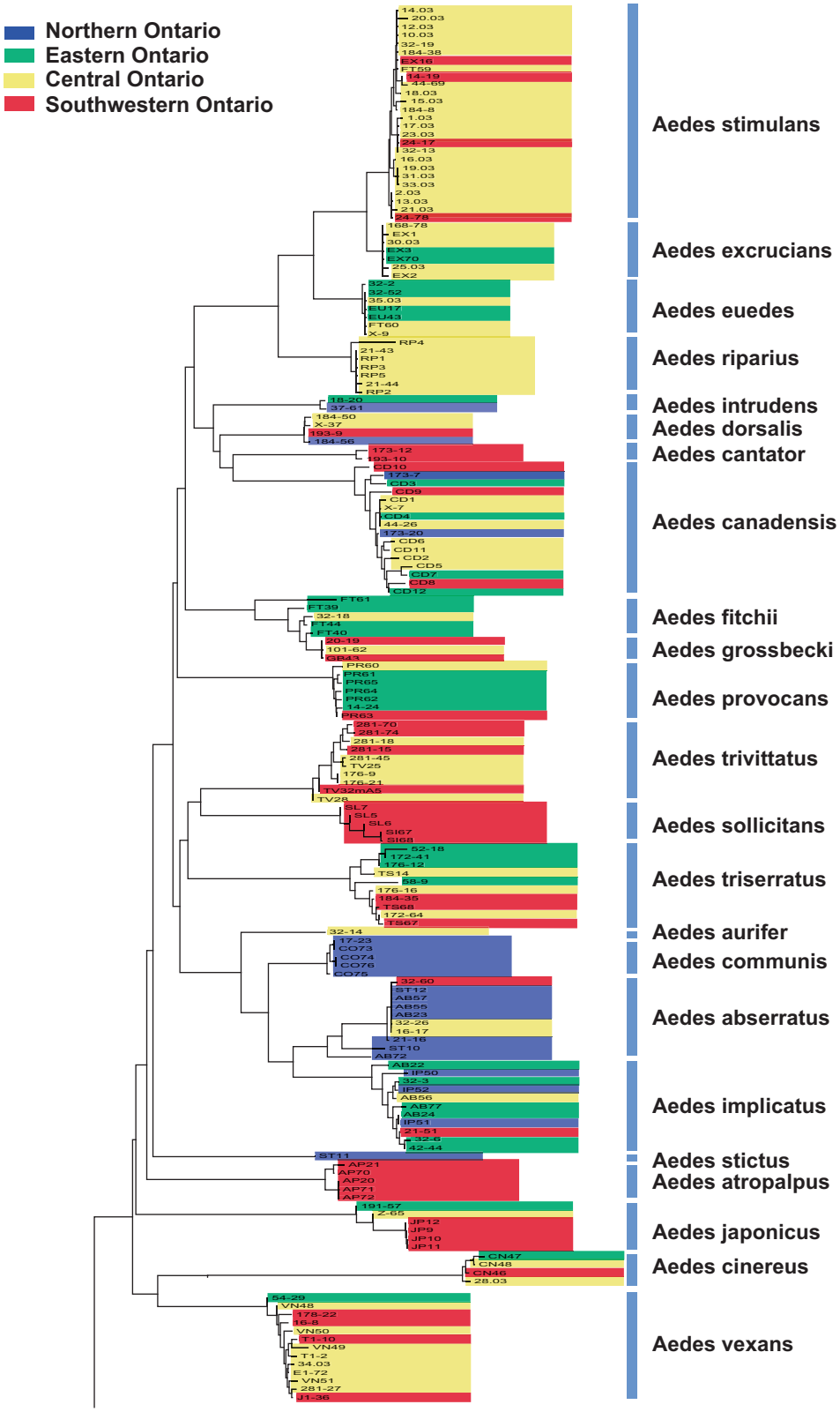

Fig. 4. Neighbour-joining analysis of the Kimura 2-parameter (K2P) distances of CO1 sequences from mosquitoes collected in Ontario. Labels indicate the collection areas in Ontario.

