# Population structure and phylogenetic relationships of *Ceutorhynchus neglectus* (Coleoptera: Curculionidae)

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**Abstract**—*Ceutorhynchus neglectus* Blatchley is a weevil that is native to, and widely distributed in, North America. It has life-history characteristics similar to its alien invasive congener, *Ceutorhynchus obstrictus* (Marsham), the cabbage seedpod weevil. Our study was undertaken to compare the population structure of *C. neglectus* in North America to that of *C. obstrictus*, which, in contrast, was introduced only recently to North America and might be expected to have a simpler population structure. We also compared the population structure of *C. neglectus* to that of *Pissodes strobi* (Peck), which is known to possess high levels of intraspecific variation and is also a Nearctic weevil. We sequenced a 790-bp fragment of mtDNA (cytochrome oxidase I (COI) gene) and a 117-bp fragment of nuclear DNA (internal transcribed spacer region 1 (ITS1)). Nested clade analysis inferred contiguous range expansion and restricted gene flow with isolation by distance. Analysis of molecular variance also supported restricted gene flow between geographically distant populations. However, within-species variation in *C. neglectus* was lower than that for other weevil species including *C. obstrictus*. We also examined DNA divergences and phylogenetic relationships among 10 species of *Ceutorhynchus* using parsimony analysis of a 2.3-kb fragment of mtDNA (COI–COII) and a 541-bp fragment of nuclear DNA (elongation factor 1$\alpha$).

**Résumé**—*Ceutorhynchus neglectus* Blatchley est un charançon indigène à l'Amérique du Nord où il a une grande répartition géographique. Les caractéristiques de son cycle biologique ressemblent à celles de son congénère exotique et envahissant, *Ceutorhynchus obstrictus* (Marsham), le charançon de la silique. Notre étude veut comparer la structure de population de *C. neglectus* en Amérique du Nord à celle de *C. obstrictus* qui doit vraisemblablement avoir une structure de population plus simple, car il n'a été introduit que récemment en Amérique du Nord. Nous avons aussi comparé la structure de population de *C. neglectus* à celle de *Pissodes strobi* (Peck) qui est bien caractérisé par une forte variation intraspécifique et qui est aussi une espèce néarctique. Nous avons séquencé un fragment de 790 pb d'ADNmt (le gène de la cytochrome C oxydase, COI) et un fragment de 117 pb d'ADN nucléaire (l'espaceur interne transcrit 1, ITS1). Une analyse cladistique emboîtée indique une expansion de l'aire de répartition par contiguïté et un flux génique restreint par l'isolement relié à la distance. Une analyse AMOVA confirme aussi le flux génique restreint entre les populations éloignées géographiquement. Cependant, la variation intraspécifique chez *C. neglectus* est plus faible que chez les autres charançons, y compris *C. obstrictus*. Nous avons

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aussi examiné les divergences de l'ADN et les relations phylogénétiques chez 10 espèces de *Ceutorhynchus* par une analyse de parcimonie d'un fragment de 2,3 kb d'ADNmt (COI–COII) et d'un fragment de 541 pb d'ADN nucléaire (le facteur d'élongation 1α).

[Traduit par la Rédaction]

## Introduction

The weevil *Ceutorhynchus neglectus* Blatchley (Coleoptera: Curculionidae) has a Nearctic distribution with a broad geographic range extending north to the Yukon and east to Quebec and Maryland (O'Brien and Wibmer 1982; Anderson 1997). It has been found as far south as Oregon in the west and south to Colorado on the eastern slopes of the Rocky Mountains. Adults are small (1 to 2 mm in length), with dark grey bodies covered in fine white scales (Blatchley and Leng 1916).

The phenology of *C. neglectus* is univoltine. Oviposition generally occurs in the spring in distal ends of developing pods of flixweed, *Descurainia sophia* (L.) Webb *ex* Prantl (Brassicaceae), and the larvae consume most of the seeds within a silique as they progress through three larval instars (Dosdall *et al.* 1999). When mature, larvae bore a hole through the pod wall, drop to the ground, burrow under the surface, and pupate (Dosdall *et al.* 1999).

In addition to *D. sophia*, *C. neglectus* has been found on a variety of brassicaceous hosts including other *Descurainia* species (R.D. Laffin, unpublished data); canola, *Brassica napus* L. and *Brassica rapa* L. (Dosdall *et al.* 1999); and marsh yellow cress, *Rorippa islandica* (Oeder) Borbas (Anderson 1997). *Polygonum* sp. (Polygonaceae) was originally thought to be a host (Blatchley and Leng 1916), but host specificity studies indicated that this report was likely accidental because *C. neglectus* appears to rely on *Descurainia* spp. or other Brassicaceae for feeding and reproduction (Dosdall *et al.* 1999). Even though *C. neglectus* occurs on canola, it is not considered an economically important pest because it causes negligible damage and population densities are not high (Dosdall *et al.* 1999).

The broad geographic range of *C. neglectus* and the overlap of its range with that of the introduced *Ceutorhynchus obstrictus* (Marsham) make it an excellent model for population structure comparisons between endemic and invasive species. The cabbage seedpod weevil, *C. obstrictus*, was accidentally introduced to North America from Europe over 70 years ago (McLeod 1953) and shares several similarities in its life history and host plant requirements with *C. neglectus* (Dosdall *et al.* 1999; Dosdall and Moisey 2004). The population structure of *C. neglectus* could be a good indicator of probable patterns of gene flow and structure that may develop in *C. obstrictus* as it continues its range expansion.

