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Population structure and variation in *Ceutorhynchus obstrictus* and *C. neglectus* (Coleoptera: Curculionidae)

by

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Abstract

Ceutorhynchus weevils comprise a worldwide genus with many economically important species. Introduced from Europe over 70 years ago, the cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Marsham), is a major pest of canola in North America, and has only recently invaded Canada east of British Columbia. I used mtDNA sequence data to examine population structure and the historical processes responsible for that structure, using both nested clade analysis and analysis of molecular variance, in order to determine the European source for the North American invasions. I also examined the population structure of *Ceutorhynchus neglectus* (Blatchley), which is native to North America, using both nuclear and mitochondrial DNA. I then compared the variation within these two *Ceutorhynchus* weevils to variation found in other weevil species. Additionally, I used parsimony analysis to construct a phylogeny of 10 *Ceutorhynchus* species to examine interspecies relationships and interspecific variation.

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1.1 The Importance, Role, and Techniques of Molecular Analysis in Population Biology and Phylogenetic Research

1.1.1 History and importance of molecular analysis

The origin of population genetics is generally attributed to theoretical studies in the 1920s and 1930s by Fisher, Haldane and Wright (Li 1977). Also, Wahlund (1928) showed that differences in genetic samples taken from members of the same species could reveal divisions in populations (Templeton 1998), and these differences were not necessarily apparent when simply examining morphological characters. At first, genetic data were used primarily for measuring similarities and differences in allele frequencies between different geographic regions to assess population differentiation and to estimate gene flow among different regions (Wright 1943). In the 1960's, protein electrophoresis, or allozyme determination, was pushed to the forefront (Avise 1994) as a method for measuring genetic variability. While this method was useful for examining variation among populations, it could not as precisely delineate relationships between those groups or give as accurate a measurement of polymorphism as DNA sequencing. About the same time that allozyme data was gaining popularity, a debate began to define the meaning of molecular variation (Avise 1994). This arose from the hypothesis that the changes detectable by gel electrophoresis were so-called neutral mutations that would not affect organismal fitness but could still become fixed in a population (Kimura 1968).

This was, of course, different from the Darwinian concept that for variation to become fixed in the population, this variable trait would have to be selected (Davison 2002). At a molecular level this is unlikely since most changes have no effect on the amino acid that would be produced. The neutral mutation hypothesis quickly gained support and is now believed to explain most genetic changes within populations (Avise 1994).

Later, restriction site surveys and DNA sequencing also provided information on evolutionary relationships among different groups (Avise 1994). This allowed for relationships between different species to be examined on a molecular scale, and not solely on the basis of morphological and behavioral characteristics.

Molecular data have been extremely useful for delineating relationships among insects at many levels, from within species to much broader studies between different families, especially when used in conjunction with morphological characters. For example, a study by Marvaldi et al. (2002) on the Superfamily Curculionoidea using a large fragment of ribosomal DNA (18S region) showed relationships between the families comprising the Superfamily and was also able to discern phylogenetic relationships between major subfamilies. In this case the use of both molecular markers and morphological characters gave a clearer picture of the relationships between groups than either character set could by itself.

However, morphological and molecular data sets may be incongruent (Patterson 1987). Incongruence is generally either due to errors in assigning morphological character states, which may be caused by convergence between different groups, or to actual differences between the evolutionary histories of different characters (DeQueiroz et al. 1995). In some cases of groups or species that are very similar morphologically,

combining molecular data with morphological characters is not insightful, so molecular data alone are used.

Molecular data have also been useful for determining phylogenies within species or between closely related species or within species that lack informative morphological characters (Landry et al. 1999, Langor and Sperling 1995, Palumbi and Benzie 1991). Sometimes, different host-plant associations (Brunner et al. 2004, Sperling et al. 1996), or pheromone types (Gooding et al. 1992, Sperling et al. 1999) can indicate different lineages within species even when no apparent morphological differences are present, but with molecular analyses these hypotheses can be checked through the identification of lineages and reconstruction of relationships between them. Within-species analyses can also be very useful for determining where lineages originated, especially in the case of range expansion or when human-aided dispersal has caused new invasions of foreign species.

However, phylogenetic estimates of intra-species relationships may be difficult to infer when multiple groups are approximately equally diverged from each other (Althoff and Pellmyr 2002). When parsimony analysis breaks down, other analytical techniques must be employed. A variety of analyses have been developed specifically for examining divisions between populations and the underlying causes for such divisions, mostly using differences in haplotype frequencies to estimate some level of relationship between groups. Aside from parsimony, the two major analyses used in this thesis are nested clade analysis (Templeton et al. 1995), and AMOVA (analysis of molecular variance) (Excoffier et al. 1992).

1.1.2 AMOVA and Nested clade analysis

AMOVA, or analysis of molecular variance, is used to measure population variation and estimate gene flow between populations based on an extension of F-statistics (Excoffier et al. 1992). F-statistics are based on the measurement of genotypic deviations from Hardy-Weinberg equilibrium proportions which may occur either in subpopulations or combined populations. F-statistics are normally measured through diallele frequencies of a given gene. F-statistics may also be estimated by analysis of variance using haplotype frequencies. Analysis of variance components allow different levels of subdivision to be tested and comprise the foundation of AMOVA determinations.

AMOVA measures genetic structuring based on a matrix of haplotype frequency differences between samples or groups of samples. One major advantage of AMOVA is that many types of molecular data can be used, such as DNA sequence, RFLP, RAPD, or allozyme data. While nested clade analysis can be a more powerful analytical tool than AMOVA, under some conditions AMOVA may outperform nested clade analysis. For example, Turner et al. (2000) showed that AMOVA was better at inferring processes at the local population level. Althoff and Pellmyr (2002) also described a situation in which AMOVA was better at detecting population structuring when division between populations was recent since it can differentiate large scale population divisions.

One of the greatest drawbacks of AMOVA has been the requirement for using a priori groupings. However, a method to test those groupings, called a spatial AMOVA (SAMOVA), was recently published by Dupanloup et al. (2002). This method aims to

maximize the total variation from differences between groups of populations. SAMOVA not only uses genetic distance and haplotype frequencies but also geographic position when determining likely population groupings. This method reduces errors in a priori groupings and will provide more optimal arrangements for population groups.

Nested clade analysis (NCA) uses the phylogenetic relationships of alleles in the context of their geographic distributions to understand population structure (Templeton 1995). NCA works by comparing the geographic distance of a haplotype or clade between different localities and a hypothetical center for each haplotype or clade. Depending on the location of the haplotype within an unrooted network of haplotypes, historical inferences can be made about haplotypes or groups of haplotypes based these geographic distances. These inferences, such as fragmentation or range expansion, are made by following an inference key described by Templeton (2004).

NCA is able to compensate for a major limitation of F_{st}-based tests. F_{st}-based tests consider divisions on a geographic scale, but they do not examine evidence of structure on a temporal scale. Also, F-statistics may not indicate what model of gene flow is appropriate for populations in a study (Hudson et al. 1992). For example, F_{st} tests cannot be used to elucidate different models of gene flow that are occurring concurrently in different areas of a species range (Templeton et al. 1995). Also, two populations arising from the same ancestry but currently showing no gene flow may confound analysis, as they may be too similar to separate using F_{st} tests.

By using a haplotype parsimony network, nested clade analysis can find associations between a phylogeny and geographic locations of the haplotypes (Templeton et al. 1995). Moreover, because different geographical associations of haplotypes will

give distinct statistical patterns, the cause of different patterns of gene flow may be determined in some cases, even if these different patterns occur simultaneously (Templeton and Sing 1993). Therefore, even if gene flow is restricted between populations because of multiple events or combinations of different historical events, nested clade analysis may still be able to uncover the different events. In addition, nested clade analysis can examine weak phylogenetic signals, like those produced by recently separated lineages, because it incorporates interior and descendant haplotypes in its analysis.

One of the greatest problems with nested clade analysis is that no statistical tests are available to assess the validity of results it produces (Knowles and Maddison 2002). Knowles and Maddison (2002) described a case where nested clade analysis failed to predict the processes used to simulate data for their tests. They suggested a coalescent approach where multiple hypotheses may be tested and error is assessed. More recently, Templeton (2004) tested the ability of nested clade analysis to detect inferences in real data sets with a priori expectations. He found that nested clade analysis performed well using real data, whereas unrealistic sampling in the simulated data from the Knowles and Maddison (2002) study caused nested clade analysis to fail.

It is not surprising that recent studies have begun to use both methods of analysis in tandem to garner as much information as possible about historical processes as well as current population structure (Turner et al. 2000, Althoff and Pellmyr 2002, Laffin et al. 2004). The advantage of using both analyses is that nested clade analyses can infer historical processes that have resulted in recent population structure, while AMOVA may better detect recent changes or current structuring of populations.

1.1.3 Mitochondrial DNA sequencing

Mitochondrial DNA (mtDNA) has several advantages for generating data for intra-species analysis. First, its relatively rapid evolutionary rate (Avise 1991) means that even within-species DNA variation is generally detectable. This makes it extremely useful for studies of population genetics, as there is usually less variation in nuclear DNA. Second, mtDNA is maternally inherited (Avise 1991) making it essentially haploid in diploid individuals. This is useful in that heterozygotes do not need to be resolved. While some evidence exists that heteroplasmy does exist in insect mtDNA (Boyce et al. 1994), this is thought to be rare. MtDNA also seems to be more resistant to degradation than nuclear DNA and is therefore easier to extract and use in further applications (Avise 1991).

Within mtDNA, the cytochrome oxidase I (COI) gene has been shown to be useful in examining phylogenetic relationships between species, and has recently been suggested as a DNA-based identification system for species (Hebert et al. 2003). COII, another mtDNA cytochrome oxidase gene, has also been used in phylogenetic analysis and has similar rates of change as COI (Simon et al. 1994, Zakharov et al. 2004).

In insects, different regions of COI seem to evolve at different rates (Lunt et al. 1996), and the 3' end of COI seems to be slightly more variable than the 5' end. Also, genetic distances between the mtDNA of closely related species of *Pissodes* bark weevils appears to evolve much faster than in other insects (Langor and Sperling 1997).

Corroboration with another weevil group (Normark 1996) suggests that mtDNA might generally evolve faster in weevils. Based on population level work on other weevils (Normark 1996, Laffin et al. 2004), the high amounts of variation in COI make it an appropriate region for examining population structure in weevils of the genus *Ceutorhynchus*.

1.2 *Ceutorhynchus obstrictus* (Marsham): the cabbage seedpod weevil

1.2.1 North American introductions

The cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Marsham) is native to Europe, where it uses a variety of Brassicaceae as hosts. Also known by its junior synonym *Ceutorhynchus assimilis* (Paykull) (Colonnelli 1990, 1993), it was introduced to North America at least once in the past century.

