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Merrill A. Peterson

Western Washington University, merrill.peterson@wwu.edu


Susanne Dobler

Jeff Holland

Lauren Tantalo

Stefanie Locke

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Behavioral, Molecular, and Morphological Evidence for a Hybrid Zone Between *Chrysochus auratus* and *C. cobaltinus* (Coleoptera: Chrysomelidae)

MERRILL A. PETERSON, SUSANNE DOBLER,¹ JEFF HOLLAND, LAUREN TANTALO, AND STEFANIE LOCKE

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ABSTRACT In this article, we describe a hybrid zone between the chrysomelid beetles, *Chrysochus auratus* (F.), and *C. cobaltinus* LeConte, which have historically been considered as having allopatric distributions. By combining field studies with surveys of museum specimens, we documented that in western North America there are two regions in which these beetles are sympatric, and four additional regions in which populations of the two species are <100 km apart. In south-central Washington, we found an ≈25 km wide area of sympatry in which the two species freely interbreed. Morphological and allozyme differences between the species allowed us to demonstrate that individuals with intermediate coloration in this area are indeed hybrids; all 22 putative hybrids we assayed for allozyme variation were heterozygous at each of three species-specific loci. Museum specimens revealed that the two species have been hybridizing in this region at least since 1952. Within the hybrid zone, ≈10–15% of the beetles is apparently F₁ hybrids. At one focal site, 22.9% of all matings involved heterospecific pairs and 20.8% of all matings involved at least one hybrid individual. Although we found no molecular evidence of introgression between the two species, morphometric results and preliminary ecological data suggest possible past introgression or weak ongoing introgression. We discuss the implications of our findings for the specific status of these two species. This system appears well suited to provide answers to long-standing questions concerning the evolution of premating barriers between hybridizing species. In addition, hybridization between these two beetle species with differing host ranges will allow us to test the hypothesis that ecologically significant traits such as diet breadth can be gained via introgression.

KEY WORDS allozymes, hybridization, mating, morphology

THE CHRYSOMELID BEETLES *Chrysochus auratus* (F.) and *C. cobaltinus* LeConte (Eumolpinae) are the sole North American representatives of this small (10 spp.) Holarctic genus (Arnett 1968, Lopatin 1984). Recently, these conspicuous beetles have been the focus of ecological and evolutionary studies of dispersal (Williams 1992, Sady 1994, Dickinson 1995), mating behavior (Dickinson 1995), feeding behavior (Dussourd and Eisner 1987), diet breadth (Dobler and Farrell 1999), and defensive chemistry (Sady 1994, Dobler et al. 1998), providing detailed documentation of their natural history.

As is typical for the genus, North American *Chrysochus* feed on dogbane plants (Apocynaceae) and the closely related milkweeds (Asclepiadaceae) (Sennblad and Bremer 1996). Specifically, *C. auratus* feeds exclusively on plants in the Apocynaceae, including *Apocynum cannabinum* L. and *Apocynum androsaemifolium* L. (Weiss and West 1921; Dussourd and Eisner 1987; Williams 1991, 1992; Dobler and Farrell 1999). Although there have been reports that this species occurs occasionally on milkweeds (Asclepiadaceae) (Weiss and West 1921), both choice and no-choice

experiments have shown that *C. auratus* from Utah, Colorado, and Georgia are unwilling to eat milkweeds (Dobler and Farrell 1999). In contrast, laboratory and field populations of *C. cobaltinus* readily eat both *Apocynum* spp. and milkweeds, including *Asclepias speciosa* Torr. and *A. eriocarpa* Benth. (Sady 1994, Dickinson 1995, Dobler and Farrell 1999). Larvae of both species are obligate root feeders, and adults eat the leaves of larval host plants. Both species produce a single generation per year, with adults emerging in early summer and persisting in host plant patches for approximately six weeks (Williams 1992, Dickinson 1995). Behavioral and population-genetic studies indicate that *C. cobaltinus* is very sedentary, with considerable population-genetic subdivision (Dickinson 1995, Dobler and Farrell 1999; unpublished data). In contrast, *C. auratus* is more vagile, and therefore features genetic homogeneity over large spatial scales (Williams 1992, Dobler and Farrell 1999).

Within most host plant patches that they occupy, both *Chrysochus* species are fairly abundant (>0.2 adults/ramet) (Williams 1992, Dickinson 1995; unpublished data). Adults of both species are distinctively colored (*Chrysochus auratus* is metallic golden-green, whereas *C. cobaltinus* is deep cobalt blue [Hatch 1971]), perch in prominent locations on host plants, and are

¹ Universität Freiburg, Institut für Biologie I, 79104 Freiburg, Germany.

lethargic compared with many beetle species, a combination of traits suggestive of aposematism (Root and Chaplin 1976). Because both species are known to sequester cardenolides obtained from host plants, it is likely that they are indeed unpalatable to many predators (Isman et al. 1977, Sady 1994, Dobler et al. 1998).