The range of *C. neglectus* is similar to that of *Pissodes strobi* (Peck), another weevil whose population structure has been examined (Laffin *et al.* 2004). In *P. strobi*, levels of divergence among haplotypes are high and population structuring is extensive. The generality of this trait among weevils can be determined by comparison with other species across the same geographic range (Avise 2000). Since *C. neglectus* is a native species, its population structure should be more similar to that of *P. strobi* than to that of *C. obstrictus*. It is unclear whether levels of variation will be as high as in *P. strobi*, but variation should be higher than in *C. obstrictus*.

In this study, gene flow and population structure were investigated using nested clade analysis (Templeton *et al.* 1995; Templeton 1998, 2004) and AMOVA (analysis of molecular variance) (Excoffier *et al.* 1992). By using these tests in tandem (Althoff and Pellmyr 2002), we sought to elucidate current and historical processes responsible for population structure. We sequenced a 790-bp fragment of mitochondrial DNA (mtDNA) corresponding to the 3′ end of the cytochrome oxidase I (COI) gene, which has been previously studied in weevils (Laffin *et al.* 2004, 2005). mtDNA is useful for comparing species because it has a relatively rapid evolutionary rate (Brown *et al.* 1979; Simon *et al.* 1994; Rokas *et al.* 2003), and mitochondria are maternally inherited so individuals may be considered haploid (Avise 2000). The 3′ end of the COI gene has been shown to have rapid rates of change in other weevil species (Normark 1996; Langor and Sperling 1997; Laffin *et al.* 2004). We also examined a fragment of nuclear DNA corresponding to the 5′ end of the internal transcribed spacer region 1 (ITS1) to examine population structure at a finer scale. We used ITS1 because it accumulates changes at a high rate in other insects

**Table 1.** Collection localities of sampled *Ceutorhynchus neglectus*.

| Locality No. | Collection locality | Latitude | Longitude | No. of specimens |
|---|---|---|---|---|
| 1 | Whitehorse, Yukon | 60°42′N | 135°04′W | 6 |
| 2 | Dawson Creek, B.C. | 55°45′N | 120°13′W | 6 |
| 3 | Sexsmith, Alta. | 55°20′N | 118°47′W | 6 |
| 4 | Rycroft, Alta. | 55°45′N | 118°42′W | 6 |
| 5 | Fahler, Alta. | 55°44′N | 117°11′W | 6 |
| 6 | Peace River, Alta. | 56°02′N | 117°08′W | 6 |
| 7 | High Prairie, Alta. | 55°34′N | 116°48′W | 6 |
| 8 | Barrhead, Alta. | 54°07′N | 114°24′W | 6 |
| 9 | Edmonton, Alta. | 53°29′N | 113°32′W | 6 |
| 10 | Vegreville, Alta. | 53°30′N | 112°05′W | 6 |
| 11 | Vermillion, Alta. | 53°21′N | 110°47′W | 6 |
| 12 | Lloydminster, Alta. | 53°07′N | 109°36′W | 6 |
| 13 | North Battleford, Sask. | 52°25′N | 109°20′W | 6 |
| 14 | Millet, Alta. | 53°05′N | 113°33′W | 6 |
| 15 | Red Deer, Alta. | 52°11′N | 113°49′W | 6 |
| 16 | Macklin, Sask. | 52°29′N | 110°48′W | 6 |
| 17 | Olds, Alta. | 51°48′N | 114°05′W | 6 |
| 18 | Airdrie, Alta. | 51°17′N | 114°01′W | 6 |
| 19 | Lethbridge, Alta. | 49°38′N | 112°47′W | 6 |
| 20 | Medicine Hat, Alta. | 49°52′N | 110°58′W | 6 |
| 21 | Missoula, Mont. | 46°58′N | 114°17′W | 6 |
| 22 | Moscow, Idaho | 46°43′N | 117°00′W | 6 |
| 23 | Manotick, Ont. | 45°13′N | 75°41′W | 6 |

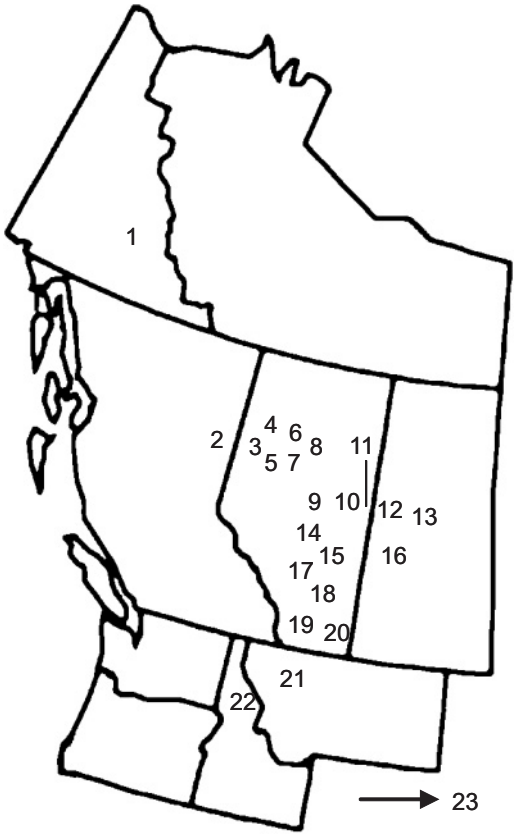
(Collins and Paskewitz 1998; Gallego and Galian 2001; Abe *et al.* 2005). It is a noncoding region of DNA, so mutations are assumed to be largely neutral, and therefore variation should be more extensive in this region than in coding regions.