In its native range, *C. obstrictus* occurs commonly in most areas that produce brassicaceous oilseed crops (Scarisbrick and Daniels 1986). Known in Europe since the early 1800s, *C. obstrictus* has become a major pest of oilseed crops in most regions (Scarisbrick and Daniels 1986). While it is unclear where in Europe this species originated, its range extends from England to Russia and the Mediterranean to Scandinavia, and it is now widespread in eastern and western Europe (Hill 1987).

In North America, *C. obstrictus* was first found in 1931 near Vancouver in British Columbia (McLeod 1953, Cárcamo et al. 2001). Since its discovery, *C. obstrictus* has been found in many locations throughout North America (McCaffrey 1992). *C.*

obstrictus has become established in the interior of British Columbia as far east as the Creston Valley (Cárcamo et al. 2001). From the Pacific Northwest, *C. obstrictus* is thought to have spread southward to California and eastward to Montana, and by 1946 was well established in those areas (Cárcamo et al. 2001). In the eastern United States, *C. obstrictus* has been established in Georgia, Alabama and Tennessee (Boyd and Lentz 1994). It is thought to occur throughout most of the continental United States, but is probably common only in regions producing brassicaceous oilseed crops or cole crops for seed.

C. obstrictus was first found in Alberta near Lethbridge in 1995 (Butts and Byers 1996). Since establishing itself in southern Alberta, the weevil has spread northward into central Alberta and eastward into Saskatchewan. Annual surveys have been undertaken to document changes in its distribution and abundance, and the CLIMEX model was used to predict that *C. obstrictus* could eventually inhabit the entire region of canola production in western Canada (Dosdall et al. 2002). *C. obstrictus* was also recently found in eastern Canada. It was first reported in Québec in 2000 (Brodeur et al. 2001) and in Ontario in 2001 (Mason et al. 2003).

The history of the *C. obstrictus* invasion of North America is not known. Most likely one or more adult females were transported across the Atlantic along with a shipment of seed, or perhaps in stored cole crops. One mystery is why *C. obstrictus* was first found on the Pacific coast rather than on the Atlantic coast, when the nearest founder populations were across the Atlantic Ocean. Since it is most commonly found in areas neighboring coastlines, one explanation could be that it was first transported to a site along the Atlantic coastal region of North America, and then was transported by land to

the Pacific coast. As for the more recent introductions to Alberta, Québec, and Ontario, it is most probable that *C. obstrictus* invasions either stemmed directly from Europe or more likely from neighboring regions that already had established populations of *C. obstrictus*.

1.2.2 Biology, damage, and control of *C. obstrictus*

Adults of *C. obstrictus* are small, about 3 to 4 mm in length, and are covered in fine white scales. Sexually immature adults overwinter along field edges, or under leaf litter in forested areas or shelterbelts (Carlson et al. 1951, Dmoch 1965). Overwintering sites may be quite distant from infested fields, as adults are strong fliers and are known to disperse long distances (up to 5 km) (Kjaer-Pedersen 1992). In early spring, adults begin to emerge from overwintering sites and disperse in search of hosts when soil temperatures rise above 15°C (Dmoch 1965). Overwintered *C. obstrictus* feed in spring on a variety of early-flowering brassicaceous hosts. In Alberta, *C. obstrictus* is commonly found infesting stands of flixweed (*Descurania sophia* [L.] Webb), hoary cress (*Lepidium draba* [L.]), stinkweed (*Thlaspi arvense* L.), wild mustard (*Sinapis arvense* L.), and volunteer canola (*Brassica napus* L. and *Brassica rapa* L.) (Fox and Dosdall 2003). Host choice is especially important as female ovaries develop at different rates, depending on the host they feed on (Fox and Dosdall 2003). Ovarian developmental rate is enhanced by feeding on racemes and pollen of plants capable of sustaining larval development (Ni et al. 1990; Fox and Dosdall 2003).

C. obstrictus generally does not invade canola until the bud stage and highest crop densities occur during flowering (Dosdall and Moisey 2004). Although canola is its primary host in western Canada, *C. obstrictus* also infests fields of other Brassicaceae such as cole crops and mustards (Brown et al. 1999, Dosdall et al. 2001).

Mated females lay eggs into immature pods, particularly those 40 to 50 mm in length (Dosdall and Moisey 2004). Deposition of the opaque, white, spheroidal eggs is preceded by exploratory and cavity formation behaviors, and followed by deposition of an oviposition deterring pheromone accompanied by brushing the abdomen along the outside of the pod (Kozlowski et al. 1983, Ferguson et al. 1999, Dosdall and McFarlane 2004). In some cases, when weevil densities are high, more than one egg may be deposited inside a single pod. Larvae develop within maturing pods and consume approximately 5 to 6 seeds during three larval instars (Cárcamo et al. 2001, Dosdall and McFarlane 2004). When ready to pupate, mature larvae chew an exit hole in the pod wall then drop to the ground and burrow into the soil. Pupation occurs within an earthen cell a few centimeters below the soil surface (Dosdall and McFarlane 2004). Adults emerge approximately 10 to 14 days after pupation (Dosdall and Moisey 2004). In western North America, pre-imaginal development of the cabbage seedpod weevil occurs more rapidly than in Europe (Dosdall and Moisey 2004), probably because of differences in crop phenologies between the two regions and the shorter growing season in North America.

Weevils from the overwintered generation gradually die off during the growing season; most have disappeared from fields by late July (Dmoch 1965). New generation adults continue to feed in fields of canola and on other brassicaceous hosts, accumulating fat reserves for overwintering. *C. obstrictus* is considered univoltine and is not known

to have multiple generations across its range. In early fall, adults disperse to overwintering sites and remain there until the following spring (Dmoch 1965).

C. obstrictus damages canola in both its larval and adult stages, and can greatly reduce yields (Dosdall et al. 2001). Larvae within seedpods do most economic damage. Developing larvae consume seeds within pods, and in outbreak years when weevil densities are high, multiple larvae within a single pod can consume all or most of the seeds (Cárcamo et al. 2001). Severe economic loss occurs even when only one larva is present per pod, as larval infestations can cause pods to dry out and shatter before harvest, causing all the seeds in those pods to be lost. This problem can be extremely prevalent in arid growing conditions like those in nonirrigated fields in southern Alberta.

Adults damage plants in several ways. First, when *C. obstrictus* adults migrate to canola fields in the spring, they feed on buds. Such feeding can destroy the vascular tissue supplying the buds, preventing further development, and can ultimately limit yield potential. Plants can compensate for early spring bud feeding by producing more buds, but only when growing conditions are favorable (Cárcamo et al. 2001), which is often an unlikely occurrence in the arid climate of southern Alberta. Canola is also damaged by new generation adults feeding late in the season through the pod walls, damaging seeds. This type of damage is of particular concern for seed producers, as damaged seeds show a lower rate of germination and a higher rate of abnormal seedling development than unaffected seeds (Buntin et al. 1995).

In addition to directly damaging canola, *C. obstrictus* can foster secondary damage by other insect pests. For example, in Europe, the pod midge, *Dasyneura brassicae* Winn. uses adult feeding holes on pods as sites for oviposition. Damage to

seeds becomes considerable when both insects infest a field, so in areas where both are present, control methods are employed at very low economic thresholds (Cárcamo et al. 2001). Feeding holes also allow access for other small insects to feed on seeds. In southern Alberta, thrips normally feed on the flowers, leaves, and outer pod walls, but have been found within pods consuming seed material, having entered via feeding holes made by *C. obstrictus* (Cárcamo et al. 2001). Holes caused by oviposition, adult feeding, or larval emergence can also leave plants vulnerable to infection by fungal pathogens (Dosdall et al. 2001).

Currently the only control strategy for the cabbage seedpod weevil involves spraying fields with chemical insecticide (Dosdall and Dolinski 2001). Chemical control is recommended when weevil density reaches 3 to 4 adults per sweep (Dosdall et al. 2001). Insecticide applications are recommended when adults are present in the field and crops are in early flower before adults have had a chance to oviposit and before pollinators are abundant. While chemical control can provide an effective means of reducing weevil populations, it does have drawbacks. Chemical control is generally not sustainable, as insects can develop resistance, and insecticides kill natural predators and parasitoids along with the pest species, as well as other beneficial insects present in fields.

Recent research on integrated management of the cabbage seedpod weevil has identified other potential control strategies, such as varietal resistance, trap cropping, and biological control by parasitoids (Dosdall et al. 2004). Kalischuk and Dosdall (2004) described the susceptibilities of different varieties and species of Brassicaceae to damage by *C. obstrictus*. Susceptibility varied greatly among species and varieties within

species. Further work on varietal resistance is underway, including crosses between resistant and susceptible varieties to develop hybrids potentially bearing genes for weevil resistance. If successful, this approach could provide the most sustainable control strategy for *C. obstrictus*.

The idea of using a trap crop to control *C. obstrictus* stems from the behavior of weevil adults to aggregate early in the season on stands of brassicaceous weeds and at field edges (Free and Williams 1979; Fox and Dosdall 2003). By planting early-flowering canola along strips at field edges, producers may attract large numbers of adult weevils that can then be controlled chemically. This would be more cost-effective and less environmentally damaging than spraying an entire field, as the areas sprayed would be much smaller, and weevils could be controlled before they entered the rest of the field to oviposit. The problem with this strategy is that *C. obstrictus* can disperse quite easily, so weevils from surrounding fields could still infest fields that had used trap cropping to reduce populations. In addition to trap cropping, the elimination of early flowering brassicaceous weeds could help reduce populations of *C. obstrictus* as suggested by Fox and Dosdall (2003) and Dosdall and Moisey (2004). In southern Alberta, weedy brassicaceous host plants are common early in the season, and control of these weeds sometimes does not occur until plants have already flowered. In addition, there are often large fields that are not cropped that can be heavily infested with these host weeds. Eliminating food sources of *C. obstrictus* early in the season could cause population levels to decline, as they need to feed soon after overwintering to replenish energy reserves.