The combination of their abundance, moderately large size (6.8–11.3 mm length [Hatch 1971]), and apparent aposematism has resulted in extensive documentation of the distributions of both species by collectors. In general, *C. auratus* has an eastern North American distribution, spanning the eastern United States and adjacent southern Canada, west to the Rocky Mountains (Arnett 1968, Dobler and Farrell 1999). At the western edge of its range, *C. auratus* extends west of the Rocky Mountains into Utah and Arizona (Dobler and Farrell 1999), as well as having populations in south-central British Columbia and the Columbia Basin of central Washington and northern Oregon (Hatch 1971). In contrast to the primarily eastern distribution of *C. auratus*, *C. cobaltinus* is found exclusively in western North America, ranging from southern California north to southern British Columbia, and east to Utah and western Montana (Dobler and Farrell 1999). To date, published accounts of these two species have not documented their co-occurrence, in spite of the extensive collections of each species.

We discovered an area along the Yakima River valley in central Washington state in which *Chrysochus auratus* and *C. cobaltinus* not only are sympatric but also engage in apparent interspecific copulation. In this article, we present behavioral, morphological, and genetic evidence that this region represents a hybrid zone (sensu Barton and Hewitt 1989, Harrison 1990) between these two beetle species.

Hybrid zones have been hailed by evolutionary biologists as "windows" through which evolutionary processes that are difficult to study may be observed (Harrison 1990). For example, hybrid zones hold tremendous promise for studying the evolution of reproductive barriers between species (Howard 1993, 1998). In addition, there has been a growing emphasis on using hybrid zones to determine the degree to which species acquire evolutionary innovations via adaptive introgression (Arnold and Emms 1998). Thus, the *Chrysochus* hybrid zone described herein provides exciting opportunities for resolving long-standing questions in evolutionary biology.

Materials and Methods

Assessment of Distributional Overlap. To document the spatial extent of overlap of *Chrysochus auratus* and *C. cobaltinus*, we sampled beetles in 30 patches of *A. cannabinum* along a 120-km stretch of the Yakima River from Ellensburg to Whitstran, WA. Patches of this plant are easy to locate in this arid region, because the growth form and color of the plant are distinctive in comparison to the riparian and sagebrush-steppe plants with which it grows (M.A.P., unpublished data). Because *A. cannabinum* is located near water sources in central Washington, we con-

centrated our efforts along the Yakima River as well as along streams, ponds, and drainage ditches near the Yakima River. In sampling each patch, we noted the identities of any *Chrysochus* adults present in the patch, and collected individuals for later genetic analyses. These latter individuals were kept alive until we stored them in the laboratory at -80°C . We sampled patches in 1998 and 1999 from mid-June through late July, a period spanning the time of peak adult activity in central Washington (unpublished data).

In addition to our survey of central Washington populations of *Chrysochus*, we obtained >8,000 specimens of *Chrysochus auratus* and *C. cobaltinus* from 13 North American entomological collections (American Museum of Natural History, California Academy of Sciences, Natural History Museum of Los Angeles County, Oregon State University, Simon Fraser University, National Museum of Natural History, University of Alberta, University of British Columbia, University of California–Berkeley, University of California–Davis, University of California–Riverside, Washington State University, and Western Washington University). Our purpose in using these specimens for analyzing the species' distributions was threefold. First, collections from the Pacific Northwest enabled us to document the extent of distributional overlap in the Columbia Basin of Washington and Oregon in more detail. Second, because many of the specimens in these regional collections were from collecting expeditions taken nearly a century ago, we were able to determine the minimum amount of time that these two species have occurred in sympatry. Finally, we used all of the collections to obtain distributional data for both species for the purpose of identifying other regions in western North America in which the distributions of the two species overlap.

Do *Chrysochus auratus* and *C. cobaltinus* Hybridize? To determine if *Chrysochus auratus* and *C. cobaltinus* indeed hybridize, we focused detailed behavioral, morphological, and allozyme studies on beetles in a patch of *A. cannabinum* located 9 km WNW of Mabton, WA, in a drainage ditch along Hwy 22 (population WA6 in Fig. 1). This patch contained large populations of each species and was the patch in which we had first noted apparent interspecific copulation.

On 21 July 1999, we determined the relative abundance of the two species in this patch, as well as the frequency of phenotypically intermediate, putative hybrids (see *Results* for a description of the putative hybrids). To do this, we sampled 699 *Chrysochus* adults on haphazardly selected plants, noting the identity of each beetle encountered, and taking care to avoid sampling the same part of the patch more than once. During this sampling, we also determined the identities of the individuals in all mating pairs we found, so that we could estimate the relative frequency of intraspecific matings, interspecific matings, and matings involving putative hybrids. *Chrysochus* individuals exhibit a form of postcopulatory mate guarding in which males ride the backs of females for extended periods after copulation. As a result, the entire duration of mating typically lasts >1.5 h (Dickinson 1995). In addition, males and females both mate multiple times