A further objective of this study was to examine divergences among multiple species of the genus *Ceutorhynchus* to better understand the phylogenetic relationships of *C. neglectus*. This genus comprises 375 species worldwide and over 70 in North America (Colonnelli 2004). We sequenced DNA from 10 *Ceutorhynchus* species to construct a preliminary framework for phylogenetic work on this economically important but poorly studied genus. We sequenced 2.3 kb of mtDNA corresponding to the COI and COII regions as well as a 541-bp region of nuclear DNA corresponding to the 5′ end of elongation factor 1 alpha (EF-1α). We used EF-1α because it has a slower rate of change and less saturation of DNA substitutions than mtDNA in other taxa (Cho *et al.* 1995; Caterino *et al.* 2001), and it also serves as an independent test of the phylogeny generated by mtDNA.

## Material and methods

Adult specimens of *C. neglectus* were collected between June 2003 and August 2004 from 23 localities in Canada and the United States (Table 1, Fig. 1). A number of other localities were sampled for *C. neglectus* without success throughout Canada (including southern British Columbia, Saskatchewan, and Manitoba) and the United States (including the Pacific Northwest and the East Coast). It is likely that in those regions individuals or populations are rare or localized in their distributions. Weevils were collected by sweep-netting individual plants or small stands of flixweed (*Descurainia* spp.) or other brassicaceous weeds. Weevils were killed either by freezing them at –70 °C or by placing them in 95%–100% ethanol; specimens were then stored at –70 °C until DNA was extracted.

**Fig. 1.** Collection localities of *Ceutorhynchus neglectus*. Numbers correspond to localities in Table 1.




Whole genomic DNA was extracted from 138 individuals of *C. neglectus* using the QIAamp DNA Mini Kit and eluted with 150 µL of Buffer AE (QIAGEN). Corresponding vouchers were placed in the Strickland Museum of Entomology at the University of Alberta after identification was verified by an independent expert (P. Bouchard, Agriculture and Agri-Food Canada, Ottawa, Ontario). DNA was stored at –20 °C before PCR amplification. A 790-bp fragment of the mtDNA COI gene was amplified and sequenced using the Jerry and Pat primers (Table 2). PCR reactions were carried out for each specimen in a reaction solution consisting of 35 µL of double-distilled water (Millipore), 5 µL of 25 mmol/L $MgCl_2$ (QIAGEN), 5 µL of 10× PCR Buffer (QIAGEN), 1 µL of 10 mmol/L dNTPs (Roche Applied Science), 1 µL of each of the two 5 pmol/µL heterologous primers, and 1 µL of extracted DNA. Taq polymerase was added right before sample amplification in a TGradient Thermocycler (Biometra, Göttingen, Germany). The thermal cycling program had an initial denaturation step of 2 min at 94 °C and then 34 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 45 °C, and extension for 2 min at 72 °C, followed by a 5-min extension at 72 °C. PCR products were visualized on agarose gels before being cleaned with the QIAquick PCR Purification Kit (QIAGEN).

Sequencing reactions were carried out, for both forward and reverse strands, using 3.5 µL of double-distilled water (Millipore), 1 µL of BigDye® Terminator Ready Reaction Mix (PE Applied Biosystems), 3 µL of 2.5× BigDye® Terminator Sequencing Buffer, 0.5 µL of one of the two previously listed primers, and 1 µL of purified PCR product. The sequencing reaction program had an initial denaturation step at 96 °C for 1 min, followed by 29 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, and was carried out using the same thermal cycler as for PCR amplification. Sequencing products were cleaned using fine-packed Sephadex G-50 columns (Amersham Biosciences) and then visualized on an ABI PRISM® 377 automated DNA sequencer (PE Applied Biosystems). Contiguous sequences were constructed and aligned manually in Sequencher 4.1 (Gene Codes Corporation 2001). Sequences were then exported for further analyses.

Two other fragments were also sequenced in 12 *C. neglectus* individuals from across the species' range in order to examine variation in independent gene regions: a 367-bp fragment of a nuclear gene, elongation factor 1 alpha (EF-1α), and a 1.2-kb fragment of the internal transcribed spacer region 1 (ITS1). PCR and sequencing reactions were carried out as above except for the use of different primers and a different annealing temperature. For EF-1α, we used the Bo and Luke primers (Table 2) and an annealing temperature of 53 °C. For the ITS1 fragment we used the ITS1 and ITS2 primers (Table 2) and an annealing temperature of 55 °C. No variation was found among the 12 EF-1α sequences, so no additional individuals were sequenced. In the ITS1 region we did find variation, so an internal primer was designed for the 5′ end of ITS1.