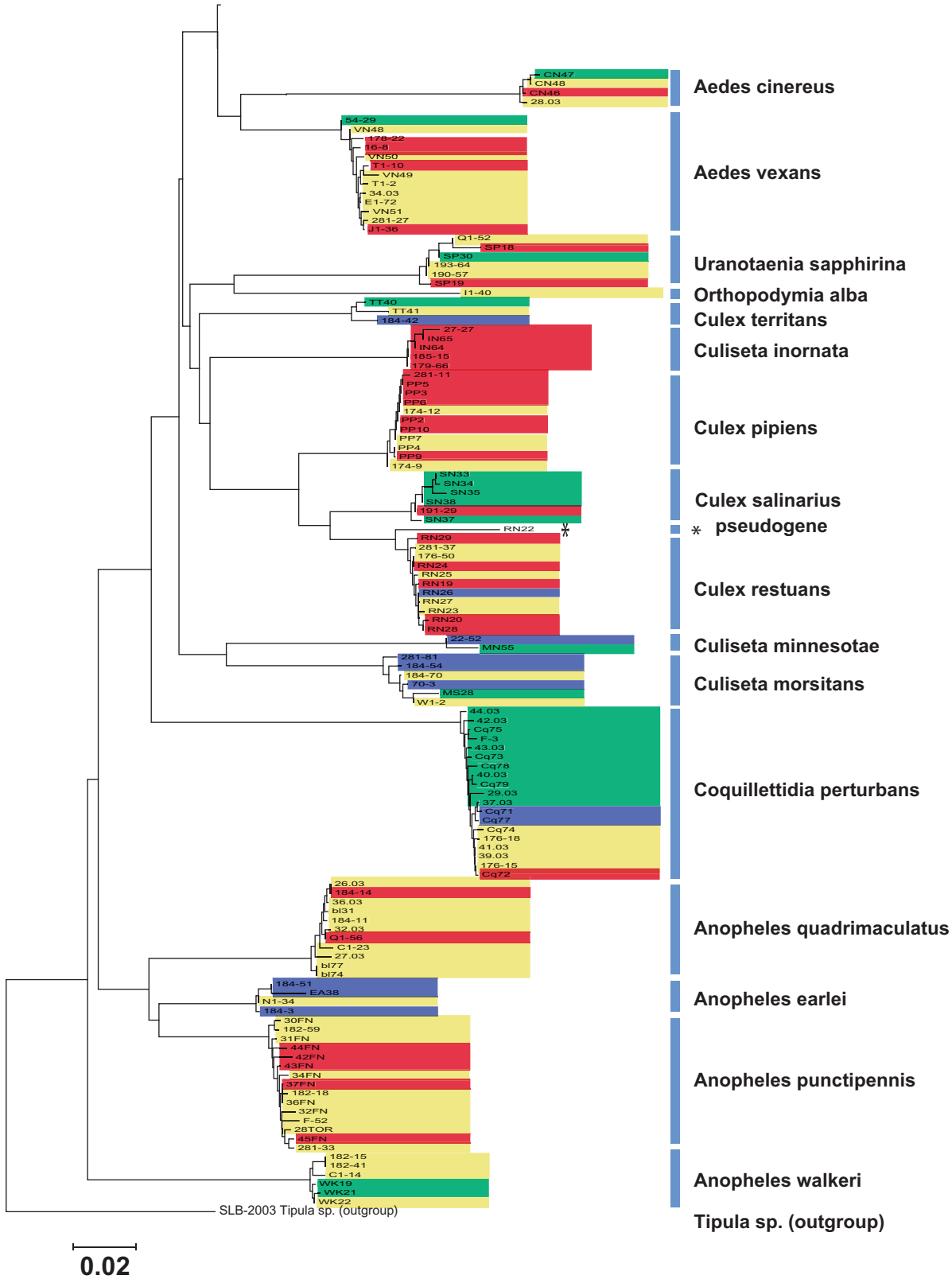

Fig. 4. Continued.