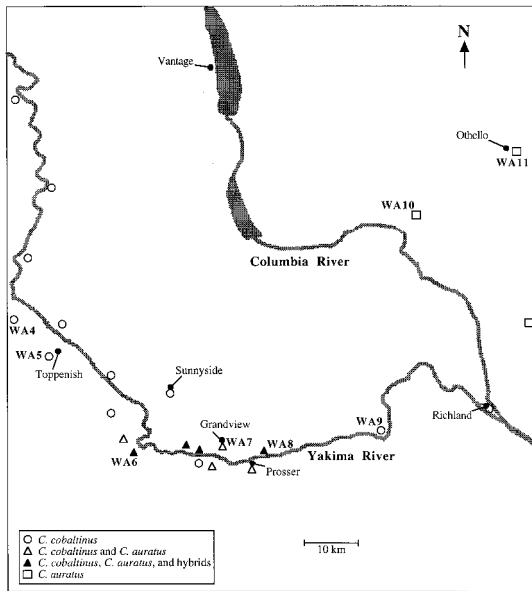


Fig. 1. Locations of *Chrysochus* populations along the Yakima River valley of south-central Washington where the distributions of *C. auratus* and *C. cobaltinus* overlap. One of the four locations from which hybrids have been recorded (WAS) is based on specimens in museum collections. The remaining three sites of known hybridization (WA6 and the two sites ≈ 5 km W of Grandview) are sites at which we observed hybrids. Site WA6 was the focal patch for our studies of hybridization. Sites with codes (WA4–WA11) indicate host plant patches from which we obtained specimens for morphometric or allozyme comparisons. Descriptive locations for all coded populations are listed in the text.

during their lives, averaging one mating per day (Dickinson 1995). Therefore, at any time, a large proportion of individuals in a population are mating, enabling one to quickly observe many matings in a population in a short period. It should be noted that although it is possible to distinguish males and females by opening their genitalic aperture, this is difficult to accomplish in the field. Thus, because males will occasionally mount males and females will occasionally mount females (M.A.P., unpublished data), it is possible that we erroneously categorized a small fraction of observed pairings as matings. However, the rarity of such same-sex mountings makes it unlikely that such errors would significantly alter our results.

To determine if hybrid individuals occurred in this patch, we performed morphological and genetic comparisons of individuals conforming to the typical coloration of *C. cobaltinus* and *C. auratus*, as well as putative hybrids (individuals that were intermediate in coloration). Typically, the flagellomeres (annuli) of *C. auratus* antennae are relatively long and narrow compared with the flagellomeres of *C. cobaltinus* (Hatch 1971). To determine if hybrids were intermediate for this species-specific morphological trait, we determined the length:width ratio of the 8th flagellomeres of 39 individuals of *C. auratus*, 34 individuals of *C. cobaltinus*, and 23 putative hybrids, all taken from

the focal patch. As with other flagellomeres, the 8th flagellomere length:width ratio of *C. auratus* is greater than that of *C. cobaltinus*, and this ratio does not vary with body size in either species (M.A.P. and J.H., unpublished data). To determine the length:width ratio of the 8th flagellomere for each individual, we digitized magnified ($80\times$) images of the flagellomeres using the analytical software, NIH Image (<http://rsb.info.nih.gov/nih-image/>). We determined the length of the flagellomere along its central axis and the width of the widest point of the flagellomere. We compared the length:width ratios of the three beetle color morphs (*auratus*, *cobaltinus*, putative hybrids) using analysis of variance (ANOVA), comparing means with least significant difference (LSD) multiple comparisons tests (SPSS 1999). We hypothesized that the putative hybrids would be morphologically intermediate to *C. auratus* and *C. cobaltinus*.

To place the length:width ratios for each species into a broader geographical context and to assess whether either species has experienced introgression, we also determined these ratios for several populations of each species located outside of the putative hybrid zone. The locations of populations used for this analysis (*C. auratus*: BC2–Kamloops, B.C.; WA1–Malott, WA; WA6–9 km WNW Mabton, WA; WA8–Whitstran, WA; WA10–Wahluke Wildlife Area, WA; WA11–Othello, WA; OR1–Umatilla, OR; MT2–Teton R., MT; AZ–Manzanita Camp, AZ; NM–Albuquerque, Bernalillo, and Silver City, NM; WI–Clintonville, Cloverleaf, Oconomowoc, Trout Lake, and Vilas Counties, WI; IL–Cook County and Lincoln Trail St. Park, IL; MO–Ft. Leonard, KS, Bridge St. Park, Jackson County, Jasper County, Jefferson County, and Moberly, MO; PA–Harrisburg, PA; NY–Flatbush, NY; *C. cobaltinus*: BC1–Creston, B.C.; WA4–Wapato, WA; WA5–Toppenish, WA; WA6–9 km WNW Mabton, WA; WA7–Grandview, WA; WA9–Benton, WA; WA12–Eastsound, WA; WA13–Vila, WA; OR2–Rowena, OR; OR3–Salem, OR; OR4–Ashland, OR; ID2–Parma, ID; MT1–24 km NE of St. Regis, MT; UT1–Salt Lake City, UT; NV2–Yerington, NV; CA2–Big Bar, CA; CA3–Davis, CA; CA4–Yosemite, CA; CA5–Dos Palos, CA; CA6–Bishop, CA; CA7–Fresno, CA; CA8–Terra Bella, CA; *C. auratus* \times *cobaltinus*: WA6–9 km WNW Mabton, WA) are indicated in Figs. 1 and 2. Due to the limited number of museum specimens from New Mexico, Wisconsin, Illinois, and Missouri, our estimates of length:width ratios for those states are based on a compilation of specimens taken from the above-listed sites. All other length:width ratios were based on samples taken from a single location. We compared flagellomere length:width ratios among species and among populations within each species using ANOVA (SPSS 1999). As an assessment of whether hybridization has led to introgression, we regressed length:width ratios for populations of each species against the distance to the nearest known population of the other species. Evidence consistent with introgression would be a tendency for length:width ratios in a species to be more similar to the other species as the distance separating populations of the two species decreased.