The 481-bp fragment of ITS1 used in this study was amplified with the new primer ITS1b. We sequenced only 117 bp cleanly for most individuals because of the large numbers of insertions or deletions (indels) contained further upstream. Because of these indels, only

**Table 2.** Primers used for PCR and sequencing.

| Primer name | Direction* | Region | Location of 3′ end | Original reference | Sequence (5′–3′) |
|---|---|---|---|---|---|
| K698 | F | COI | 1460 | Simon *et al.* 1994 | TAC AAT TTA TCG CCT AAA CTT CAG CC |
| Ron | F | COI | 1751 | Simon *et al.* 1994 | GGA TCA CCT GAT ATA GCA TTC CC |
| K699 | R | COI | 1840 | Sperling *et al.* 1995 | AGG AGG ATA AAC AGT TCA C/TCC |
| Jerry | F | COI | 2183 | Simon *et al.* 1994 | CAA CAT TTA TTT TGA TTT GG |
| Nancy | R | COI | 2192 | Simon *et al.* 1994 | CCCTTT GGT AAA ATT AAA ATA TAA ACT |
| Brian III | F | COI | 2495 | Laffin *et al.* 2004 | CCT CCT CTT TAT GAT CAA TTG G |
| K741 | R | COI | 2578 | Caterino and Sperling 1999 | TGG AAA TGT GCA ACT ACA TAA TA |
| Mila | R | COI | 2659 | Simon *et al.* 1994 | GCT AAT CCA GTG AAT AAT GG |
| Geoish | F | COII | 2733 | New | AAT AAG AAT TTT TTA TTT TAT |
| George | F | COI | 2792 | Bogdanowicz *et al.* 1993 | ATA CCT CGA CGT TAT TCA GA |
| Pat | R | COI | 3014 | Simon *et al.* 1994 | TCC AAT GCA CTA ATC TGC CAT ATT A |
| Pierre | F | COII | 3138 | Simon *et al.* 1994 | AGA GCC TCT CCT TTA ATA GAA CA |
| Marilyn | R | COII | 3389 | Simon *et al.* 1994 | TCA TAA GTT CAR TAT CAT TG |
| Marish | R | COII | 3425 | New | TTT CAT CTA AAA TAT ATA ATA |
| Barbara | R | COII | 3494 | Simon *et al.* 1994 | GGT AAA ACT ACT CGA TTA TCA AC |
| Eva | R | COII | 3782 | Bogdanowicz *et al.* 1993 | GAG ACC ATT ACT TGC TTT CAG TCA TCT |
| Starsky | F | EF-1α | 0 | Cho *et al.* 1995 | CAC ATY AAC ATT GTC GTS ATY GG |
| Papsky | F | EF-1α | 15 | Reed and Sperling 1999 | CGG ACA CGT CGA CTC CGG |
| Steve | F | EF-1α | 56 | New | CGT AGA TTC TGG TAA ATC TAC |
| Bo | F | EF-1α | 174 | Cho *et al.* 1995 | GCT GAG CGY GAR CGT GGT ATC AC |
| Cho | F | EF-1α | 234 | Reed and Sperling 1999 | GTC ACC ATC ATY GAC GC |
| Hutch | R | EF-1α | 238 | Cho *et al.* 1995 | CTT GAT GAA ATC YCT GTG TCC |
| Nadine | R | EF-1α | 494 | New | CAG GGT TGT AAC CAA TTT TCT |
| Luke | R | EF-1α | 541 | Cho *et al.* 1995 | CAT RTT GTC KCC GTG CCA KCC |
| ITS1 | F | ITS1 | 0 | White *et al.* 1990 | TCC GTA GGT GAA CCT GCG G |
| ITS1b | R | ITS1 | 523 | New | CAG GCC GAC CCG TCC GAA AAC |
| ITS2 | R | ITS1 | 1183 | White *et al.* 1990 | GCT GCG TTC TTC ATC GAT GC |

**Note:** Positions are relative to *Drosophila yakuba* (Clary and Wolstenholme 1985) for mtDNA and *Heliothodes diminutivus* (Cho *et al.* 1995) for EF-1α.
*F, forward; R, reverse.

**Table 3.** Collection localities of sampled *Ceutorhynchus* and outgroup species.

| No. | Species | GenBank mtDNA No. | GenBank EF-1α No.* | Collector | Collection locality |
|---|---|---|---|---|---|
| 1 | *Ceutorhynchus obstrictus* (Marsham, 1802) | DQ058695 | NA | R.D. Laffin | Lethbridge, Alta. |
| 2 | *Ceutorhynchus subpubescens* LeConte, 1876 | DQ058696 | DQ058706 | R.D. Laffin | Calgary, Alta. |
| 3 | *Ceutorhynchus neglectus* Blatchley, 1916 | DQ058697 | DQ058707 | R.D. Laffin | Lethbridge, Alta. |
| 4 | *Ceutorhynchus erysimi* (Fabr., 1787) | DQ058698 | DQ058708 | R.D. Laffin | Millet, Alta. |
| 5 | *Ceutorhynchus fallax* (Boheman) | DQ058699 | NA | B.A. Korotyaev | Pachyphragma, Turkey |
| 6 | *Ceutorhynchus gallorhenanus* Hoffman, 1954 | DQ058700 | NA | B.A. Korotyaev | Pachyphragma, Turkey |
| 7 | *Ceutorhynchus filirostris* (Reitter) | DQ058701 | DQ058709 | B.A. Korotyaev | Pachyphragma, Turkey |
| 8 | *Ceutorhynchus rapae* (Gyllenhal, 1837) | DQ058702 | DQ058710 | B.A. Korotyaev | Novoaleksandrovsk, Russia |
| 9 | *Ceutorhynchus cochleariae* (Gyllenhal, 1813) | DQ058703 | DQ058711 | B.A. Korotyaev | Adegea, Russia |
| 10 | *Ceutorhynchus querceti* (Gyllenhal, 1813) | DQ058704 | NA | B.C. Schmidt | Whitehorse, Yukon |
| 11 | *Pissodes strobi* (Peck, 1817) | U77976 | NA | D. Langor | Swan Hills, Alta. |
| 12 | *Omphalapion hookeri* (Kirby, 1808) | DQ058705 | NA | R.D. Laffin | Edmonton, Alta. |

*NA, not available.

forward strands could be sequenced. To estimate haplotypes from the diploid sequences, we used haplotype subtraction (Clark 1990; Xu *et al.* 2002). Since only one direction could be sequenced, heterozygotes were scored only when obvious, so there may be a sampling bias in favor of homozygotes.