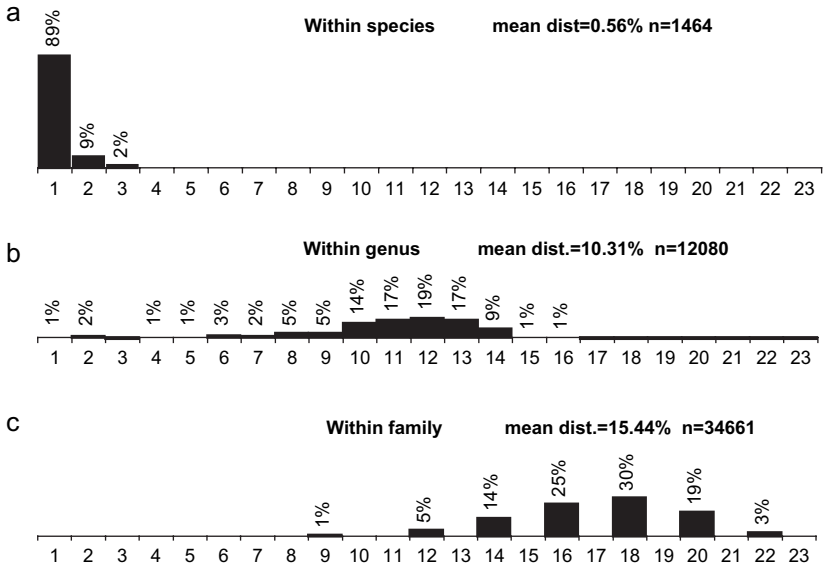

Fig. 5. Pairwise comparisons between CO1 sequences among mosquito species separated into three categories: (a) intraspecific; (b) intragenic, and (c) intergenic differences between individuals.

morphologically distinctive subspecies of *Aedes vexans (Ae. vexans vexans* (Meigen) and *Ae. vexans nipponi* (Theobald), 0−1.11%), but deeper divergences were detected in two species. One specimen of *Aedes fitchii* (Felt & Young) showed 3.6−3.9% divergence from other conspecific individuals, and specimens of *Aedes abserratus* (Felt & Young) were separated into three clusters showing 2.6–3.2% sequence divergence. A single case involving *Ae. fitchii* and *Aedes grossbecki* Dyar & Knab was detected where the three individuals of *Ae. grossbecki* were closer to some individuals of *Ae. fitchii* than to some of the latter's own conspecifics, but they still possessed distinct barcodes. There was generally high bootstrap support (90−100%) for the terminal branches at the species level, with the exception of a few records for *Anopheles* species, obtained from GenBank and represented by only one or two individuals.

Plots of the total number of transitions (ts) + transversions (tv) at all sites against the sequence divergence (Fig. 6) showed a rapid increase in transitions at the conspecific level and partially at the congeneric levels to a max of 30–40 ts substitutions at around 7% divergence. The incidence of transitions levelled off at the border between the congeneric and the intergeneric levels. By contrast, transversions increased steadily from zero substitutions at the conspecific level to $<$ 10 substitutions at around 7% divergence at the congeneric level, and then, after a sudden jump to 20 substitutions, grew rapidly to a max of ~ 60 substitutions among more distantly related sequences.

The neighbour-joining analysis of nucleotide and amino acid sequences showed that most mosquito species separated into distinct clusters (Figs 2, 3 and 4). Species in genera represented by more than one taxon usually formed cohesive assemblages.

Discussion

An effective DNA-based identification system requires the satisfaction of three conditions: (a) it must be possible to recover the target DNA from all species; (b) the sequence information must be easily analysed, and (c) the information content of the target sequence must be sufficient to enable species-level identification. All three of these requirements were met in this study. We were able to recover and align the targeted CO1 fragment from all mosquito species we examined. Furthermore, although