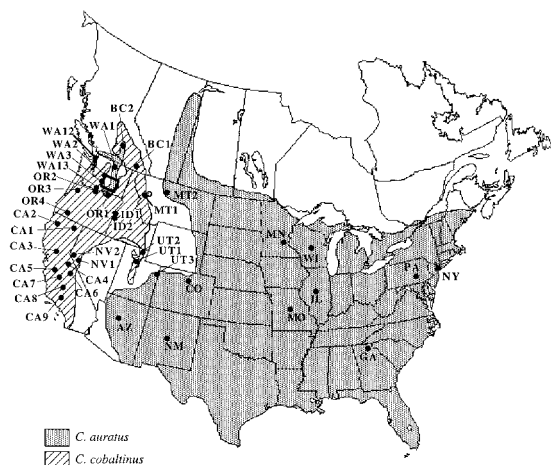


Fig. 2. Distribution of *C. auratus* and *C. cobaltinus* in the United States and Canada, based on the literature and our surveys of museum specimens. Distributions of these two species overlap in south-central British Columbia, and south-central Washington/north-central Oregon. In addition, the ranges of these beetles come into close proximity in Utah, western Montana, north-central Washington, and northwest Washington/southwest British Columbia. The box in central Washington indicates the region that is shown in detail in Fig. 1. The locations of populations used for morphometric or allozyme surveys are indicated with population codes. Descriptive locations for these populations are listed in the text. The map positions for sites in New Mexico, Wisconsin, Illinois, and Missouri are not intended to indicate actual locations of sites, as our samples from these states were compilations of individuals across multiple sites. For all other sites, map positions do indicate the actual positions of populations.

To supplement our morphological analyses of hybridization, we performed allozyme electrophoresis on individuals from the focal population, as well as

pure populations of each species from central Washington and other parts of the ranges of each species (Table 1). The locations of these populations (*C. auratus*: WA6–9 km WNW Mabton, WA; WA8–Whitstran, WA; WA10–Wahluke Wildlife Area, WA; WA11–Othello, WA; UT3–Dinosaur National Monument, UT; CO–Left Hand Cyn., CO; MN–Forest Lake; GA–Mt. Yonah, GA; *C. cobaltinus*: WA2–Ellensburg, WA; WA3–Gleed, WA; WA4–Wapato, WA; WA5–Toppenish, WA; WA6–9 km WNW Mabton; ID1–Payette, ID; UT2–Logan, UT; NV1–Gardnerville, NV; CA1–Canby, CA; CA9–Frazier Park, CA; *C. auratus* × *C. cobaltinus*: WA6–9 km WNW Mabton, WA) are indicated in Figs. 1 and 2. We initially surveyed these populations for allozyme loci that were diagnostic for the two *Chrysochus* species. This survey revealed that three loci (aspartate aminotransferase [AAT], isocitrate dehydrogenase [IDH], and malate dehydrogenase [MDH]) were reliably scorable and had no alleles shared by the two species. Subsequently, we determined if the putative hybrids were uniformly heterozygous for each of these species-specific markers, a pattern that would be expected if they were all hybrids. In contrast to the expectation for hybrids, only 12.5% of first-generation backcrossed individuals and fewer than 2% of more advanced generations of backcrossed individuals would be expected to be heterozygous at each of three species-specific loci (Bocklen and Howard 1997). In performing the allozyme analyses, we followed the cellulose-acetate gel electrophoresis protocols of Hebert and Beaton (1993).

Results

Distributional Overlap. By surveying populations of beetles occupying *A. cannabinum* patches in central Washington and by examining museum specimens, we

Table 1. Morphometric comparisons of the eight antennal flagellomere of *C. auratus*, *C. cobaltinus*, and hybrids

Species/Population	n	Mean l:w ratio (SE)	Species/Population	n	Mean l:w ratio (SE)
<i>Chrysochus auratus</i> ^a			<i>Chrysochuscobaltinus</i> ^a		
BC2	15	1.79 (0.041)	BC1	20	1.37 (0.020)
WA1	20	1.92 (0.027)	WA4	6	1.16 (0.032)
WA6 ^b	34	1.96 (0.017)	WA5	12	1.27 (0.022)
WA8	7	1.89 (0.017)	WA6 ^b	39	1.25 (0.011)
WA9	17	1.96 (0.020)	WA7	20	1.28 (0.017)
WA10	8	1.95 (0.035)	WA11	20	1.19 (0.019)
OR1	12	2.08 (0.037)	OR2	13	1.38 (0.028)
MT	12	2.04 (0.053)	OR3	8	1.25 (0.029)
AZ	13	2.09 (0.031)	OR4	8	1.19 (0.035)
NM	16	2.04 (0.048)	ID2	16	1.23 (0.025)
WI	17	1.99 (0.024)	UT1	12	1.37 (0.026)
IL	18	2.00 (0.033)	NV2	20	1.20 (0.019)
MO	18	2.05 (0.031)	CA2	20	1.22 (0.018)
PA	20	1.98 (0.022)	CA3	20	1.16 (0.015)
NY	16	2.01 (0.032)	CA4	20	1.21 (0.016)
			CA5	20	1.19 (0.015)
<i>C. auratus</i> × <i>C. cobaltinus</i>			CA6	20	1.22 (0.016)
WA6 ^b	23	1.62 (0.025)	CA7	16	1.24 (0.021)
			CA8	20	1.19 (0.020)

^a Overall comparison of *C. auratus* and *C. cobaltinus*: $P < 0.001$, among-population comparisons in each species: $P < 0.001$.