In Europe and some parts of North America, adult and larval *C. obstrictus* populations are partially kept in check by a suite of hymenopterous parasitoids. Two species with some promise for weevil control in southern Alberta include *Microctonus melanopus* (Ruthe), a braconid that parasitizes adults, and *Trichomalis lucidus* (Walker), a pteromalid larval ectoparasite (Fox et al. 2004; Dosdall unpublished data). *M. melanopus* is a solitary endoparasitoid of adult *C. obstrictus*. The parasitoid attacks newly emerged adults late in the season, and overwinters as a first instar within the adult host. During the following spring, the parasitoid larva emerges from the adult weevil and pupates in the soil (Speyer 1925; Jourdheuil 1960). *T. lucidus* has been reared from *C. obstrictus* at several sites in western North America (Dosdall, unpublished data). It is also known from Europe (Noyes 2002). *Trichomalus perfectus* (Walker) and *Mesopolobus morys* L. are the principal chalcid parasitoids of *C. obstrictus* in Europe, where parasitism rates typically exceed 50%, can reach 90%, and be great even at low pest densities (Buntin 1998, Murchie and Williams 1998).

Recent work on *M. melanopus* suggests that while it may help control populations of *C. obstrictus* elsewhere in its range, it is not yet and may never be sufficiently abundant in southern Alberta to be an effective biocontrol agent (Fox et al. 2004). Currently, *T. perfectus* is being evaluated for release in Canada as a potential biocontrol agent (Kuhlmann et al. 2002). Whether it will be able to effectively reduce populations of *C. obstrictus* in Canada remains to be seen.

In addition to looking for parasitoids in southern Alberta, some attention should be given to other areas that will eventually be infested by *C. obstrictus*. For example, there may be parasitoid species found in the Peace River region that are currently

found on native species of Ceutorhynchinae that may be effective for controlling populations of *C. obstrictus*. Since the climate is so different in northern Alberta than in southern Alberta, it would not be surprising if other parasitoid species would be found there, or even if species ineffective at controlling *C. obstrictus* in southern Alberta, like *M. melanopus*, might be more effective in northern Alberta. Potential parasitoids could also be searched for in other newly invaded areas, like Ontario and Québec.

1.2.3 Importance of determining source populations of North American introductions

Research to identify European source populations for the North American introductions of *C. obstrictus* has not been undertaken previously. This is partly due to the difficulty in finding characters on which to base a comparison between populations in different regions. In other insects, even with wide geographic ranges, differences in morphological characters do not help in determining where introductions originated. In some species, regional differences in behavior, such as host plant associations, or pheromone types, may provide clues as to where introductions originated, but no such characters have been noted for *C. obstrictus*. Consequently, molecular markers were used to help in determining the origins of *C. obstrictus* in North America.

Determining source populations for North American introductions is important because it has implications for finding the most effective management strategies for *C. obstrictus*. For biological control agents, biotypes of the control agent need to be matched up with those found in the area of origin of North American introductions. Also, in other weevil species, varietal resistance of host plants has been shown to differ

among geographic regions (Tomlin et al. 1997). Management strategies that work well in specific regions of Europe could be matched with North American populations of *C. obstrictus*. If more than one introduction into North America occurred, then more than one management strategy may need to be developed for *C. obstrictus*.

1.3 *Ceutorhynchus neglectus* (Blatchley)

1.3.1 Biology

Ceutorhynchus neglectus (Blatchley) adults are small, only 1 to 2 mm in length, with red-brown legs and dark grey bodies covered in white scales. *C. neglectus* is commonly found on flixweed (*D. sophia*) in western North America. It is native to North America, with a transcontinental range (Anderson 1997). In the west it can be found as far north as Yukon, and as far south as Oregon, while in the east its range extends from Québec to Maryland. It also ranges southward along the eastern slopes of the Rocky Mountains as far as Colorado (Anderson 1997).

Originally thought to use *Polygonum* sp. as hosts (Blatchley and Leng 1916), *C. neglectus* has been found on a variety of *Descurania* species, *Rorippa islandica* (Oeder) Borbas (Anderson 1997), and canola, *B. rapa* and *B. napus* (Dosdall et al. 1999). It is noteworthy that although *C. neglectus* prefers flixweed as a host (Dosdall et al. 1999), its range is not as extensive in North America as that of its host. Flixweed is widespread throughout North America, but *C. neglectus* has a restricted range, especially in the

United States. One possible explanation is that flixweed was introduced from Europe, but *C. neglectus* has not yet expanded its range to match that of this host.

The life cycle of *C. neglectus* is similar to that of *C. obstrictus*. Adults oviposit in the distal region of pods (Dosdall et al. 1999). Developing larvae feed on seeds within pods and progress through three larval instars. Usually almost all seeds within infested pods are eaten. When larvae are mature, they chew a hole through the pod wall and exit, dropping to the ground. Larvae burrow about 2 cm into the soil, construct an earthen cell and pupate. New generation adults emerge to feed before finding overwintering sites. *C. neglectus* is considered univoltine, and overwintered adults probably die off before the next fall.

1.3.2 Importance of *C. neglectus*

Although *C. neglectus* can be found in fields of canola, it is only considered a minor pest of the crop in Canada (Dosdall et al. 1999). This is mostly due to its preference for other hosts, like flixweed, and because its feeding on canola seedlings does little to reduce host fitness.

C. neglectus can be a useful model for examining population structure and DNA sequence variability in a native weevil species, since it is a native species closely related in both biology and host plant requirements to the introduced *C. obstrictus*. It also invites comparison of population structure between invasive and endemic congeneric species. As an introduced species, *C. obstrictus* can be expected to have a population structure that reflects the characteristics of its North American founding population. Moreover,

if it spread from its point of introduction without human disturbance, its population structure may eventually be similar to that produced by any historical range expansion in native *C. neglectus*. Also, the range of *C. neglectus* is very similar to that of another weevil species, *Pissodes strobi* (Peck), and therefore may have some similar constraints on its distribution, for example temperature for overwintering survival.

Similar research on population structure and genetic variability has been conducted on *P. strobi* (Laffin et al. 2004). In *P. strobi*, extensive population structuring was found as well as high levels of genetic divergence between haplotypes. This may be a trait of mtDNA in weevils in general, but there has not been enough work on other groups of weevils to verify this hypothesis. By examining another native weevil with a similar range to *P. strobi*, I should be able to see whether mtDNA in weevils is more variable than in other insects, or if it is just a pattern common to *P. strobi* and perhaps a few others.

1.4 *Ceutorhynchus* phylogeny

1.4.1 Species of *Ceutorhynchus*

The genus *Ceutorhynchus* is very diverse with a worldwide distribution that comprises over 370 described species (Colonnelli 2004). In North America, species of *Ceutorhynchus* can be found almost anywhere, and over 80 species have been recorded (Colonnelli 2004). This genus contains many economically important species, both as

invasive species introduced accidentally that have become pests of crops, and as species introduced from Europe or Asia for classical biological control of weeds.

Ceutorhynchus species use a variety of hosts for feeding or oviposition. Most species with recorded biology have larval development occurring within plants, either within heads of developing flowers, within seedpods, or within the stems of maturing plants.

In a broader context, the genus belongs in the subfamily Ceutorhynchinae, and although phylogenetic work on this group is currently underway, there have been no molecular studies on the higher phylogeny of the subfamily published to this point. Almost no research of any kind on the relationships among species within this genus has been conducted. The placement of Ceutorhynchinae within Curculionidae is also unclear (Marvaldi et al. 2002).

1.5 Overview

This thesis contains two data chapters. The first deals with the potential source populations for the North American introductions of *C. obstrictus*, and the second describes population structure and mtDNA variation in *C. neglectus* as well as variation between different species within this genus.

Chapter 2 examines a 475 bp fragment of the COI gene for over 170 individuals in 16 localities across its native European and introduced North American range. Both nested clade analysis and AMOVA were used to determine historical processes

responsible for population structure, as well as to examine current levels of gene flow between populations.

In Chapter 3, a 790 bp fragment of the COI gene and a 117 bp fragment of the ITS1 region were sequenced in over 130 individuals from 23 localities across the range of *C. neglectus*, with extensive sampling in the west. ITS1 was used because it accumulates changes much more rapidly than mtDNA (Gallego and Galian 2001). Preliminary analysis of EF-1α (Cho et al. 1995), was used to determine if it also varied across the range of *C. neglectus*. Population structure and within-species sequence variation was examined for *C. neglectus* and compared to other groups.

Mitochondrial and nuclear DNA variation was also examined among available species within the genus *Ceutorhynchus*. A total of 2.3 kb of the mitochondrial COI and COII genes was sequenced, as well as 541 bp of the nuclear gene EF-1α, a gene primarily useful for genus level phylogenies because of its slower rate of change when compared to mtDNA (Cho et al. 1995). Parsimony analysis was used to resolve phylogenetic relationships for ten species of *Ceutorhynchus* and two outgroup species.

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Chapter 2: Population structure of the cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Marsham)(Coleoptera Curculionidae): Origins of North American introductions.

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2.1 Introduction

The cabbage seedpod weevil *Ceutorhynchus obstrictus* (Marsham), also known by the junior synonym *Ceutorhynchus assimilis* (Paykull) (Colonnelli 1990, 1993), is native to Europe, where it is a widespread, serious pest of canola or oilseed rape (*Brassica napus* L.). The species is oligophagous on Brassicaceae, with hosts that also include cole crops, mustards, and weeds (Cárcamo et al. 2001).

Adults of *C. obstrictus* overwinter beneath leaf litter, often in shelterbelts, and emerge in the spring to feed on early-flowering hosts (Dmoch 1965; Dosdall and Moisey 2004). In western Canada, adults migrate to canola when buds develop, and their feeding reduces flower and pod production (Dosdall and Moisey 2004). Eggs are deposited into immature pods, and most economic damage is caused by larvae consuming seeds within the siliques. Damage can be exacerbated by premature pod shattering which occurs more frequently in infested than noninfested pods (Dosdall et al. 2001). Mature larvae bore out of the pods and drop to the ground where they burrow down and pupate. New generation

adults emerge in late summer and feed on maturing pods, causing further loss of yield and crop quality (Dosdall et al. 2001).

In North America, *C. obstrictus* was first discovered near Vancouver in British Columbia in 1931 (McLeod 1953, Cárcamo et al. 2001). It is now widespread in interior British Columbia and the Pacific Northwest and is believed to have dispersed throughout most of the United States (McCaffrey 1992). *C. obstrictus* was first found in southern Alberta in 1995 (Cárcamo et al. 2001) and has since spread to central Alberta and western Saskatchewan (Dosdall et al. 2002). In eastern Canada, *C. obstrictus* was found in Québec in 2000 (Brodeur et al. 2001) and in Ontario in 2001 (Mason et al. 2004).

C. obstrictus is found throughout eastern and western Europe in regions producing oilseed crops, and has been known there since the early 1800s (Scarisbrick and Daniels 1986). The European source areas for the North American populations of *C. obstrictus* are unknown.