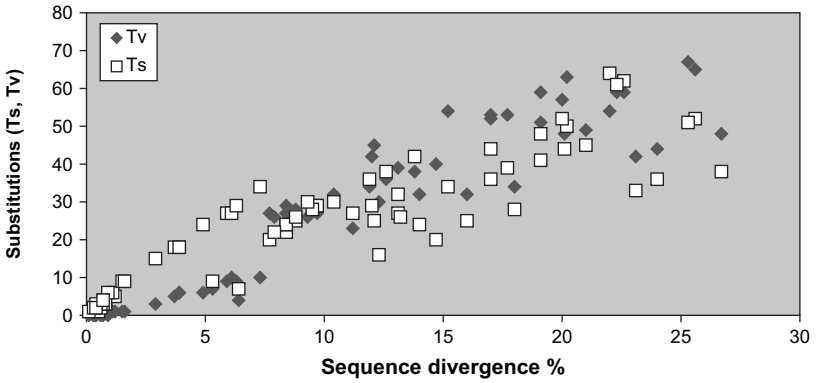

Fig. 6. Observed numbers of transitions (ts) and transversions (tv) for the CO1 gene plotted against sequence divergence. The ts saturation begins to level off at around 7.5% sequence divergence. Tv increases steadily from the conspecific level of ~ 7% divergence, then, after a sudden jump, continues to grow more rapidly among more distantly related taxa.

we amplified CO1 from total genomic DNA, we detected only a single nuclear pseudogene. In addition, specimens of all species formed distinctive clusters and, with the exception of a single species pair, barcode divergences were relatively large between taxa. Finally, species boundaries were congruent with those established by morphological taxonomic work.

DNA-based species identification systems depend on the ability to distinguish intraspecific from interspecific variation. In the present study, CO1 sequence differences among congeneric species were, on average, almost 20 times higher than the average differences within species. The average conspecific K2P divergence for mosquito species (0.55%) in this study is slightly higher than those earlier reported for North American birds (0.27%; Hebert *et al.*, 2004) and moths (0.25%; Hebert *et al.*, 2003a). However, this difference reflects our detection of deep intraspecific divergence in two taxa (*Ae. fitchii, Ae. abserratus*), instances that may indicate overlooked sibling species.

Interestingly, two morphologically distinctive subspecies, *Ae. vexans vexans* and *Ae. vexans nipponi* (dissimilar coloration of scales on the abdominal sternites) show barcode congruence. The latter subspecies was brought to the U.S.A. in 1999, probably from Korea.

We detected one case of low interspecific sequence divergence, involving the *Ae. fitchii/Ae. grossbecki* complex. *Ae. grossbecki* is rare in Ontario, although common in nearby northwestern Ohio (Venard & Mead, 1953). Adults of this species were collected from the Windsor–London area, in the region of its first recorded presence in Canada (Helson *et al.*, 1978), and specimens of *Ae. fitchii* were collected further to the north-east. The latter individuals showed morphological evidence of hybridization in that two types of scales were present on the wings of single individuals: large triangular scales, typical of *Ae. grossbecki*, were observed on the anterior half of their wings and elongate scales, typical of *Ae. fitchii,* occurred posteriorly. In cases such as this, indicating possible hybridization, as well as in those cases characterized by incomplete sorting of mitochondrial lineages, more detailed morphological and genetic examinations will be required. The examination of faster evolving mitochondrial genes, such as the control region or ND4, as well as analysis of nuclear regions, such as internal transcribed spacers (ITS), may aid in establishing species boundaries in at least some of the cases that cannot be resolved though CO1.

The effective application of DNA sequence data to molecular diagnostics depends on patterns of nucleotide substitution and the rate of variation among sites (Blouin *et al.*, 1998). The CO1 region in mosquitoes is characterized by a high rate of transitional saturation along the sequence divergence axis, particularly at silent sites. The ts saturation begins to level off at around 7.5% sequence divergence, suggesting caution in the interpretation of pairwise comparisons at the congeneric and intergeneric levels, unless silent sites are excluded from analysis.

Although our studies on the mosquito fauna of eastern Canada provide an early indication of the patterns of CO1 sequence divergence within and among species, GenBank sequences for *Anopheles earlei* Vargas from South Africa and *Anopheles quadrimaculatus* Say from the U.S.A. grouped closely with other individuals of their species from Ontario. Moreover, other GenBank sequences from 'exotic' species, in the genera *Culex, Culiseta* and *Anopheles*, grouped with allied taxa in our NJ analysis, but formed distinct, tight sequence clusters. On this basis, we anticipate that further growth in taxon and geographical coverage will not seriously alter the conclusions drawn from this study. In short, we expect that congeneric species will regularly show sequence divergences in the CO1 region averaging ~ 10% and that divergence values for conspecific individuals will usually fall below 0.5%.