Nested clade analysis and AMOVA were completed using the ITS1 fragment. mtDNA was not used because variation within it was too low to be useful in these analyses. Nested clade analysis was performed using TCS 1.18 (Clement *et al.* 2004) and GeoDis 2.2 (Posada and Templeton 2004). TCS was used to create a parsimony network with a 95% confidence limit and GeoDis was used to calculate geographic distances between haplotypes and their centers using the coordinates in Table 1. AMOVA was carried out using SAMOVA 1.0 (Dupanloup *et al.* 2002) to find optimal population groupings. Variation among populations and Fst values were estimated with Arlequin 2.0 (Schneider *et al.* 2000).

To provide a framework for understanding the evolutionary relationships between *C. neglectus* and *C. obstrictus*, phylogenetic analysis was performed on sequence data from 10 species of *Ceutorhynchus* from North America and Europe and two outgroups (Table 3). All specimens were collected between June 2002 and July 2004. We sequenced 2.3 kb of mtDNA corresponding to the COI–COII region for all species, and 541 bp of nuclear DNA corresponding to the 5′ end of EF-1α for 6 species where DNA quality was sufficiently high. Species were identified by P. Bouchard and B.A. Korotyaev (Zoological Institute of Russian Academy of Sciences, St. Petersburg). DNA extraction, PCR amplification, and sequencing were carried out using the methods described above and primers in Table 2.

Parsimony analysis and bootstrapping were performed with PAUP* version 4.0 beta 10 (Swofford 2002) for each gene separately and all genes combined. Exhaustive parsimony analysis was carried out to obtain the best tree using *Omphalapion hookeri* (Kirby) and *P. strobi* as outgroups. Bootstrapping values were calculated from 100 replicates. Percent divergence was also calculated using PAUP*.

## Results

Seven mitochondrial or nuclear gene haplotypes (GenBank Nos. DQ058688– DQ058694)

**Table 4.** Distribution of haplotypes across localities; locality numbers correspond to those in Table 1.

| Haplotype | Locality | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
| COI 1 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | |
| COI 2 | | | | | | | | | | | | | | | | | | | | | | | 6 |
| ITS1 A | 3 | 4 | 5 | 4 | 5 | 1 | 7 | 7 | 3 | 5 | 8 | 5 | 4 | 3 | 4 | 6 | 6 | 5 | 8 | 4 | 8 | 7 | 4 |
| ITS1 B | 5 | 5 | 1 | 3 | 1 | 1 | 3 | | 9 | 2 | 3 | 1 | 5 | 6 | 5 | 1 | 2 | 5 | 2 | 3 | 4 | 5 | |
| ITS1 C | | | | | | | | | | | | 2 | 2 | | | 1 | | | 1 | 1 | | | 8 |
| ITS1 D | 3 | 3 | 6 | 5 | 3 | 8 | 2 | 4 | | 2 | 1 | 2 | | 2 | 1 | 3 | 3 | 2 | | 4 | | | |
| ITS1 E | 1 | | | | 3 | 2 | | 1 | | 3 | | 2 | 1 | 1 | 2 | 1 | 1 | | 1 | | | | |

were found among the 138 individuals sampled (Table 4). The COI fragment yielded only two haplotypes, and these were different at a single nucleotide position, giving a sequence divergence of 0.13%. The first haplotype comprised almost all individuals sampled. The second haplotype was restricted to the Manotick, Ontario, locality and was found in all the individuals there. Because of the low haplotype variation and its simple geographic distribution, no further analysis was performed using COI.

Five haplotypes were found in the ITS1 DNA fragment and all but one were homozygous in at least one specimen (Table 5). In all, 47% of individuals were homozygous. For the 53% that were heterozygous, 24% had alleles that differed at 1 bp, 25% differed at 2 bp, and 4% differed at 3 bp.

Sequence divergence among haplotypes ranged from 0.9% to 3.4% and the overall nucleotide diversity among haplotypes was 1.5%. All localities contained more than one haplotype: 3 localities had two haplotypes, 6 had three, 12 had four, and 2 had five. Haplotype A was the most common haplotype and was the only one found at all localities. A total of 116 haplotype A alleles were found across all localities and accounted for 8%–67% of alleles at a given locality. Haplotype C was the rarest of the haplotypes, with only 15 alleles of this haplotype found across all localities, but it was the main haplotype in Manotick.

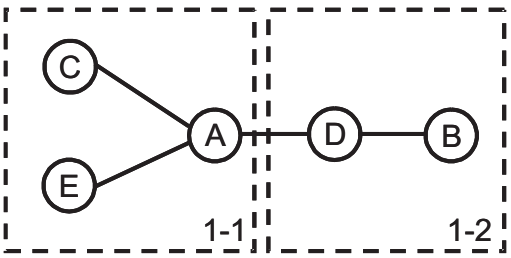
The haplotype network (Fig. 2) yielded by TCS 1.18 (Clement *et al.* 2004) had a maximum of three mutational steps between any two haplotypes and no missing haplotypes. Clade 1–1 consisted of haplotypes from all localities except Manotick, while clade 1–2 comprised haplotypes from all localities. This observation is congruent with the results from the COI analysis.