^b Pairwise comparisons of *C. auratus*, *C. cobaltinus*, and hybrids from WA6: all $P < 0.001$ (LSD test).

discovered that the ranges of *C. auratus* and *C. cobaltinus* either overlap or come in close proximity in six regions in western North America (Fig. 2). In two of these regions, the beetles are sympatric. One such region was revealed by museum specimens of both species that had been collected in different years from Kamloops, British Columbia. The other region of sympatry is an ≈ 25 km stretch of the Yakima River valley (Fig. 1), where we documented eight sites in which the two *Chrysochus* species co-occur. Two of these sites (Prosser and WA7) are based on museum records of individuals collected in different years, but the remaining six are sites at which we observed *C. auratus* and *C. cobaltinus* in the same host plant patch at the same time.

In addition to these regions of sympatry, there are four other regions where populations of the two species are separated by small distances (Fig. 2). In Utah, the range of *C. cobaltinus* extends as far south as Austin, which is <90 km from the nearest known population of *C. auratus*, in Capitol Reef National Park. In western Montana, an apparently disjunct population of *C. auratus*, located on the west side of the Rocky Mountains at Perma, occurs within 15 km of a population (MT1) of *C. cobaltinus* (Fig. 2). In north-central Washington, a population of *C. auratus* at Malott (WA1) is within 50 km of a known population of *C. cobaltinus*, north of Winthrop. Similarly, the westernmost populations of *C. auratus*, at Langley, British Columbia, are within 60 km of the nearest known population of *C. cobaltinus*, at Eastsound, WA (WA12). Further documentation of the precise range limits of both species in these regions is necessary to determine if any of these regions represent additional zones of contact between the two species.

Evidence of Hybridization. In the large focal patch (WA6) in which *C. auratus* and *C. cobaltinus* co-occurred, we observed a high frequency of interspecific copulation. In total, 192 of the 699 individuals (27.5%) we observed on 21 July 1999 were mating when we encountered them, collectively composing 96 mating pairs. Of these 96 observed matings, 22 (22.9%) involved a *C. auratus* individual mating with a *C. cobaltinus* individual. Intraspecific matings accounted for 54 (56.3%) of the observed matings. Presumably, because *C. auratus* was approximately twice as abundant as *C. cobaltinus* at this site (59 versus 29% of all beetles encountered), most (77.8%) of these intraspecific matings involved *C. auratus* individuals.

In this patch, as well as in two patches located ≈ 5 km W of Grandview (Fig. 1), we also found individuals that had coloration intermediate to the golden-green of *C. auratus* and the cobalt blue of *C. cobaltinus*. These individuals ranged in color from metallic bluish green to dark brownish purple with minimal iridescence. The abdominal sterna of most of these individuals were brown unlike the green or blue coloration of the abdominal sterna of typical *C. auratus* or *C. cobaltinus*. In addition to having distinctive coloration, these putative hybrids had a slightly different pattern of behavior from either *Chrysochus* species. Although typical *C. auratus* and *C. cobaltinus* adults were almost

invariably found feeding, mating, or ovipositing on the foliage of the host plants, the putative hybrids were frequently near the center of the plant, perching quiescently along one of the main stems of the plant. Despite their relative inactivity, putative hybrids were successful in finding mates, because 20 of the 96 observed matings (20.8%) involved at least one putative hybrid. We encountered these putative hybrids mating with other putative hybrids (two of 20 matings), as well as with *C. auratus* (13 of 20 matings) and *C. cobaltinus* (5 of 20 matings).

In all three patches in which we found these phenotypically intermediate individuals (the two patches ≈ 5 km W of Grandview and the focal patch, WA6), they were less abundant than *C. auratus* and *C. cobaltinus*. Nonetheless, they were not rare in these patches, accounting for four of 28 individuals we encountered in one patch (14.3%), five of 32 individuals in a second patch (15.6%), and 82 of 699 individuals (11.7%) in the patch in which we conducted our focal studies.

Morphometric measurements of the 8th antennal flagellomere were consistent with the hypothesis that these putative hybrids were indeed hybrids. In focal patch WA6, where the two species are sympatric, *C. auratus* individuals had a length:width ratio (1.96 ± 0.017) that was significantly greater than that of sympatric *C. cobaltinus* (1.25 ± 0.011 ; $P < 0.001$). Putative hybrids collected from this patch had length:width ratios (1.62 ± 0.025) that were intermediate to and significantly different from the ratios of *C. auratus* ($P < 0.001$) and *C. cobaltinus* ($P < 0.001$) (based on LSD tests).