In summary, this study has provided the first CO1 barcodes for Canadian mosquitoes and has established their effectiveness in discriminating species of mosquitoes recognized through prior taxonomic work. Specimens of single species formed barcode clusters with tight cohesion that were usually clearly distinct from those of allied species. Sequence divergences were, on average, nearly 20 times higher for congeneric species than for members of a species, ensuring that species identifications within this local species assemblage were robust. As an effort has now been launched to gather DNA barcodes for all known mosquito species, a full evaluation of the effectiveness of DNA barcoding for members of the family Culicidae should soon be available.

Acknowledgements

This work was supported by grants from NSERC to F. F. Hunter and P. D. N. Hebert. We are grateful to Aynsley Thielman and Jim Edsall for mosquito identifications and to Monty Wood and Richard Darsie for verifying taxonomic assignments. We thank Angela Hollis for assistance with DNA sequencing, Jim Edsall of the New Brunswick Museum, Trudy Stanfield of Health Canada-First Nation & Inuit Health Branch, various units of the Ontario Ministry of Health and the West Nile Virus Surveillance Programme for providing some specimens examined in this study.

References

Beebe, N.W., Foley, D.H., Cooper, R.D., Bryan, J.H. & Saul, A. (1996) DNA probes for the *Anopheles punctulatus* complex. *American Journal of Tropical Medicine and Hygiene*, **54**, 395–398.

Beebe, N.W. & Saul, A. (1995) Discrimination of all members of the *Anopheles punctulatus* complex by polymerase chain reaction-restriction fragment length polymorphism analysis. *American Journal of Tropical Medicine and Hygiene*, **53**, 478–481.

Besansky, N.J., Severson, D.W. & Ferdig, M.T. (2003) DNA barcoding of parasites and invertebrates disease vectors: what you don't know can hurt you. *Trends in Parasitology*, **19**, 545–546.

Bisby, F.A., Ruggiero, M., Edwards, J. & Haeuser, C. (2002) Taxonomy at the click of the mouse. *Nature*, **418**, 367.

Blouin, M.S., Yowell, C.A., Courtney, C.H. & Dame, J.B. (1998) Substitution bias, rapid saturation, and use of mtDNA for nematode systematics. *Molecular Biology and Evolution*, **15**, 1719–1727.

Cohuet, A., Simard, F., Toto, J.-C., Kengne, P., Coetzee, M. & Fontenille, D. (2003) Species identification with the *Anopheles funestus* group of malaria vectors in Cameroon and evidence for a new species. *American Journal of Tropical Medicine and Hygiene*, **69**, 200–205.

Condotta, S.A., Hunter, F.F. & Bidochka, M.J. (2004) West Nile virus infection rates in pooled and individual mosquito samples. *Vector-Borne and Zoonotic Diseases*, **4**, 198–203.

Cook, S., Diallo, M., Sall, A.A., Cooper, A. & Holmes, E.C. (2005) Mitochondrial markers for molecular identification of *Aedes* mosquitoes (Diptera: Culicidae) involved in transmission of arboviral disease in West Africa. *Journal of Medical Entomology*, **42**, 19–28.

Cooper, R.D., Waterson, D.G.E., Frances, S.P., Beebe, N.W. & Sweeney, A.W. (2002) Speciation and distribution of the members of the *Anopheles punctulatus* (Diptera: Culicidae) group in Papua New Guinea. *Journal of Medical Entomology*, **39**, 16–27.

Crampton, J.M. & Hill, S.M. (1997) Generation and use of species-specific DNA probes for insect vector identification. *The Molecular Biology of Insect Disease Vectors: a Methods Manual* (ed. by J.M. Crampton, C.B. Beard & C. Louis), pp. 384–398. Chapman & Hall, London.

Darsie, R.F. Jr & Ward, R.A. (2005) Identification and geographical distribution of the mosquitoes of North America, north of Mexico. *Journal of the American Mosquito Control Association*, **21**, 1–383.