Nested contingency analysis of ITS1 sequences using GeoDis 2.2 (Posada and Templeton 2004) revealed significant associations between clades and sampling locations (Table 6). Both clades as well as the entire cladogram showed a significant association between geography and clades. There were two patterns inferred from nested clade analysis of the ITS1 fragment (Table 7). The first, which was found in both clades, was contiguous range expansion, and the second, which was found for the entire cladogram, was restricted gene flow with isolation by distance.

**Table 5.** Frequencies of genotypes for all ITS1 sequences.

| Haplotype | A | B | C | D | E |
|---|---|---|---|---|---|
| A | 0.232 | | | | |
| B | 0.195 | 0.116 | | | |
| C | 0.036 | 0.021 | 0.022 | | |
| D | 0.079 | 0.058 | 0 | 0.101 | |
| E | 0.065 | 0.014 | 0.007 | 0.051 | 0 |

**Fig. 2.** Haplotype network and nested clade design for five haplotypes detected in ITS1 (internal transcribed spacer region 1) sequences. Letters in circles represent haplotypes and lines between haplotypes represent one-step mutational changes. Dashed boxes represent one-step clades.




AMOVA detected very little structuring of *C. neglectus* populations based on ITS1. SAMOVA 1.0 (Dupanloup *et al.* 2002) indicated that the optimal population structuring showed two distinct groups: one comprising the Manotick locality and the other made up of all other localities. Variation among populations within groups was lower than variation between the two groups, which suggests restricted gene flow between the two groups (Table 8). About 38% of the total variation detected was between the two groups of populations, and only 3.5% of the variation was between populations within groups. A significant overall Fst value was obtained using both the variance estimate (Fst = 0.41) and the traditional genotype frequencies under Hardy–Weinberg equilibrium (Fst = 0.38). This further supports the inference of restricted gene flow between the populations.

Parsimony analysis of the 2.3-kb mtDNA fragment from the 10 *Ceutorhynchus* species and 2 outgroup species yielded a single tree with a length of 1349 steps (Fig. 3). Of these 12 species, those that are native to western North America are distributed throughout the tree. Two species with a stem-mining lifestyle in larvae, *C. subpubescens* LeConte and *C. rapae* (Gyllenhal), are monophyletic and are quite divergent from the other species examined. Two species that appear to be very similar in morphology, *C. neglectus* and *C. querceti* (Gyllenhal), are quite divergent with respect to mtDNA.

**Table 6.** Nested contingency analysis of geographical associations for ITS1 sequence data from *Ceutorhynchus neglectus*.

| Clade | Permutational $\chi^2$ statistic | Probability |
|---|---|---|
| 1–1 | 47.74 | 0* |
| 1–2 | 88.57 | 0* |
| Total | 40.22 | 0.007* |

*Significant at the 0.05 level.

The EF-1α sequence could not be obtained for four of the *Ceutorhynchus* species. This may have been due to a combination of specimen preservation and extraction methods that lowered DNA quality so that regions of EF-1α could not be amplified. For species where a fragment of EF-1α could be obtained, a single tree was found with a length of 37 steps (Fig. 4) and the topology was fully congruent with that found using only mtDNA. The combined analysis also yielded a single tree that matched the topology of the other trees.

Uncorrected genetic distances between species of *Ceutorhynchus* ranged from 4.4% to 15.0% for mtDNA and from 0.2% to 4.6% for EF-1α (Table 9). For those species where both nuclear and mtDNA sequences were obtained, the genetic distance in mtDNA ranged from 3.6 to 13.8. On average, variation in COI–COII was about 6 times greater (ranging from 2.5 to 22 times greater) than variation in EF-1α based on comparisons where both sequences were available.

## Discussion

Analysis of mtDNA in *C. neglectus* populations determined that the only locality that differed significantly from the others was Manotick, Ontario. Even though sequence divergence between the haplotype from Manotick and

**Table 7.** Demographic inferences from nested clade distance analysis (Templeton *et al.* 1995; Templeton 1998) of ITS1 in *Ceutorhynchus neglectus*.

| Clade | Inference chain | Inferred pattern* |
|---|---|---|
| Haplotypes in 1–1 | 1-2-11-12-No | A |
| Haplotypes in 1–2 | 1-2-11-12-No | A |
| One-step clades in 2–1 | 1-2-3-4-No | B |

*A, contiguous range expansion; B, restricted gene flow with isolation by distance.

**Table 8.** AMOVA results for tests of genetic divisions between populations of *Ceutorhynchus neglectus*.

| Source of variation | Variance components | Percent of variation |
|---|---|---|
| Among regions | 0.321 | 37.58 |
| Among populations within regions | 0.03 | 3.54 |
| Within populations | 0.503 | 58.87 |
| Overall Fst | 0.41* | |

*Significant at 0.05 level.

the major haplotype was only 0.13%, the discovery of a complete haplotype replacement indicates that *C. neglectus* in Manotick probably stemmed from a different source than the populations at other localities in the study. This is not surprising, considering that the geographic distance between Manotick and the next nearest study locality is about three times greater than the distance between any of the other localities. Without further sampling or more information about variation, data from mtDNA could not provide further insight into population structure.