All North American populations originated either from independent introductions from Europe or by spreading from neighboring areas in North America previously colonized by the weevil. Unless new introductions occurred, the Alberta population likely originated from migrants from either British Columbia or Montana, and populations in Québec and Ontario may have originated from the eastern United States. Identifying the origins of North American populations has important management implications, because control strategies derived from the same source areas may be more effective than those which were not, particularly for the introduction of biological control agents (Mackauer et al. 1990).

Mitochondrial DNA (mtDNA) sequence has been used to elucidate taxonomy, population structure and phylogenetic relationships of other weevil species (Normark 1996, Langor and Sperling 1997, Laffin et al. 2004). One major advantage of using mtDNA is its relatively rapid evolutionary rate (Brown et al. 1979, Simon et al. 1994), which allows genetic differences to be detected between populations that diverged relatively recently. This is especially important for introduced species where variation in new areas might be low and differences have not had time to accumulate (DeSalle et al. 1987). There is also little within-individual variation in protein coding regions of mtDNA of one other weevil where this has been examined (Boyce et al. 1994), and individuals may be considered haploid because the marker is inherited maternally.

Generally, species with broad geographic ranges have substantial genetic variation (Avise 1994). However, for invading or introduced species, low variation might be expected due to founder effects and genetic bottlenecks (Nei et al. 1975, Hufbauer et al. 2004) because colonizing populations are often made up of only a few individuals that do not represent the range of genetic variability of source populations. In other curculionid species, mtDNA has been shown to be highly variable (Normark 1996, Langor and Sperling 1997, Laffin et al. 2004), so variation should be detectable in the native range of *C. obstrictus*. This variation should allow us to identify areas in Europe where the North American populations originated, and to estimate the origins of the populations in Alberta, Québec, and Ontario through comparison of sequence variation between populations in Europe and North America.

In this study I estimated levels of maternal gene flow and examined population structure across the range of *C. obstrictus*. I sequenced a 475-base pair fragment of

mtDNA from the cytochrome oxidase I gene, corresponding to part of a region previously examined in another curculionid (Langor and Sperling 1997) that showed high within-species sequence divergence. Sequences provided information that was used to identify areas with genetically distinct populations, as well as to uncover historical processes that were potentially responsible for current population structure. By using both AMOVA (Excoffier et al. 1992) and nested clade analysis (Templeton et al. 1995; Templeton, 1998; Templeton 2004) in tandem, I were better able to understand current and historical processes responsible for population structure than if either test was used alone (Althoff and Pellmyr 2002).

2.2 Material and Methods

Collections of *C. obstrictus* were made from 16 localities across North America and Europe (Fig. 2-1, Table 2-1). Adult weevils were collected between May 2002 and June 2003 by sweep netting stands of canola or brassicaceous weeds. Specimens were killed either by extended freezing at -20°C or by placing them in 70-100% ethanol, then storing them at -70°C before DNA extraction. Specimens were identified based on morphological characters and host plant association.

Mitochondrial DNA was extracted from 176 individuals using the QiaAmp DNA Mini Kit and eluted with 200μL of Buffer AE (Qiagen), and corresponding vouchers were placed in the Strickland Museum of Entomology at the University of Alberta. Extracted DNA was stored at -20°C prior to amplification via polymerase chain reaction (PCR). A 475-bp fragment of the mitochondrial gene, cytochrome oxidase I,

corresponding to nucleotide positions 2184-2658 of *Drosophila yakuba* mtDNA (Clary and Wolstenholme 1985) was amplified. Most PCR reactions were carried out in a reaction solution consisting of 35μL of double distilled water (Millipore), 5μL of 25mM $MgCl_2$ (Quiagen), 5μL of 10X buffer (Qiagen), 1μL of 10mM dNTPs (Roche), 1μL each of two 5pM/μL heterologous primers C1-J-2183 (5'-CAACATTTATTTTGATTTTTTG) (Simon et al. 1994) and C1-N-2659c (5'-ACTAATCCTGTGAATAAAGG) (modified from Simon et al. 1994), and finally 1μL of extracted DNA. In cases where initial amplification failed (<10%), the PCR reaction solution was altered by increasing the amount of extracted DNA by 2-5μL and decreasing the amount of double distilled water by a corresponding amount. DNA Taq polymerase was added as a final step before samples were placed into a Biometra T-Gradient PCR Thermal Cycler (Gottingen, Germany). The PCR thermal cycling program was as follows: an initial denaturation for 2 minutes at 94°C, followed by 34 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 45°C, and extension for 2 minutes at 72°C, then a final extension at 72°C for 5 minutes. Products obtained from PCR amplification were visualized on agarose gels to verify fragment sizes and assess quality, then cleaned using the QIAquick PCR purification kit (Qiagen).

Both forward and reverse strands were sequenced using the same primers as for initial PCR amplification. In a few cases, where sequencing reactions did not produce clean sequences, a larger PCR fragment was produced, with the 475-bp fragment nested within, using the heterologous primers C1-J-2183 and TL2-N-3014 (5'TCCAATGCACTAATCTGCCATATTA) (Simon et al. 1994). Sequencing reaction solutions consisted of 3.5μL of double distilled water (Millipore), 1μL of BigDye

terminator cycle sequencing mix, 3μL of 2.5X sequencing buffer (PE Applied Biosystems), 0.5μL of one of the three previously listed primers and 1μL of purified PCR product. The reaction program consisted of an initial step at 96°C for 1 minute, followed by 29 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Sequencing products were cleaned using Sephadex G-50 fine packed columns (Amersham Biosciences), and visualized on an ABI 377 automated DNA sequencer (PE Applied Biosystems).

Contiguous sequences were constructed using Sequencher 4.1 (Gene Codes Corporation 2001). No insertions or deletions were seen, so sequences could be aligned manually using Sequencher 4.1.

Nested clade analysis was conducted for only European samples and again for all samples. Nested clade analysis was completed using two programs: TCS 1.18 (Clement et al. 2004) and GeoDis 2.2 (Posada and Templeton 2004). TCS 1.18 was used to construct a network of haplotypes with 95% confidence limit for parsimony (Templeton et al. 1995), and a cladograph showing nesting structure of the haplotypes. GeoDis 2.2 was then used to calculate distances between populations where haplotypes or clades were found, based on the cladograph generated by TCS 1.18 and coordinates of sampling localities (Table 2-1).

AMOVA (analysis of molecular variance) and F_{ST} values were also calculated for the dataset using Arlequin 2.0 (Schneider et al. 2000) to estimate genetic variation between populations and groups of populations. To generate optimal groupings, SAMOVA 1.0 (Dupanloup et al. 2002) was used. Four different groups were identified, one made up of only the German samples, the second comprised of the Québec

population, the third of the Swedish and Russian samples and the fourth comprised of the remainder of the North American samples in addition to England and Finland.

2.3 Results

Eleven haplotypes were found in the 176 individuals sequenced (Genbank accession #AY692327-AY692337). Eight of the haplotypes occurred at single localities, leaving three haplotypes shared at more than one locality (Table 2-2). Most commonly, population samples were comprised of a single haplotype, as was the case in seven localities. All but one of these seven localities was North American, with the exception being the Lapinjarvi, Finland population. Three samples contained two haplotypes, five samples had three haplotypes, and one was comprised of four haplotypes. Three North American populations had more than one haplotype: Kelowna, British Columbia (two haplotypes), Hereward, Ontario (two haplotypes) and Quatre-Chemins, Québec (three haplotypes). Haplotype 1, which was the most common and consisted of 125 individuals, was found in all populations at frequencies ranging from 10-100%. Haplotype 6 was the second most common haplotype represented by 27 individuals. The other haplotypes were represented by 1-12 individuals, with four haplotypes found only once. Sequence divergence between haplotypes ranged from 0.21-1.05%, and overall nucleotide diversity for all haplotypes was 0.39%.

The haplotype network yielded by TCS 1.18 (Clement et al. 2004) had a maximum of five mutational steps between any two haplotypes, and only one missing haplotype (Fig. 2-2). The network had three clades: 1-1, 1-2, and 1-3. Clade 1-1

contains haplotype 1 which is found in all populations, clade 1-2 consists of haplotypes found only in Swedish and Russian populations, and clade 1-3 contains haplotypes found only in Germany.

Nested contingency analysis from GeoDis 2.2 (Posada and Templeton 2004) showed significant associations between clades and sampling locations only when testing all the samples (Table 2-3). However, only clade 1-1 and the whole network showed this significant relationship between geography and clades.

Using the clade distances generated by GeoDis 2.2 (Posada and Templeton 2004) and the GeoDis 2.2 inference key (Templeton et al. 1995, Templeton 2004), nested clade distance analysis of just the European samples gave three patterns from three clades (Table 2-4). The three patterns detected were restricted gene flow, contiguous range expansion, and long distance colonization and/or past fragmentation. When considering all samples, three patterns were also detected (Table 2-5). These patterns were allopatric fragmentation, past fragmentation and restricted gene flow with some long-range dispersal.

AMOVA detected significant structuring between the different populations (Table 2-6). Variation between populations within groups was lower than variation between groups, suggesting restricted gene flow among populations. Almost 72% of the variation detected was between the different groups of populations, and only 0.02% of the variation was between populations within the different groups. A significant F_{ST} value also supports the structuring of populations of *C. obstrictus*.

2.4 Discussion

Of the 11 haplotypes found in this study, only haplotype 1 occurred at all localities sampled. However, parsimony analysis suggests that it is not representative of the ancestral lineage, although the phylogenetic reconstruction was too poorly resolved to be conclusive (data not shown). In Europe, populations included individuals of haplotype 1 but were also made up to a large extent of individuals of other haplotypes. Unlike North America, only two of the European localities were predominantly haplotype 1, Rothamsted, England, and Lapinjarvi, Finland. Of the other European localities all except the Gottingen, Germany locality had similar haplotype frequencies. The German locality had two haplotypes that were not found elsewhere, and these made up the majority of those samples. Samples were collected primarily from northern Europe, and it is possible that additional haplotypes may exist in southern European populations. It is also possible that more extensive sampling would show that the haplotypes found in German populations are not as unique as indicated here.

Nested clade analysis of the European samples and of all samples inferred three different processes responsible for the current genetic structure of *C. obstrictus* populations. At the one-step clade level, past fragmentation and allopatric fragmentation was inferred, although when looking at the other one-step clade level, contiguous range expansion and past fragmentation were inferred (Table 2-4, Table 2-5). These inferences are not contradictory; instead they reinforce one another. Fragmentation of source populations would be inferred for the lower level clades because historically, before rapeseed crops were widespread, *C. obstrictus* would probably have occurred on randomly dispersed, temporary stands of brassicaceous weeds and mustards. Past gene

flow among populations is expected, making genetic structure unstable because small populations existing in a weed stand would need to disperse whenever that stand disappeared, leading to gene flow among multiple smaller populations. This would account for the contiguous range expansion seen in the nested clade analysis of European samples. *C. obstrictus* can disperse long distances (Ankersmit 1956; Kjaer-Pedersen 1992), so gene flow is expected between neighboring populations even if separated. Once production of rapeseed began on a broad geographical scale in the 17th century (Scarisbrick and Daniels 1986), *C. obstrictus* no longer had as great a need to disperse, and genetic structure of smaller populations might have stabilized as part of a larger population centered in areas of rapeseed production. This may have led to lower gene flow between populations.