Dusfour, I., Linton, Y.M., Cohuet, A. *et al*. (2004) Molecular evidence of speciation between island and continental populations of *Anopheles (Cellia) sundaicus* (Diptera: Culicidae), a principal malaria vector taxon in Southeast Asia. *Journal of Medical Entomology*, **41**, 287–295.

Edwards, F.W. (1932) *Genera Insectorum. Diptera, Family Culicidae. Fascicle 194*. Desmet-Verteneuil, Brussels.

Fairley, T.L., Póvoa, M.M. & Conn, J.E. (2002) Evaluation of the Amazon River delta as a barrier to gene flow for the regional malaria vector, *Anopheles aquasalis* (Diptera: Culicidae) in northeastern Brazil. *Journal of Medical Entomology*, **39**, 861–869.

Fairley, T.L., Renaud, T.M. & Conn, J.E. (2000) Effects of local geographic barriers and latitude on population structure in *Anopheles punctipennis* (Diptera: Culicidae). *Journal of Medical Entomology*, **37**, 754–760.

Favia, G., Lanfrancotti, A., Spanos, L., Siden-Kiamos, I. & Louis, C. (2001) Molecular characterization of ribosomal DNA polymorphisms discriminating among chromosomal forms of *Anopheles Gambiae s.s. Insect Molecular Biology*, **10**, 19–23.

Foley, D.H., Cooper, R.D. & Bryan, J.H. (1995) A new species within the *Anopheles punctulatus* complex in Western Province, Papua New Guinea. *Journal of the American Mosquito Control Association*, **11**, 122–127.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294–299.

Garros, C., Harbach, R.E. & Manguin, S. (2005) Systematics and biogeographical implications of the phylogenetic relationships between members of the *funestus* and *minimus* groups of *Anopheles* (Diptera: Culicidae). *Journal of Medical Entomology*, **42**, 7–18.

Godfray, H.C.J. (2002) Challenges for taxonomy. *Nature*, **417**, 17–19.

Goswami, G., Raghavendra, K., Nanda, N., Gakhar, S.K. & Subbarao, S.K. (2005) PCR-RFLP of mitochondrial cytochrome oxidase subunit II and ITS2 of ribosomal DNA: markers for the identification of members of the *Anopheles culicifacies* complex (Diptera: Culicidae). *Acta Tropica*, **95**, 92–99.

Green, C.A., Munstermann, L.E., Tan, S.G., Panyim, S. & Baimai, V. (1992) Population genetic evidence for species A, B, C, and D of the *Anopheles dirus* complex in Thailand and enzyme electromorphs for their identification. *Medical and Veterinary Entomology*, **6**, 29–36.

Hackett, B.J., Gimnig, J., Guelbeogo, W., Constantini, C. & Koekemoer, L.L. (2000) Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* and *Anopheles rivulorum*, and uncover a cryptic taxon. *Insect Molecular Biology*, **9**, 369–374.

Harbach, R.E. & Kitching, I.J. (1998) Phylogeny and classification of the Culicidae Diptera). *Systematic Entomology*, **23**, 327–370.

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

Hebert, P.D.N., Ratnasingham, S. & deWaard, J.R. (2003b) Barcoding animal life: CO1 divergences among closely allied species. *Proceedings of the Royal Society of London, Series B*, **270,** 596–599.

Hebert, P.D.N., Stoeckle, M.Y., Zemlak, T.S. & Francis, C.M. (2004) Identification of birds through DNA barcodes. *Public Library of Science, Biology*, **2**, 1657–1663.

Helson, B.V., Surgeoner, G.A., Wright, R.E. & Allan, S.A. (1978) *Culex tarsalis, Aedes sollicitans, Aedes grossbecki*: new distribution records from southwestern Ontario. *Mosquito News*, **38**, 137–138.

Kampen, H. (2005) Integration of *Anopheles beklemishevi* (Diptera: Culicidae) in a PCR assay diagnostic for palaearctic *Anopheles maculipennis* sibling species. *Parasitology Research*, **97**, 113–117.