ITS1 proved more useful than mtDNA for assessing population structure in *C. neglectus* owing to the greater variation in the examined ITS1 sequences. The difference between Manotick and the other localities was less obvious for ITS1 than for mtDNA, since Manotick shared ITS1 haplotypes with the other localities. Nevertheless, Manotick was the only locality where the majority of haplotype copies were haplotype C, which was the least common haplotype found at the other localities. The parsimony network and nested clade analysis yielded by TCS 1.18 (Clement *et al.* 2004) showed that Manotick was the only locality where haplotypes from both clades were not present. Nested contingency analysis indicated a significant association between geography and clades. This suggests low levels of gene flow between populations, even between those that are geographically close.

Nested clade analysis of the ITS1 region inferred two processes responsible for the current genetic structure of populations. At the one-step clade level, contiguous range expansion was inferred. This may be due to the fact that flixweed, the primary host plant of *C. neglectus*, is an opportunistic weed that readily invades disturbed habitats. These habitats are often temporary; for example, weed treatment of agricultural fields or exclusion by more competitive species will greatly reduce localized stands of flixweed. Stands of flixweed may be temporary and may not even last an entire season, so *C. neglectus* would need to disperse to different stands during the year or to new sites after overwintering. Since *C. neglectus* probably disperses randomly away from old feeding sites or overwintering sites, contiguous range expansion is plausible because collection localities would be populated by random individuals. Individual sample localities that are near each other could have different haplotype ratios because of founder effects at each temporary locality.

When the entire network is examined, restricted gene flow with isolation by distance is inferred. This result stems from the difference between the Manotick population and other populations. Since *C. neglectus* in Manotick primarily had the least common haplotype, and

**Fig. 3.** Phylogram of *Ceutorhynchus* spp. generated from PAUP* exhaustive parsimony analysis of 2.3 kb of mtDNA (COI–COII region). Bootstrap values are indicated on the tree.

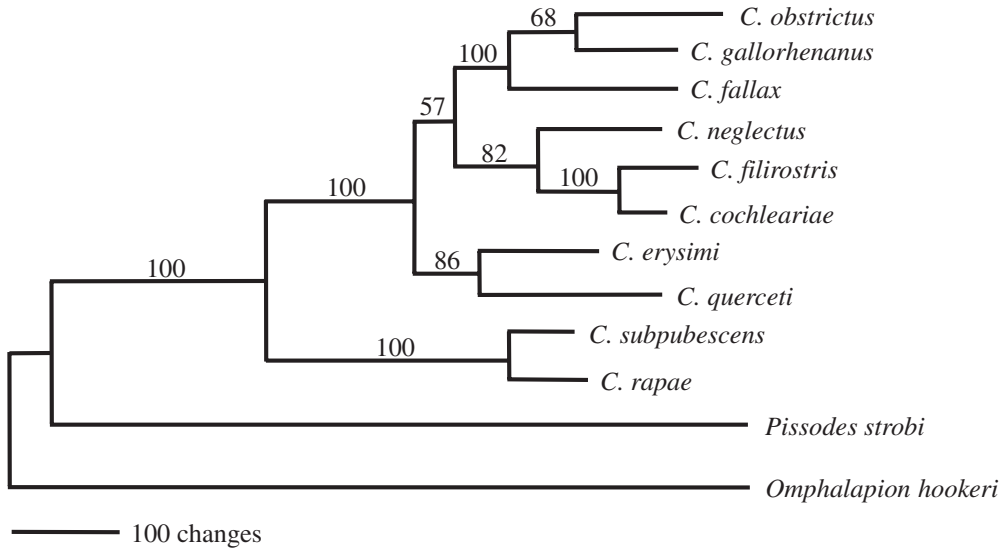
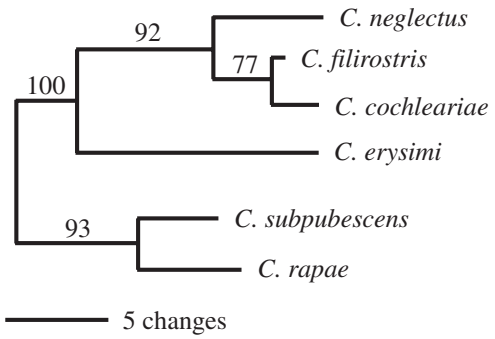



**Fig. 4.** Phylogram of *Ceutorhynchus* spp. generated from PAUP* exhaustive parsimony analysis of 541 bp of nuclear DNA (EF-1α). Bootstrap values are indicated on the tree.




that haplotype was restricted to Manotick and only a few other localities in southern Alberta or Saskatchewan, it is likely that gene flow is restricted between geographically distant populations.

Fst analysis supported nested clade analysis, as significant Fst values were found between many populations and the overall Fst value was significant. Significant Fst values indicate a subdivision of the population into distinct groups that do not interbreed. This result is not surprising because of the large geographic distance between the two groups (over 1700 km).

AMOVA detected two distinct genetic groups based on genetic distance and geography, and again the division was between Manotick and the other localities sampled. Even without further sampling in central North America, it is clear that at least some populations in the east and west are different, especially when the congruent results from the ITS1 and mtDNA data are considered. The genetic differences between populations most likely resulted from different founders dispersing to those areas within the past 10 000 years, after the Pleistocene deglaciation. An early dispersal would have relied on a native host plant, probably another *Descurainia* species (Dosdall *et al.* 1999), whereas dispersal within the past 200 years could have followed the introduction and spread of flixweed across North America (Best 1977).