Restricted gene flow was detected by nested clade analysis, potentially due to the lack of need for dispersal in agricultural areas, or to geographic barriers separating many of the populations. For populations in Europe, some populations are separated by water, mountain ranges, or geographic expanses where suitable host plants may not occur. For example, populations in Scandinavia probably mix with populations in the rest of Europe, excluding human-assisted transport, only by crossing the Baltic Sea at a narrow point between Denmark and Sweden or moving through northern Europe then heading southward over several generations. It seems less likely that individuals frequently cross large bodies of water, which probably serve as barriers restricting gene flow between populations. Passive dispersal using wind currents in Europe has been shown in other insect species such as the diamondback moth (*Plutella xylostella* [L.] Lepidoptera: Plutellidae) (French and White 1960), but it is unlikely that *C. obstrictus* disperses in

the same way. Unlike *P. xylostella*, *C. obstrictus* adults crawl down within the crop canopy when winds are strong (Ankersmit 1956). Long-range dispersal is indicated primarily because of the human assisted introduction of *C. obstrictus* to North America, but this inference would also explain why the population in Finland is so dissimilar to the populations geographically nearest to it, especially considering the long periods of time required for such an amount of genetic difference to build up (DeSalle et al. 1987). The fixed haplotype in Finland is most likely due to a founder effect from a relatively recent introduction.

AMOVA and nested contingency analysis also showed significant associations between haplotypes or clades and specific populations, and detected low levels of gene flow among different groups of populations. In the AMOVA analysis only 0.02% of total variation was between populations within the same genetically distinct group. Variation between different groups was much higher, and accounted for about 72% of total variation. This, along with a significant overall F_{ST} value, suggests that there are three genetically differentiated populations with reduced levels of gene flow between them. As previously stated, geographic barriers could at least partially explain restricted gene flow, but this could also be due to selection for short rather than long distance dispersal behavior when rapeseed production greatly increased and became concentrated in certain areas.

The differences between populations in Europe also allow us to make statements about population structure in North America. For all localities except Québec, populations were almost completely homogeneous and consisted of the same haplotype. The different genetic composition of the Québec population suggests that there have

been two separate introductions of *C. obstrictus* to North America. The oldest introduction, first reported in British Columbia in the 1930s (Cárcamo et al. 2001), most likely originated from a population genetically similar to those from Rothamsted, England or Lapinjarvi, Finland, since they are the only two European populations made up predominantly of haplotype 1. The current genetic diversity in western North America indicates that at least two individuals were introduced, since one individual of haplotype 4 was found at Kelowna, BC, although this haplotype could have been introduced later. The same can be said for haplotype 5 in Hereward, Ontario. Neither of these haplotypes were found in Europe, although they may be present at very low levels and were not detected. Though unlikely, they may even have originated independently in North America, because they differ from haplotype 1 by only one nucleotide. Between England and Finland, England is a more likely candidate as a source for the original introduction to North America because England has had continuous rapeseed production since the 1800s, while rapeseed was not cultivated in Finland until the 1950s (Scarisbrick and Daniels 1986), after *C. obstrictus* had been introduced to North America. It is conceivable that the similarity between Finland and England is due to an introduction from England to Finland shortly after Finland began cultivating rapeseed. This could also explain the dissimilarity between the Swedish and Finnish populations.

The Québec population represents a second introduction to North America, and is most similar to the population from Kolbeck, Sweden. Kolbeck is the only other sample to share all three of Québec's haplotypes. The difference in haplotype ratio between the two localities may simply be due to sampling effects, founder events or the limited sample that was assayed. The other Swedish locality or the two Russian localities

indicate that this second introduction could have originated anywhere within a broad region, because samples from these localities lack only one haplotype that may be present there but was not recovered in this study. I do not have information to say precisely where in North America this population was first introduced. The introduction to Québec could have originated directly from a European population, or from a neighboring area with subsequent dispersal to Québec. It does seem that the Québec and Ontario populations came from different source populations.

This study could only determine that the Alberta or Ontario populations originated in any of a broad range of areas of Europe, due to the low amount of variation in these populations as well as surrounding populations. More precise identification of European source areas would require further work. This could include using molecular techniques able to detect finer amounts of variation, such as AFLP or microsatellite analysis, further DNA sequencing, or more sampling in Europe.

Finding likely sources for the North American populations has important implications for developing integrated management practices for *C. obstrictus* in North America. First, because there have been multiple introductions, different regions of North America may need to implement different management practices if the genetic differences detected are representative of differences in phenology or susceptibility to parasitoids. If differences in phenology exist then researchers should look to the source populations for better management strategies for weevils in their areas, for example variability in host plant resistance in different plant lines has been shown for other weevil species (Tomlin et al. 1997). This means that for Alberta or Ontario, management practices that more closely follow those of England or regions with genetically similar

weevils such as Finland might be more effective, while Québec growers might benefit by more closely following those of Sweden or parts of Russia. It may also focus the search for biocontrol agents for possible release into North America. For example, the pteromalid *Trichomalus perfectus* (Walker) is a promising biocontrol agent of *C. obstrictus* that is currently under evaluation for potential release in Canada (Kuhlmann et al. 2002). It is widespread in its range throughout Europe (Szczepanski 1972, Laborius 1972, Murchie 1996), and this study shows that if approved for release, the best match of biotypes would be different for Québec than other regions of the country.

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Table 2-1. Collection localities of sampled *Ceutorhynchus obstrictus*.

Locality Number	Collection Locality	Lattitude	Longitude	No. of Specimens
1	Kelowna, British Columbia	49 56 N	119 29 W	12
2	Moscow, Idaho	46 47 N	116 54 W	10
3	Creston, British Columbia	49 05 N	116 35 W	10
4	Lethbridge, Alberta	49 41 N	112 47 W	12
5	Conrad, Montana	48 10 N	111 56 W	10
6	Madison, Alabama	34 41 N	86 44 W	14
7	Elora, Ontario	43 41 N	80 26 W	10
8	Hereward, Ontario	43 49 N	80 19 W	14
9	Quatre Chemins, Québec	46 36 N	71 04 W	11
10	Rothamsted, England	51 49 N	0 22 W	15
11	Kolbeck, Sweden	59 49 N	0 22 W	10
12	Gottingen, Germany	51 32 N	9 57 E	8
13	Oland, Sweden	56 85 N	13 30 E	10
14	Lapinjarvi, Finland	60 65 N	26 23 E	10
15	Adygea, Russia	45 01 N	38 58 E	10
16	Novoaleksandrovsk, Russia	45 29 N	41 13 E	10

Table 2-2. Distribution of haplotypes shown against localities, with locality numbers corresponding to those in Table 1, localities 1-9 are North American and 10-16 are European.

Haplotype	Locality															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	11	10	10	12	10	14	10	13	2	13	2	2	1	10	4	1
2										1						
3										1						
4	1								8				3			
5								1								
6									1		6		6		6	8
7																1
8												2				
9												4				
10											1					
11											1					

Table 2-3. Nested contingency analysis of geographical associations of all samples for COI sequence data from *Ceutorhynchus obstrictus*. A clade with no geographical variation is not shown.

Clade[a]	Permutational χ^2 statistic	Probability
1-1	123.91	0.031*
1-2	8.18	0.9170
Total	247.63	0*

*Significant at the 0.05 level
[a]From Fig. 2

Table 2-4. Demographic inferences from nested clade distance analysis (GeoDis 2.2 inference key) of European samples of mtDNA in *Ceutorhynchus obstrictus*.

Clade[a]	Inference chain	Inferred pattern
Haplotypes in 1-1	1-2-3-5-6-7-Yes	A
Haplotypes in 1-2	1-2-11-12-No	B
Whole network	1-2-3-5-6-13-14-No	C

[a]From Fig. 2

A= Restricted gene flow

B = Contiguous range expansion

C= Long distance colonization and/or past fragmentation

Table 2-5. Demographic inferences from nested clade distance analysis (GeoDis 2.2 inference key) of all samples of mtDNA in *Ceutorhynchus obstrictus*.

Clade[a]	Inference chain	Inferred pattern
Haplotypes in 1-1	1-2-3-4-9-10-Yes	A
Haplotypes in 1-2	1-2-3-5-15-No	B
Whole network	1-2-3-5-6-7-Yes	C

[a]From Fig. 2

A = Allopatric fragmentation

B = Past fragmentation

C = Restricted gene flow with some long range dispersal

Table 2-6. AMOVA results for tests of genetic divisions between populations of *Ceutorhynchus obstrictus*.

Source of Variation	Variance components	Percent of variation
Among regions	0.521	71.58
Among populations within regions	0.001	0.02
Within populations	0.207	28.40
Overall F_{ST}	0.71*	

* Significant at 0.05 level

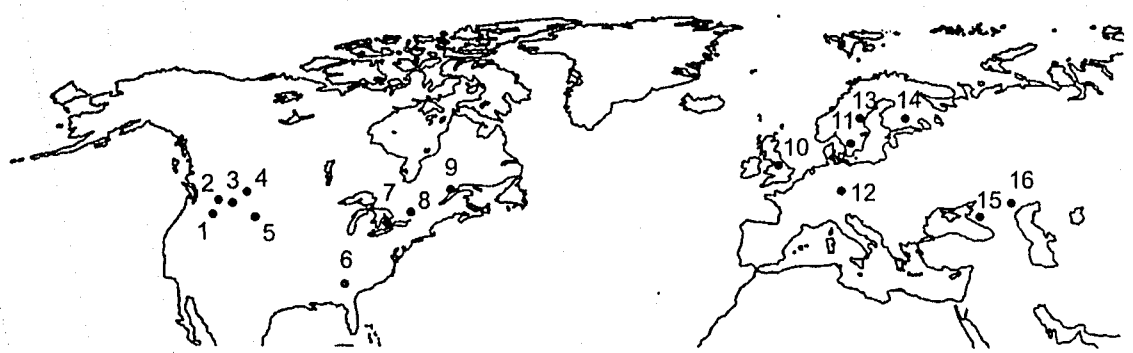

Figure 2-1. Study localities for *Ceutorhynchus obstrictus* in North America and Europe. Locality numbers correspond to those in Table 1.