To place this result in a broader geographical context, we must consider overall differences between the species, as well as regional variation within each species (Table 1). We found that the length:width ratio of the 8th flagellomere is greater in populations of *C. auratus* (mean across populations \pm SE: 1.98 ± 0.008) than in *C. cobaltinus* (1.24 ± 0.014), when comparing populations taken from throughout the majority of each species' range ($P < 0.001$). However, within both species, there was significant variation among populations in flagellomere length:width ratios (*C. auratus*: $P < 0.001$; *C. cobaltinus*: $P < 0.001$) (Table 1). For *C. auratus*, the length:width ratio decreased as the distance to the nearest population of *C. cobaltinus* decreased ($P = 0.024$), suggesting introgression of genes from *C. cobaltinus* into nearby *C. auratus* populations (Fig. 3). However, there was no evidence suggesting introgression of *C. auratus* genes into *C. cobaltinus*, as the mean length:width ratio of *C. cobaltinus* flagellomeres was not greater in populations close to the nearest *C. auratus* populations (Fig. 3, $P = 0.57$).

Allozyme evidence supported the morphometric results in verifying the hybrid status of phenotypically intermediate beetles. In all populations, except the population in which we conducted our focal observations (WA6), the only alleles we recorded for each species were species-specific alleles (Table 2). In other words, populations of *C. auratus* from outside of the zone of overlap featured only *C. auratus* alleles,

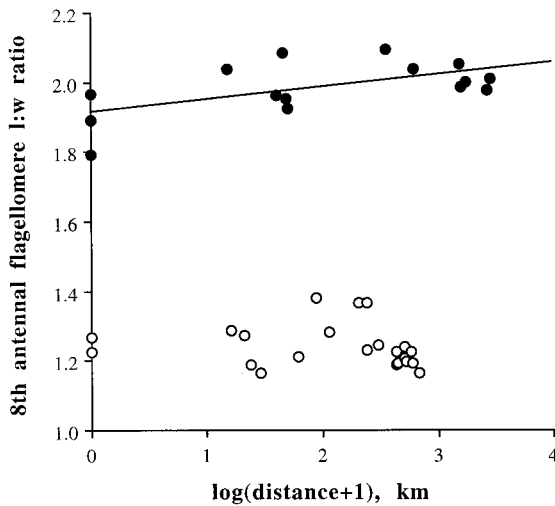


Fig. 3. Change in antennal morphology in both *C. auratus* and *C. cobaltinus* in relation to increasing proximity to populations of the other species. For this analysis, the distance to the nearest population of the other species was used, rather than the distance to the hybrid zone. As the distance to the nearest population of *C. cobaltinus* decreases, the length:width ratio of the 8th antennal flagellomere in *C. auratus* (closed circles) is decreased ($y = 0.035x + 1.194$, $P = 0.024$). In contrast, the length:width ratio of the 8th flagellomere of *C. cobaltinus* (open circles) does not vary with distance from the nearest *C. auratus* population ($P = 0.57$).

and populations of *C. cobaltinus* from outside of the zone of overlap featured only *C. cobaltinus* alleles. This was true for all three loci. However, this pattern broke

down for the one site of sympatry (WA6) from which we sampled beetles. At this location, five of the 52 specimens we sampled that had coloration typical of *C. cobaltinus* were heterozygous at all three loci, featuring both *C. cobaltinus* and *C. auratus* alleles. Such a result would be expected for F_1 hybrids, but unexpected for backcrossed individuals, in which no $>12.5\%$ of individuals should be heterozygous for each of three species-specific genetic markers (Boecklen and Howard 1997). Indeed, based on this probability, there is only a 0.003% probability (0.125^3) that a set of five backcrossed individuals would all be heterozygous for all three loci. These individuals also had flagellomere length:width ratios (1.58 ± 0.059) intermediate to the typical values for each species. Taken together, these results strongly indicate that these five beetles were hybrids between *C. auratus* and *C. cobaltinus*, rather than pure *C. cobaltinus* or backcrossed individuals, and thus we treated them as hybrids in the analyses shown in Tables 1 and 2. The rest of the *C. cobaltinus* individuals were homozygous for *C. cobaltinus* alleles. None of the 60 *C. auratus* we sampled from site WA6 featured *C. cobaltinus* alleles. We had identified 17 of the individuals that we collected for allozyme analysis from this site as putative hybrids. All of these putative hybrids were heterozygous for all three loci, with an allele from each *Chrysochus* species (Table 2). These results strongly support our initial assignment of these beetles as hybrids.

Our examination of specimens from museum collections revealed two putative hybrids (as evidenced by coloration) that were collected in 1952 at a fourth site, 5 km NE of Prosser. Antennal morphometrics

Table 2. Allele frequencies at three allozyme loci for populations of *Chrysochus auratus*, *C. cobaltinus*, and hybrids

Locus	Allele	Species/Population									Hybrids ^a
		<i>C. auratus</i>				<i>C. cobaltinus</i>					
		WA6	WA8	WA9	WA10	UT3	CO	MN	CA	WA6	
AAT	S	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50	
	M	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	
	F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
IDH	S	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50	
	F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	
MDH	S	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	
	F	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50	
<i>n</i>		60	30	30	30	12	12	10	5	22	
		WA2	WA3	WA4	WA5	WA6	ID1	UT2	NV1	CA1	CA9
AAT	S	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	M	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	0.87	0.71
	F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.13	0.29
IDH	S	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	F	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MDH	S	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>n</i>		30	30	30	30	47	30	12	12	12	12

^a All hybrids were heterozygous at each locus.

confirmed that these putative hybrids were indeed hybrids (length:width ratios of the 8th flagellomere were 1.55 and 1.56 for these two specimens). It is noteworthy that one of the patches (WA8) in which we documented co-occurrence in 1999 was also ≈ 5 km NE of Prosser, suggesting that at this site the two beetles have co-occurred for at least 47 yr. We found no other individuals that had the combination of antennal morphometrics and coloration that is typical of most hybrids. However, we did find single museum specimens of *C. cobaltinus* from Kamloops, BC (BC2 in Fig. 1), and Austin, UT (the southernmost limit of the distribution of *C. cobaltinus* in Utah), that had flagellomere length:width ratios (1.45 and 1.51, respectively) that were atypically high for *C. cobaltinus*.