Kent, R.J., West, A.J. & Norris, D.E. (2004) Molecular differentiation of colonized human malaria vectors by 28S ribosomal DNA polymorphism. *American Journal of Tropical Medicine and Hygiene*, **71**, 514–517.

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.

Koekemoer, L., Lochouarn, L., Hunt, R.H. & Coetzee, M. (1999) Single-strand conformation polymorphism analysis for identification of four members of the *Anopheles funestus* (Diptera: Culicidae) group. *Journal of Medical Entomology*, **36**, 125–130.

Krzywinski, J. & Besansky, N.J. (2003) Molecular systematics of *Anopheles*: from subgenera to subpopulations. *Annual Review of Entomology*, **48**, 111–139.

Krzywinski, J., Sangaré, D. & Besansky, N.J. (2005) Satellite DNA from the Y chromosome of the malaria vector *Anopheles gambiae*. *Genetics*, **169**, 185–196.

Krzywinski, J., Wilkerson, R.C. & Besansky, N.J. (2001) Toward understanding Anophelinae (Diptera, Culicidae) phylogeny: insights from nuclear single copy genes and the weight of evidence. *Systematic Biology*, **50**, 540–556.

Linton, Y.M., Harbach, R.E., Seng, C.M., Anthony, T.G. & Matusop, A. (2001) Morphological and molecular identity of *Anopheles (Cellia) sundaicus* (Diptera: Culicidae), the nominotypical member of a malaria vector species complex in Southeast Asia. *Systematic Entomology*, **26**, 357–366.

Linton, Y.M., Smith, L., Koliopoulos, G., Samanidou-Voyadjoglou, A., Zounos, A.K. & Harbach, R. (2003) Morphological and molecular characterization of *Anopheles (Anopheles) maculipennis* Meigen, type species of the genus and the nominotypical member of the *maculipennis* complex. *Systematic Entomology*, **28**, 39–55.

Lipscomb, D., Platnik, N. & Wheeler, Q. (2003) The intellectual content of taxonomy: a comment on DNA taxonomy. *Trends in Ecology and Evolution*, **18**, 65–66.

Mallet, J. & Willmott, K. (2003) Taxonomy: renaissance or Tower of Babel? *Trends in Ecology and Evolution*, **18**, 57–59.

Manonmani, A., Townson, H., Adeniran, T., Jambulingam, P., Sahu, S. & Vijayakumar, T. (2001) rDNA-ITS2 polymerase chain reaction assay for the sibling species of *Anopheles fluviatilis*. *Acta Tropica*, **78**, 3–9.

Marrelli, M.T., Floeter-Winter, L.M., Malafronte, R.S., Tadei, W.P., Lourenco-De-Oliveira, R., Flores-Mendoza, C. & Marinotti, O. (2005) Amazonian malaria vector anopheline relationships interpreted from ITS2 rDNA sequences. *Medical and Veterinary Entomology*, **19**, 208–218.

Mitchell, A., Sperling, F.A.H. & Hickey, D.A. (2002) Higher-level phylogeny of mosquitoes (Diptera: Culicidae): mtDNA data support a derived placement for *Toxorhynchites*. *Insect Systematics and Evolution*, **33**, 163–174.

Moriais, I. & Severson, D.W. (2003) Intraspecific DNA variation in nuclear genes of the mosquito *Aedes aegypti*. *Insect Molecular Biology*, **12**, 631–639.

Nguyen, D.M., Tran, D.H., Harbach, R.E., Elphick, J. & Linton, Y.M. (2000) A new species of the *Hyrcanus* group of *Anopheles*, subgenus *Anopheles*, a secondary vector of malaria in coastal areas of southern Vietnam. *Journal of the American Mosquito Control Association*, **16**, 189–198.

Phuc, H.K., Ball, A.J., Son, L. *et al*. (2003) Multiplex PCR assay for malaria vector *Anopheles minimus* and four related species in the Myzomyia Series from Southeast Asia. *Medical and Veterinary Entomology*, **17**, 423–428.

Proft, J., Maier, W.A. & Kampen, H. (1999) Identification of six sibling species of the *Anopheles maculipennis* complex (Diptera: Culicidae) by a polymerase chain reaction assay. *Parasitology Research*, **85**, 837–843.