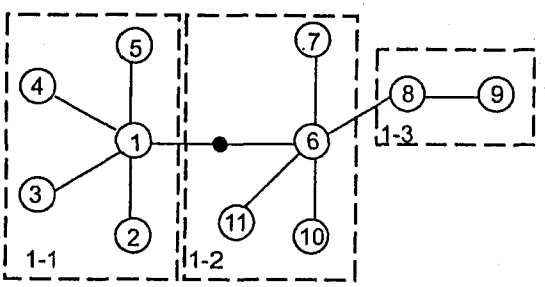

Figure 2-2. Haplotype network and nested clade design of the 11 haplotypes detected in this study. Numbers in circles represent haplotypes. Filled circle represents an intermediate hypothesized haplotype connecting observed haplotypes, and lines between haplotypes represent one-step mutational change. Dashed boxes represent one-step clades. Each clade is designated by a hyphenated number, the first indicating the number of steps within the clade.

Chapter 3: Population structure and phylogenetic relationships of *Ceutorhynchus neglectus* (Blatchley) (Coleoptera: Curculionidae)

*A version of this chapter has been submitted for publication. Laffin, Dosdall & Sperling. 2005. The Canadian Entomologist.

3.1 Introduction

Ceutorhynchus neglectus (Blatchley) (Coleoptera: Curculionidae) has a Nearctic distribution with a broad geographic range extending north to the Yukon and east to Québec and Maryland (Anderson 1997; O'Brien and Wibmer 1982). 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, 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 (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 (Dosdall et al. 1999) since it causes negligible damage and population densities are not high.

The broad geographic range of *C. neglectus* and overlap of its range with 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 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 geographic 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 were high and population structuring was extensive. The generality of this trait among weevils can be determined by comparison with other species across the same geographic range.

In this study, gene flow and population structure were investigated using nested clade analysis (Templeton et al. 1995; Templeton, 1998; Templeton 2004) and AMOVA (analysis of molecular variance) (Excoffier et al. 1992). By using these tests in tandem

(Althoff and Pellmyr 2002), I sought to elucidate current and historical processes responsible for population structure. I sequenced a 790 bp fragment of mitochondrial DNA (mtDNA) corresponding to the 3' end of cytochrome oxidase I (COI) gene which has been previously studied in weevils (Laffin et al. 2004, Laffin et al. 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 COI has been shown to have rapid rates of change in other weevil species (Normark 1996, Langor and Sperling 1997, Laffin et al. 2004). I also examined a fragment of nuclear DNA corresponding to the 5' end of the internal transcribed spacer region 1 (ITS 1) to examine population structure at a finer scale. I used ITS 1 because it accumulates changes at a high rate in other insects (Collins and Paskewitz 1998, Gallego and Galian 2001, Abe et al. 2005). It is a non-coding 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 species of *Ceutorhynchus* in order to better understand the phylogenetic relationships of *C. neglectus*. I sequenced DNA from 10 *Ceutorhynchus* species to construct a preliminary framework for phylogenetic work on this economically important but poorly studied genus. I 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). I used EF-1α because it generally has a slower rate of change and less

saturation of DNA substitutions, compared to mtDNA, and it also serves as an independent test of the phylogeny generated by mtDNA.

3.2 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 3-1, Fig. 3-1). A number of other localities were sampled without success for *C. neglectus* throughout Canada (including southern British Columbia, Saskatchewan, and Manitoba) and the U.S. (including the Pacific Northwest and the East Coast). It is likely that in those regions individuals or populations are rare or highly 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 either brought to the lab alive and killed by freezing at -70°C or killed by placing them in 95 to 100% ethanol; specimens were then stored at -70°C until DNA was extracted.

Whole genomic DNA was extracted from 138 individuals, 6 from each location, of *C. neglectus* using the QiaAmp DNA Mini Kit and eluted with 150μL of Buffer AE (Qiagen). Corresponding vouchers were placed in the Strickland Museum of Entomology at the University of Alberta after identification of a subsample was verified by an independent expert (P. Bouchard). DNA was stored at -20°C before PCR amplification. A 790 base pair fragment of the mtDNA gene COI was amplified and sequenced using the Jerry and Pat primers (Table 3-2). PCR reactions were carried out for each specimen in a reaction solution consisting of 35μL double distilled water

(Millipore), 5μL 25mM $MgCl_2$ (Qiagen), 5μL 10X buffer (Qiagen), 1μL 10mM dNTPs (Roche), 1μL each of the two 5pM/μL heterologous primers and 1μL of extracted DNA. Taq polymerase was added right before sample amplification using a Biometra T-Gradient PCR Thermal Cycler (Göttingen, Germany). The thermal cycling program had an initial denaturation of 2 minutes at 94°C and then 34 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 45°C, and extension for 2 minutes at 72°C followed by a 5 minute 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 3.5μL double distilled water (Millipore), 1μL BigDye terminator cycle sequencing mix, 3μL 2.5X sequencing buffer (PE Applied Biosystems), 0.5μL of one of the two previously listed primers and 1μL of purified PCR product. The sequencing reaction program had an initial denaturation at 96°C for 1 minute, followed by 29 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes, and was carried out using the same thermal cycler as for PCR amplification. Sequence products were cleaned using Sephadex G-50 fine packed columns (Amersham Biosciences), and then visualized on an ABI 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 individuals from across the range of *C. neglectus*, 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 (ITS1) region. PCR and sequencing reactions were

carried out as above except for the use of different primers and a different annealing temperature. For EF-1α, I used the Bo and Luke primers (Table 3-2), and an annealing temperature of 53°C. For the ITS1 fragment I used the ITS1 and ITS1b primers (Table 3-2) and an annealing temperature of 55°C. No variation was found in the 12 EF-1α sequences so no additional individuals were sequenced. In the ITS1 region I did find variation, so an internal primer was designed for the 5' end of ITS1.

The fragment of ITS1 used in this study used the new primer ITS1b which amplified a fragment that was 481 bp long. I 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 forward strands could be sequenced. To estimate haplotypes from the diploid sequences I used haplotype subtraction (Clark 1990, Xu et al. 2002). Since only one direction could be sequenced, heterozygotes were only scored 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 F_{ST} values were estimated with Arlequin 2.0 (Schneider et al. 2000).

In order 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 3-3). All specimens were collected between June 2002 and July 2004. I 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 six species where DNA quality was sufficiently high. Species were identified by P. Bouchard (Agriculture and Agri-Food Canada) and B.A. Korotyaev (Zoological Institute of the Russian Academy of Sciences). DNA extraction, PCR amplification, and sequencing were carried out using the methods described above and primers in Table 3-2.

Parsimony analysis and bootstrapping were performed with PAUP version 4.0 beta 9 (Swofford 2002) for each gene separately and combined. Exhaustive parsimony analysis was carried out to obtain the best tree. Bootstrapping values were calculated from 100 replicates. Percent divergence was also calculated using PAUP.

3.3 Results

Seven mitochondrial or nuclear gene haplotypes (Genbank #to be inserted here) were found in the 138 individuals of *C. neglectus* sampled (Table 3-4). COI 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, ON 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 was present as a homozygote in at least one specimen (Table 3-5). In all, 47% of individuals were homozygous. For the 53% that were heterozygous, 24% differed at one bp between alleles, 25% differed at 2 bp, and 4% differed at 3 bp.

Sequence divergence among haplotypes ranged from 0.9-3.4% and overall nucleotide diversity for haplotypes was 1.5%. All locality samples contained more than one haplotype: three localities had two haplotypes, six had three haplotypes, 12 had four, and two had five. Haplotype A was found most commonly and was the only one found at all localities. A total of 116 haplotype A alleles were found across all localities and comprised from 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, ON.

The haplotype network (Fig. 3-2) 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, ON, while clade 1-2 was comprised of haplotypes from all localities.

Nested contingency analysis of ITS1 sequences using GeoDis 2.2 (Posada and Templeton 2004) revealed significant associations between clades and sampling locations (Table 3-6). 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 3-7). 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.

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 3-8). 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 F_{ST} value was obtained using both the variance estimate (F_{ST} =0.41), as well as using traditional genotype frequencies under Hardy-Weinberg equilibrium (F_{ST} =0.38). This further supports the inference of restricted gene flow between the populations.

Parsimony analysis of the 2.3 kb mtDNA fragment from 10 *Ceutorhynchus* species and the two outgroup species yielded a single tree with a length of 1349 steps (Fig. 3-3). Of these, the species 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*, are quite divergent with respect to mtDNA.

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 which 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. 3-4) and the topology was the same as that found using only mtDNA for the same species. Combined data 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 3-9). For those species where both nuclear and mtDNA were obtained the genetic distance in mtDNA ranged from 3.6 to 13.8. On average, variation in COI/COII was about six times greater and ranged from 2.5 times to 22 times greater than in EF-1α based on comparisons where both sequences were available.

3.4 Discussion

Analysis of mtDNA in *C. neglectus* populations determined that the only locality that differed significantly from the others was Manotick ON. Even though sequence divergence between the haplotype from Manotick and 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 other localities in the study. This is not surprising, considering the geographic distance between Manotick and the next nearest study locality. 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* due to the greater variation in ITS1 sequences that I examined. The

difference between Manotick and other localities was less obvious than for mtDNA since Manotick shared haplotypes with other localities. Nevertheless, Manotick was the only population where the majority of haplotype copies were haplotype C, which was the least common haplotype found at 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 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 may be near each other could have different haplotype ratios because of founder effects at each temporary locality.

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

F_{ST} analysis supported nested clade analysis as significant F_{ST} values were found between many populations and the overall F_{ST} value was significant. Significant F_{ST} 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.

AMOVA detected two distinct genetic groups based on genetic distance and geography, and again this difference was between Manotick and the other localities sampled. Even without further sampling in central North America, it is clear that populations in the east and west are different, especially when considering the congruent results of ITS1 and mtDNA data. The genetic differences between populations most likely resulted from different founders dispersing to those areas after the Pleistocene deglaciation or more recently with the spread of flixweed across North America.

Phylogenetic analysis provided insight into relationships among the species of *Ceutorhynchus* that I 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 morphology, the two species appear superficially similar in some parts of their

range, but are quite divergent genetically. Even within the small number of *Ceutorhynchus* species I examined, they are clearly not each other's closest relative.