Discussion

From detailed distributional data for both *Chrysochus auratus* and *C. cobaltinus* in western North America, we have identified two regions in which these beetles are sympatric as well as four other regions in which their ranges are in close proximity. Furthermore, we found that along a 25-km stretch of the Yakima River valley of south-central Washington, these two beetle species frequently engage in interspecific matings in host plant patches in which they co-occur. In such patches, morphologically intermediate individuals exist that are heterozygous for a suite of three species-specific allozyme markers. Taken together, these data provide compelling evidence that in south-central Washington, *C. auratus* and *C. cobaltinus* form a hybrid zone (sensu Barton and Hewitt 1989, Harrison 1990).

Whether the Yakima River valley is the only region in which *C. auratus* and *C. cobaltinus* hybridize remains unresolved. There is a distinct possibility of hybridization in the vicinity of Kamloops, British Columbia (BC2), where both species have been collected. The low flagellomere length:width ratio of *C. auratus* at this site suggests recent or ongoing introgression of *C. cobaltinus* alleles into *C. auratus*. In addition, the high length:width ratio for the single specimen of *C. cobaltinus* that we examined from this site may also be the result of introgression from *C. auratus*. Furthermore, it is possible that there are as-yet undiscovered hybrid zones in those regions in which the ranges of the two beetles come in close proximity. For example, the fact that *C. cobaltinus* from Utah (both UT1 and the single specimen from Austin, UT) have among the highest flagellomere length:width ratios for the species, suggests introgression from nearby populations of *C. auratus*. If this is the case, the introgression must be weak, because none of the Utah specimens of either species showed allozyme evidence of introgression (Table 2). Indeed, Boecklen and Howard (1997) have shown that in first-generation backcrossed individuals, only 12.5% of individuals would be expected to be homozygous for a suite of three species-specific genetic markers. However, in fourth and fifth generation backcrosses, >80% of individuals would be expected to have this pattern of

allelic variation. Thus, the fact that we only found individuals in Utah that were homozygous for the three species-specific loci strongly suggests that both F_1 hybrids and first-generation backcrossed individuals must be extremely rare. However, without data from many more loci, we cannot rule out the possibility that advanced backcrossed individuals exist in Utah or British Columbia populations of either species (Boecklen and Howard 1997). It would be interesting to examine additional central Utah and south-central British Columbia populations of both species to determine if a hybrid zone exists in either region.

The existence of hybrid zones raises the question of whether the hybridizing species are truly distinct species (Harrison 1993). In this particular case, we are compelled to argue that *C. auratus* and *C. cobaltinus* are distinct, based primarily on analyses of molecular differences between the species. Dobler and Farrell (1999) sequenced a 1264-bp fragment of mtDNA containing a portion of the cytochrome oxidase I gene, a tRNA leucine gene, and most of the cytochrome oxidase II (COII) gene. They found that in this portion of the mtDNA the sequence for *C. auratus* was diverged by $\approx 6.5\%$ from the sequences seen in *C. cobaltinus*. This degree of sequence divergence suggests that these two taxa have been diverged for a long time. Indeed, the molecular clock for the cytochrome oxidase I (COI) gene in arthropods is ≈ 1.7 – 2.1% sequence divergence per million years (Brower 1994, Funk et al. 1995; B. Farrell, unpublished data). The COII gene in many insects evolves perhaps as much as 60% faster than the COI gene (Simon et al. 1994), but in *Chrysochus* they appear to evolve at similar rates (S.D., unpublished data). Depending on whether the region sequenced by S.D. and B. Farrell (1999) has evolved at a rate matching that of typical insect COI or COII, it appears that *C. auratus* and *C. cobaltinus* have been diverged for ≈ 2 – 3 million years. The degree of mitochondrial DNA sequence divergence seen between *C. auratus* and *C. cobaltinus* is consistent with the hypothesis that the Yakima River hybrid zone is the result of secondary contact between two long-isolated species. One avenue by which this secondary contact might have arisen is if *C. auratus* crossed the continental divide via a river corridor such as the Peace River in British Columbia, and then followed the Fraser and Columbia River drainages south into south-central British Columbia and eastern Washington. However, in the absence of collection records from northern British Columbia, this hypothesis remains speculative.

Our second reason for arguing that these two species are distinct is that in surveys of diagnostic allozyme loci we did not find any individuals with variation that would indicate successful reproduction by hybrids (i.e., homozygous for one diagnostic locus and heterozygous for another diagnostic locus). Given that we sampled 129 *Chrysochus* individuals from a site at which 11.7% of the individuals were hybrids, it appears that if hybrids do reproduce, they do so only rarely. Indeed, we have recently documented that, compared with the parental species, hybrid females

have low survival, fecundity, and fertility (unpublished data). Thus, postzygotic barriers between the species are apparently well established in this region of sympatry, reducing the extent to which introgression between the species might occur.