Phylogenetic analysis provided insight into relationships among the 10 species of *Ceutorhynchus* that we examined. First, the stem-mining weevils, *C. rapae* and *C. subpubescens*, group together and are distant from all other species sampled. This could mean that the stem-mining habit of larvae evolved once in this genus. A second discovery of interest is the genetic distance between *C. neglectus* and *C. querceti*. On the basis of external morphology, the two species appear superficially similar in some parts of their range, but are quite divergent genetically. Little or no published information is available on other character systems, such as internal morphology, for these species. Even though we examined only a small number of the more than 375 species of *Ceutorhynchus*

known worldwide, these two species are clearly not each other's closest relative.

mtDNA divergences between closely related species of weevils are thought to be higher than those among other insects (Normark 1996; Langor and Sperling 1997), and variation within some weevil species has also been shown to be higher than in other insects such as dipterans, hemipterans, or other coleopterans, even across a very small geographic distance (Langor and Sperling 1997; Althoff and Pellmyr 2002). We saw high divergence between species but this may be due to the small number of species sampled in this large genus. Nuclear DNA divergences in our study were similar to those found in other curculionids (Jordal *et al.* 2000). It may be that mtDNA in weevils evolves at a relatively higher rate than in other insect taxa, which would account for the greater difference in rates between mtDNA and EF-1α. A greater diversity of curculionid species groups will need to be examined to test this hypothesis.

Within-species variation is much higher in the white pine weevil, *P. strobi*, than in *C. neglectus* (Laffin *et al.* 2004). Variation in *C. obstrictus* is also higher than in *C. neglectus*, but is not as high as in *P. strobi* (Laffin *et al.* 2005). Low variation in *C. neglectus* contradicts our expected results. The high level of variation within *P. strobi* suggested that weevils generally have higher intraspecific divergences than other insects (Laffin *et al.* 2004), but our study of *Ceutorhynchus* weevils has shown that this is not correct. It is unclear whether only *P. strobi* has high intraspecific variation or whether other weevils also exhibit this trait even though *C. obstrictus* and *C. neglectus* do not. Also, other coleopterans such as chrysomelids do not exhibit as high a rate of intraspecific variation as *P. strobi* (Sota *et al.* 2004).

Variation in mtDNA within and between *C. neglectus* populations is much lower than in the other weevils we examined across similar geographic distances (Laffin *et al.* 2004, 2005). This may be due to a relatively recent range expansion of *C. neglectus* following the spread of flixweed across North America or an earlier expansion after the retreat of Pleistocene glaciation as long as 10 000 years ago. Additionally, the range of *P. strobi* overlaps greatly with that of *C. neglectus* and the two species may have had similar biogeographic histories, and yet their population genetic structures are very different. Thus, there appear to be other processes

**Table 9.** Uncorrected percent divergence between species for mitochondrial and nuclear DNA.

| Species | *C. obs* | *C. sub* | *C. neg* | *C. ery* | *C. fal* | *C. gal* | *C. fil* | *C. rap* | *C. coc* | *C. que* | *P. str* | *O. hoo* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. obstrictus* | — | | | | | | | | | | | |
| *C. subpubescens* | 12.2 | — | 3.8 | 4.6 | | | 2.9 | 0.8 | 3.3 | | | |
| *C. neglectus* | 13.9 | 13.2 | — | 3.9 | | | 1.2 | 3.5 | 1.4 | | | |
| *C. erysimi* | 12.3 | 11.6 | 12.6 | — | | | 3.5 | 3.7 | 3.7 | | | |
| *C. fallax* | 7.0 | 12.2 | 12.9 | 12.7 | — | | | | | | | |
| *C. gallorhenanus* | 5.8 | 12.2 | 12.4 | 11.7 | 6.5 | — | | | | | | |
| *C. filirostris* | 13.3 | 15.0 | 12.3 | 13.8 | 13.1 | 12.9 | — | 3.3 | 0.2 | | | |
| *C. rapae* | 12.8 | 8.3 | 13.2 | 13.1 | 12.9 | 12.3 | 15.0 | — | 3.5 | | | |
| *C. cochleariae* | 13.1 | 13.9 | 11.8 | 13.4 | 12.8 | 12.8 | 4.4 | 14.9 | — | | | |
| *C. querceti* | 13.3 | 12.7 | 13.0 | 11.7 | 13.2 | 13.0 | 13.0 | 15.0 | 12.8 | — | | |
| *P. strobi* | 20.6 | 19.1 | 19.5 | 19.3 | 20.0 | 19.7 | 21.1 | 19.6 | 20.3 | 20.4 | — | |
| *O. hookeri* | 20.7 | 20.1 | 20.1 | 19.8 | 20.2 | 19.7 | 20.6 | 21.1 | 20.2 | 21.0 | 21.8 | — |

**Note:** Below diagonal, 2.3 kb of COI + COII mtDNA; above diagonal, 541 bp of EF-1α.

acting on the population structure of native species of Curculionidae. These processes may be due to differences in biology, dispersal mechanisms, or host phenology. For example, *P. strobi* generally does not disperse great distances, while *C. neglectus* may need to disperse long distances to find new temporary stands of its host; therefore, differences between separated populations would not build up as easily in *C. neglectus*. A comparison of *P. strobi* with more closely related weevil species should yield more information on whether the high rate of intraspecific variation is restricted to *P. strobi* or is expressed in some closely related groups as well. Also, an expanded sampling of *C. neglectus* may indicate possible founder populations for the two mtDNA haplotypes we found in this study.

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