MtDNA divergences between species of closely related weevils are thought to be higher than in other insects (Langor and Sperling 1997, Normark 1996), and variation within some weevil species has also been shown to be higher than in other insects even across a very small geographic distance (Laffin et al. 2004). I 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 this study were similar to those found in other beetles (Jordal et al. 2000). It may be that mtDNA in weevils evolves at a relatively higher rate than in other species, 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). The high level of variation within *P. strobi* suggested that weevils generally have higher intraspecific divergences than other insects (Laffin et al. 2004), but further study of *Ceutorhynchus* weevils has shown that this is not correct. It is unclear whether only *P. strobi* has high intraspecific variation, or if other weevils also exhibit this trait even though *C. obstrictus* and *C. neglectus* do not.

Variation in mtDNA within and between *C. neglectus* populations is much lower than in other weevils across similar geographic distances (Laffin et al. 2004, Laffin et al. 2005). This may be due to a relatively recent expansion of *C. neglectus* into its current

range from regions where the species resided during Pleistocene glaciations when much of its range was covered by ice. However, the range of *P. strobi* overlaps greatly with that of *C. neglectus* and the two species may have had a similar biogeographic history, and so there appear to be other unknown processes acting on population structure in native species of Curculionidae.

3.5 Acknowledgements

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Table 3-1. Collection localities of sampled *Ceutorhynchus neglectus*.

Locality #	Collection Locality	Latitude	Longitude	# Specimens Sequenced
1	Whitehorse, YK	60 42 N	135 04 W	6
2	Dawson Creek, BC	55 45 N	120 13 W	6
3	Sexsmith, AB	55 20 N	118 47 W	6
4	Rycroft, AB	55 45 N	118 42 W	6
5	Fahler, AB	55 44 N	117 11 W	6
6	Peace River, AB	56 02 N	117 08 W	6
7	High Prairie, AB	55 34 N	116 48 W	6
8	Barrhead, AB	54 07 N	114 24 W	6
9	Edmonton, AB	53 29 N	113 32 W	6
10	Vegreville, AB	53 30 N	112 05 W	6
11	Vermillion, AB	53 21 N	110 47 W	6
12	Lloydminster, AB	53 07 N	109 36 W	6
13	North Battleford, SK	52 25 N	109 20 W	6
14	Millet, AB	53 05 N	113 33 W	6
15	Red Deer, AB	52 11 N	113 49 W	6
16	Macklin, SK	52 29 N	110 48 W	6
17	Olds, AB	51 48 N	114 05 W	6
18	Airdrie, AB	51 17 N	114 01 W	6
19	Lethbridge, AB	49 38 N	112 47 W	6
20	Medicine Hat, AB	49 52 N	110 58 W	6
21	Missoula, MT	46 58 N	114 17 W	6
22	Moscow, ID	46 43 N	117 00 W	6
23	Manotick, ON	45 13 N	75 41 W	6

Table 3-2. Primers used for PCR and sequencing.

Primer Name	Forward/ Reverse	Region	Location of 3' end	Original Reference	Sequence (5'-3')
K698	F	CO I	1460	Simon et al. 1994	TAC AAT TTA TCG CCT AAA CTT CAG CC
Ron	F	CO I	1751	Simon et al. 1994	GGA TCA CCT GAT ATA GCA TTC CC
K699	R	CO I	1840	Sperling et al. 1995	AGG AGG ATA AAC AGT TCA C/TCC
Jerry	F	CO I	2183	Simon et al. 1994	CAA CAT TTA TTT TGA TTT TTT GG
Nancy	R	CO I	2192	Simon et al. 1994	CCC GGT AAA ATT AAA ATA TAA ACT
Brian III	F	CO I	2495	Laffin et al. 2004	CCT CCT CTT TAT GAT CAA TTG G
K741	R	CO I	2578	Caterino and Sperling 1999	TGG AAA TGT GCA ACT ACA TAA TA
Mila	R	CO I	2659	Simon et al. 1994	GCT AAT CCA GTG AAT AAT GG
Geoish	F	CO II	2733	New	AAT AAG AAT TTT TTA TTT TAT
George	F	CO I	2792	Bogdanowicz et al. 1993	ATA CCT CGA CGT TAT TCA GA
Pat	R	CO I	3014	Simon et al. 1994	TCC AAT GCA CTA ATC TGC CAT ATT A
Pierre	F	CO II	3138	Simon et al. 1994	AGA GCC TCT CCT TTA ATA GAA CA
Marilyn	R	CO II	3389	Simon et al. 1994	TCA TAA GTT CAR TAT CAT TG
Marish	R	CO II	3425	New	TTT CAT CTA AAA TAT ATA ATA
Barbara	R	CO II	3494	Simon et al. 1994	GGT AAA ACT ACT CGA TTA TCA AC
Eva	R	CO II	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
ITS 1	F	ITS 1	0	White et al. 1990	TCC GTA GGT GAA CCT GCG G
ITS 1b	R	ITS 1	523	New	CAG GCC GAC CCG TCC GAA AAC
ITS 2	R	ITS 1	1183	White et al. 1990	GCT GCG TTC TTC ATC GAT GC

Note: positions relative to *Drosophila yakuba* (Clary and Wolstenholme 1985) for mtDNA, and *Heliothodes diminutivus* (Cho et al. 1995) for EF-1α.

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

#	Species	Genbank mtDNA #	Genbank EF-1α #	Collector	Collection Locality
1	*Ceutorhynchus obstrictus* (Marsham)	DQ058695	N/A	R.D. Laffin	Lethbridge, AB
2	*Ceutorhynchus subpubescens* (LeConte)	DQ058696	DQ058706	R.D. Laffin	Calgary AB
3	*Ceutorhynchus neglectus* (Blatchley)	DQ058697	DQ058707	R.D. Laffin	Lethbridge AB
4	*Ceutorhynchus erysimi* (Fabricius)	DQ058698	DQ058708	R.D. Laffin	Millet, AB
5	*Ceutorhynchus fallax* (Boheman)	DQ058699	N/A	B.A. Korotyaev	Pachyphragma, Turkey
6	*Ceutorhynchus gallorheanus* (Hoffman)	DQ058700	N/A	B.A. Korotyaev	Pachyphragma, Turkey
7	*Ceutorhynchus filirostris* (Reitter)	DQ058701	DQ058709	B.A. Korotyaev	Pachyphragma, Turkey
8	*Ceutorhynchus rapae* (Gyllenhal)	DQ058702	DQ058710	B.A. Korotyaev	Novoaleksandrovsk, Russia
9	*Ceutorhynchus cochleariae* (Gyllenhal)	DQ058703	DQ058711	B.A. Korotyaev	Adegea, Russia
10	*Ceutorhynchus querceti* (Gyllenhal)	DQ058704	N/A	B.C. Schmidt	Whitehorse YK
11	*Pissodes strobi* (Peck)	U77976	N/A	D. Langor	Swan Hills AB
12	*Omphalapion hookeri* (Kirby)	DQ058705	N/A	R.D. Laffin	Edmonton AB

Table 3-4. Distribution of haplotypes shown against localities, with locality numbers corresponding 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				

Table 3-5. 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

Table 3-6. Nested contingency analysis of geographical associations for ITS1 sequence data from *Ceutorhynchus neglectus*.

Clade	Permutational X^2 statistic	Probability
1-1	47.74	0*
1-2	88.57	0*
Total	40.22	0.007*

*Significant at the 0.05 level

Table 3-7. 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 3-8. 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 F_{ST}	0.41*	

* Significant at 0.05 level

Table 3-9. 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. gallorheanus	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	--

Below diagonal: 2.3 kb of COI + COII mtDNA
Above diagonal: 541 bp of EF-1α

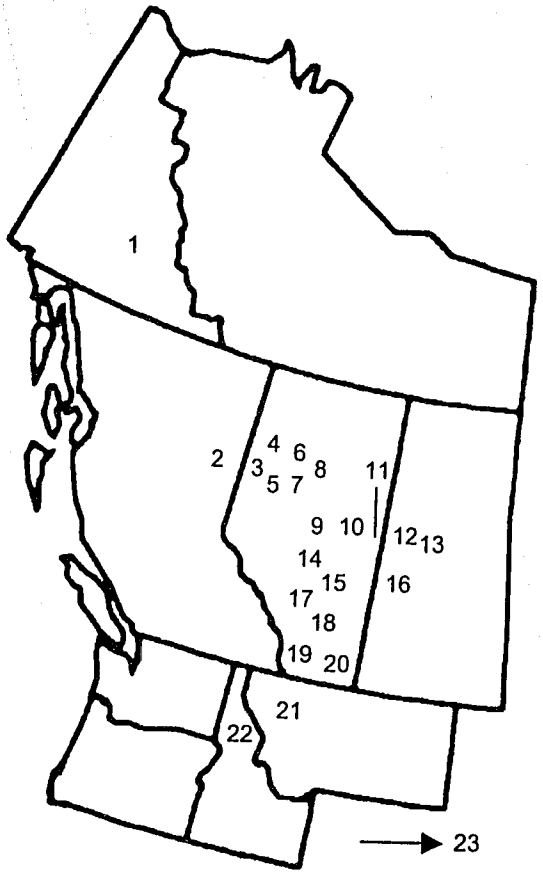

Figure 3-1. Collection localities of *C. neglectus*. Numbers correspond to localities in Table 1.

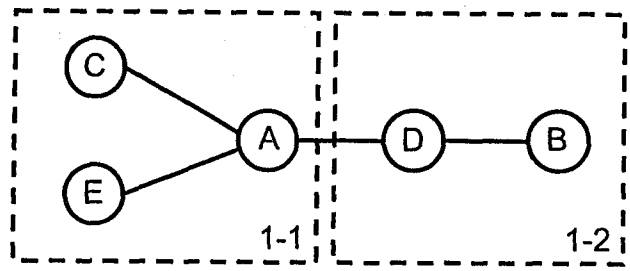

Figure 3-2. Haplotype network and nested clade design for 5 haplotypes detected in ITS1 sequences. Letters in circles represent haplotypes and lines between haplotypes represent one-step mutational change. Dashed boxes represent one-step clades.