Evidence that these postzygotic barriers might not be complete, allowing some introgression of genes across species boundaries, comes from our intraspecific comparisons of antennal morphology, as well as from preliminary studies of diet breadth and mobility in the hybrid zone (unpublished data). Morphometric comparisons revealed that the mean 8th flagellomere length:width ratio in *C. auratus* was reduced in populations lying in close proximity to populations of *C. cobaltinus*, though the opposite was not true in *C. cobaltinus*. Thus, it appears that there may have been some leakage of genes across species boundaries in areas of near or actual distributional overlap. An alternative explanation for this result is that antennal morphology in each species varies in relation to a cline in some environmental factor. We view this explanation as unlikely, given that the morphometric regression involved relating antennal morphology to the distance to the nearest population of the other species, rather than to the distance from the hybrid zone. Thus, for example, the morphology of Washington populations of *C. auratus* was plotted in relation to their distance from Washington populations of *C. cobaltinus*, and the morphology of central Rocky Mountain populations of *C. auratus* was plotted in relation to their distance to the nearest populations of *C. cobaltinus*, not the Washington populations. Thus, this analysis included multiple geographic gradients, making it much less likely that the morphometric results for *C. auratus* might be due to an underlying environmental gradient. Our hypothesis that introgression has occurred is supported by preliminary ecological data. We have documented that within the hybrid zone *C. auratus* and *C. cobaltinus* have similar mobilities (unpublished data) despite the general tendency for *C. auratus* to be more mobile than *C. cobaltinus* outside of the hybrid zone (Williams 1992, Dickinson 1995, Dobler and Farrell 1999). This result suggests the possibility that within the hybrid zone introgression has eroded the differences in mobility that typify these species. At this point, this conclusion remains speculative, and would best be resolved by mapping genes associated with mobility, and looking for introgression of those genes across the hybrid zone.

In a similar vein, we have found that within the hybrid zone a small proportion of *C. auratus* individuals will eat milkweed (unpublished data). This result stands in sharp contrast to results from populations elsewhere in western North America, where beetles are unwilling to eat milkweed, even in the absence of a choice (Dobler and Farrell 1999). Taken together, these morphological and ecological results are suggestive of ongoing introgression across species boundaries in this hybrid zone, or are perhaps testimony to a legacy of past introgression. We are currently performing studies to further analyze whether mobility

and diet breadth traits have in fact introgressed between these two species.

Introgression of ecologically significant traits would be significant because, in theory, novel traits gained by either species could lead to evolutionary innovations in that species (Harrison 1993). Although hybrid zones have generally been disregarded by zoologists as sources of evolutionary innovation (Harrison 1993), we view the introgression of ecologically significant traits as one of the most likely means by which such innovations could result from hybridization. For example, Futuyma et al. (1995) have argued that in the chrysomelid genus *Ophraella* a lack of genetic variation in the ability to feed on different host plants has constrained host shifts in this genus. The failure of typical *C. auratus* populations to exploit milkweeds may be due to a similar lack of genetic variation, but in regions of hybridization with *C. cobaltinus*, introgression of genes from *C. cobaltinus* could supply *C. auratus* with the necessary variation to expand its diet to include milkweeds. Indeed, such genes could move easily across species boundaries, even in the face of reduced hybrid fitness, if they conferred an adaptive advantage (Barton and Bengtsson 1986). If this were the case, it may still prove impossible to detect introgression of neutral markers, which would introgress much less freely (Barton and Bengtsson 1986), making it difficult to rigorously establish that any introgression had occurred. However, if the genes underlying the adaptively significant traits could be determined, it would be possible to study their introgression. If such knowledge of the genetic basis of diet breadth and mobility were available, the *Chrysochus* hybrid zone would provide an excellent opportunity to assess the potential evolutionary significance of hybridization in animals.

In addition, this hybrid zone promises to be well-suited for studying the evolution of reproductive barriers between hybridizing species. Reinforcement theory (Dobzhansky 1937) predicts the evolution of prezygotic mating barriers in response to selection imposed by postzygotic mating barriers between hybridizing taxa. In its strictest sense, this theory has been applied to hybridization between populations for which postzygotic barriers are not complete (Butlin 1989). Recently, however, Howard (1993) has argued that the use of the term "reinforcement" should be broadened to include those cases in which prezygotic barriers evolve in response to hybridization between species with complete postzygotic barriers (Butlin [1989] has referred to this scenario as "reproductive character displacement"). Consensus on this issue appears to be building; Butlin (1995) has recently acknowledged that the apparently trivial distinction between complete and nearly complete postzygotic barriers suggests that the narrow definition of reinforcement may be overly strict. Although reinforcement theory is intuitively appealing, its acceptance by evolutionary biologists has been far from universal, in part because of the difficulties in studying the mating behaviors of hybridizing populations (Howard 1993, Noor 1999). Our allozyme data re-

vealed that if hybrids do successfully reproduce, they do so rarely, suggesting low hybrid fitness and the possibility for reinforcement. Because the mating behaviors of these beetles are so amenable to study (Dickinson 1995), this system offers a unique opportunity to test the predictions of reinforcement theory in the field.

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