Rey, D., Despres, L., Schaffner, F. & Meyran, J.C. (2001) Mapping of resistance to vegetable polyphenols among *Aedes* taxa (Diptera, Culicidae) on a molecular phylogeny. *Molecular Phylogenetics and Evolution*, **19**, 317–325.

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

Sallum, M.A., Schultz, T.R., Foster, P.G., Aronstein, K., Wirtz, R.A. & Wilkerson, K.C. (2002) Phylogeny of Anophelinae (Diptera: Culicidae) based on nuclear ribosomal and mitochondrial DNA sequences. *Systematic Entomology*, **27**, 361–382.

Santomalazza, F., Della Torre, A. & Caccone, A. (2004) Short report: a new polymerase chain reaction-restriction fragment length polymorphism method to identify *Anopheles arabiensis* from *An. gambiae* and its two molecular forms from degraded DNA templates or museum samples. *American Journal of Tropical Medicine and Hygiene*, **70**, 604–606.

Scott, J.A., Brogdon, W.G. & Collins, F.H. (1993) Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene*, **49**, 520–529.

Seberg, O., Humphries, C.J., Knapp, S., Stevenson, D.W., Petersen, G., Scharff, N. & Andersen, N.M. (2003) Shortcuts in systematics? A commentary on DNA-based taxonomy. *Trends in Ecology and Evolution*, **18**, 63–65.

Shaikevitch, E.V. & Vinogradova, E.B. (2004) Molecular genetic methods for the identification of the urban mosquito *Culex pipiens pipiens F. molestus* (Diptera, Culicidae). *Parazitologia*, **38**, 406–412.

Singh, O.P., Chandra, D., Nanda, N. *et a*l. (1997) Differentiation of *Anopheles minimus* species complex by RAPD-PCR technique. *Journal of the Medical Association of Thailand*, **80**, 598–602.

Smith, J.L. & Fonseca, D.M. (2004) Rapid assays for identification of members of the *Culex (Culex) pipiens* complex, their hybrids, and other sibling species (Diptera: Culicidae). *American Journal of Tropical Medicine and Hygiene*, **70**, 339–345.

Sukowati, S., Baimai, V., Harun, S., Dasuki, Y., Andris, H. & Efrivati, M. (1999) Isozyme evidence for three sibling species in the *Anopheles sundaicus* complex from Indonesia. *Medical and Veterinary Entomology*, **13**, 408–414.

Tautz, D., Arctander, P., Minelli, A., Thomas, R.H. & Vogler, A.P. (2003) A plea for DNA taxonomy. *Trends in Ecology and Evolution*, **18**, 70–74.

Van Bortel, W., Trung, H.D., Manh, N.D., Roelants, P., Verle, P. & Coosemans, M. (1999) Identification of two species within the *Anopheles minimus* complex in northern Vietnam and their behavioural divergencies. *Tropical Medicine and International Health*, **4**, 257–265.

Venard, C.E. & Mead, F.W. (1953) An annotated list of Ohio mosquitoes. *Ohio Journal of Science*, **53**, 327–331.

Walton, C., Handley, J.M., Kuvangkadilok, C., Collins, F.H. & Harbach, R.E. (1999) Identification of five species of the *Anopheles dirus* complex from Thailand, using allele-specific polymerase chain reaction. *Medical and Veterinary Entomology*, **13**, 24–32.

Weeto, M.M., Koekemoer, L.L., Kamau, L., Hunt, R.H. & Coetzee, M. (2004) Evaluation of the species-specific PCR assay for the *Anopheles funestus* group from 11 African countries and Madagascar. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **98**, 142–147.

Wood, D.M., Dang, P.T. & Ellis, R.A. (1979) The mosquitoes of Canada. Diptera: Culicidae. In: *The Insects and Arachnids of Canada*, Part 6. *Agriculture Canada Publication*, 1–390.

Zavortink, T.J. (1990) Classical taxonomy of mosquitoes – a memorial to John N. Belkin. *Journal of the American Mosquito Control Association*, **6**, 593–599.

Accepted 13 September 2006