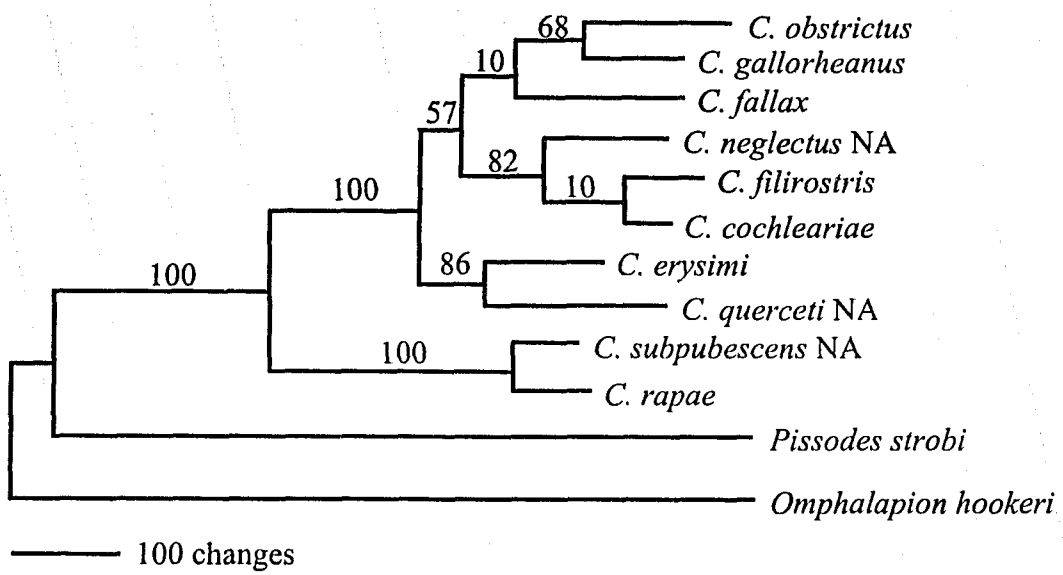

Figure 3-3. Phylogram of *Ceutorhynchus* spp. generated from PAUP exhaustive parsimony analysis of 2.3 kb of mtDNA (COI/COII regions). Bootstrap values are indicated on tree.

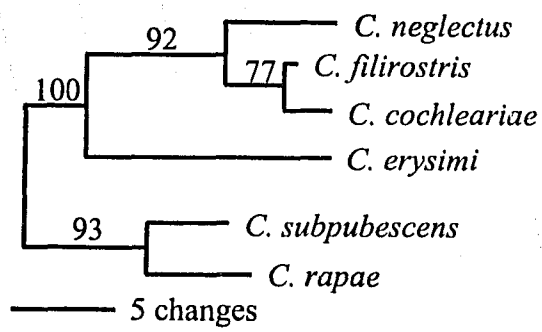

Figure 3-4. Phylogram of *Ceutorhynchus* spp. generated from PAUP exhaustive parsimony analysis of 541 bp of nuclear DNA (EF-1α). Bootstrap values are indicated on tree.

Chapter 4: Discussion and Conclusions

4.1 Discussion

Ceutorhynchus is a widely distributed genus of the beetle family Curculionidae which contains several economically important pest species, as well as some species used in classical biocontrol of weeds. In Chapter 2, an analysis of genetic variation in *Ceutorhynchus obstrictus* (Marsham), a recently introduced invasive pest of canola crops in Alberta (Cárcamo et al. 2001), was conducted in an attempt to determine the source population of the Albertan and other North American invasions.

The origins of the invasive population in Alberta could not be determined precisely as there was little or no variation among individuals of *C. obstrictus* across most of North America. But even though a specific source was not found for the Alberta population, there are two most likely scenarios for the introduction. It is possible that *C. obstrictus* migrated to Alberta from Montana in the south. This would have required adult weevils to disperse northward through an expanse of hundreds of kilometers where canola is not commonly grown and cruciferous weed stands are few and patchy in their distributions. Even though the flight and migratory capabilities of *C. obstrictus* should enable it to bridge these geographical distances (Kjaer-Pedersen 1992), a more likely scenario is that *C. obstrictus* was introduced from the Creston Valley, British Columbia, where it is known to have been established much longer than it has been in Montana (Cárcamo et al. 2001). This would still require dispersion of the weevil through an area where obvious host plants are not common, but the weevil could have been transported

by humans across the Rocky Mountains via the Crowsnest Pass and into southern Alberta. Human-assisted transport could explain the weevil invasion of Alberta from both Montana and the Creston Valley. Since *C. obstrictus* has been in the Creston Valley longer and transport from British Columbia to Alberta, especially of agricultural products, would occur more frequently than across the U.S.-Canada border, I believe the Alberta population probably originated from Creston. A less likely scenario for the Alberta invasion is an introduction from a geographically separate region. This would have also required human-assisted dispersal, but is less likely because the distance traversed would be much further than if the weevil was transported from Montana or Creston. Also, while the European populations contain the haplotype found in Alberta, the absence of other haplotypes in Alberta make most European sources unlikely.

Beyond Alberta, I found evidence for a second separate introduction of *C. obstrictus* into North America. The first time *C. obstrictus* was reported in North America was from near the west coast in British Columbia (McLeod 1953), and the species is now well established on the east coast of North America, in the southern United States (McCaffrey 1992). Almost all populations in North America are made up of the most common haplotype I found. Thus *C. obstrictus* may have traversed the North American continent from west to east, or another, undetected introduction occurred from a source population similar to that from which the first introduction was derived.

The second North American introduction from a different source population is indicated by genetic differences between *C. obstrictus* weevils from Québec and individuals found in the rest of North America. *C. obstrictus* was only recently found in Québec (Brodeur 2001) and was also found in Ontario for the first time only a year

later (Mason et al. 2003), yet they appear to be from distinct source populations. Populations in Ontario and Québec are presently geographically disjunct (Mason et al. 2003), but are probably expanding their ranges toward each other. Therefore it is fortunate that this study was conducted now, before the two populations merge and interbreeding obscures any genetic distinctions between the two populations.

The European sources for the two North American populations could not be determined precisely because there was insufficient genetic variation among the different sample localities in the study. Regardless, England was the most likely source for the first introduction of *C. obstrictus* to North America because England has had established oilseed production longer than Finland (Scarisbrick and Daniels 1986). Also, transport of goods between England and North America would have been more common than between Finland and North America. As for the introduction to Québec, there is no obvious explanation to help identify its European origin. While the sample from Québec is most similar to the samples from Sweden and Russia it is unknown which location is the most likely origin, or if there is another unsampled area in Europe from which the Québec invasion originated.

Chapter 3 dealt primarily with the population structure of *Ceutorhynchus neglectus* (Blatchley) and also included a small phylogenetic analysis of 10 of the described species of *Ceutorhynchus*. For *C. neglectus*, only two distinct groups of populations were found among all localities examined, and both were detected by sequencing mtDNA and ITS1. The difference between east and west in *C. neglectus* could be explained because they came from one source and are different due to founder effects, or because they stemmed from two different source populations not sampled

in this study. The variation among the two different *C. neglectus* populations is considerably less than that observed for two other weevil species that have been examined in detail. In its native range, *C. obstrictus* has much greater genetic variation over similar geographic distances. This low level of genetic variation in *C. neglectus* could be partly due to the transient nature of flixweed, its primary host. Since flixweed generally occurs in small stands that may not be present year after year, constant range expansion into areas where flixweed appears could create a founder effect every time *C. neglectus* reenters an area. Under these conditions it is possible that only the most common haplotype would generally survive, which could explain why I found no difference in mtDNA of *C. neglectus* throughout western North America. Because nested clade analysis inferred both contiguous range expansion and restricted gene flow it is unclear whether or not the lack of variation is partially due to host plant availability. Continual founder effects could actually also preserve variation as long as gene flow between subpopulations occurs (Nurnberger and Harrison 1995).

Variation observed in *C. neglectus* was also considerably less than that found in *Pissodes strobi* (Peck) (Laffin et al. 2004). This was unexpected since the ranges of both species are similar (Anderson 1997, Laffin et al. 2004) and both are native to North America. Both species would have dispersed into their current ranges after the Pleistocene deglaciation, so it is likely that the greater genetic variation seen in *P. strobi* is at least partly due to a greater genetic diversity in its source population.

Because of the similarities between *C. obstrictus* and *C. neglectus* (Dosdall et al. 1999, Dosdall and Moisey 2004), I had thought that the eventual population structure of *C. obstrictus* could be estimated by examining the current population structure of *C.*

neglectus. However, this is unlikely to be the case, because of differences in the distributions and abundances of host plants between the two species. Even though each weevil species can occur on the primary host of the other, the population structure of *C. obstrictus* is not likely to mirror that of *C. neglectus* because the availabilities of flixweed and canola are so different in most areas.

The higher level phylogenetic analysis of *Ceutorhynchus* provided two discoveries of interest. First, the phylogenetic separation of the two species with stem-mining larvae from the other species in this study suggests that a stem-mining larval lifestyle may have only evolved once in this genus. However, more species need to be examined, and the lifestyles of more basal species within this genus need to be determined to validate this idea. The phylogenetic analysis also revealed an unexpected genetic divergence between two species, *C. neglectus* and *C. querceti* (Gyllenhal), whose morphologies are superficially quite similar in the eastern part of their range

It is unfortunate that there has not been more research conducted on the population structure of *Ceutorhynchus* species as well as on relationships among different species of *Ceutorhynchus*. Considering that there are so many species that are either pests of crops or have been investigated as potential classical biocontrol agents of introduced weeds, it is probable that substantial benefits can arise from additional research on these taxa. If variation exists within other species of the genus, as in the case of *C. obstrictus*, then different management strategies may be more or less effective in different geographic regions. This would potentially include the introduction of different parasitoids that are successful at controlling populations in different areas.

Any management strategy for pest species should also take into account the effects on closely related species, so that the impact on non-target species can be minimized. For example, if releases of European parasitoids are considered for North America to enhance biological control of *C. obstrictus*, the impact of the candidate parasitoids should be evaluated for closely related endemic weevils. It would be difficult to conduct assessments of closely related species because phylogenetic relationships remain unresolved for most species of *Ceutorhynchus*.

This phylogenetic analysis should provide a useful framework for further studies on this genus. Future studies could easily incorporate more species and investigate other regions of the genome than those examined in this thesis. These molecular analyses can then either validate or refute some of the ideas proposed in this thesis.

The two population structure studies reported in this thesis could probably be examined at a finer scale using different techniques than the ones used so far. Even though microsatellite analysis could not be completed for *C. obstrictus* due to difficulty in developing markers, it may be possible to develop microsatellites for both *C. obstrictus* and *C. neglectus* if more resources were available for this work. Additionally, AFLP analysis might be used to develop markers to differentiate populations. With more markers and wider population sampling, it may be possible to pinpoint the exact sources of the North American introductions as well as the origins of recent Alberta and Ontario introductions.

Eventually, the genetic variation I detected in *C. obstrictus* in North America may relate to a difference in response to different control strategies. In this case, other researchers could use this information to help develop a more precise suite of

management strategies for the different populations that have invaded North America, whether by introducing different parasitoids or using different varieties of canola. Thus the most important findings of this thesis may be its potential to impact the economic and environmental sustainability of canola production in regions where cabbage seedpod weevil infestations are causing, or have the potential to cause, economic losses to producers.

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