Living on the edge: Comparative phylogeography and phylogenetics of Oreohelix land snails at their range edge in Western Canada

Zach Dempsey
University of Lethbridge

Cameron Goater
University of Lethbridge

Theresa Burg (✉ Theresa.burg@uleth.ca)
University of Lethbridge

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Living on the edge: Comparative phylogeography and phylogenetics of *Oreohelix* land snails at their range edge in Western Canada

Z. W. Dempsey⁴, C. P. Goater⁴, and T. M. Burg⁴

Running title: *Oreohelix* Phylogeography in Canada

Abstract

**Background:** The biodiversity and distributions of terrestrial snails at local and regional scales are influenced by their low vagility and microhabitat specificity. The accessibility of large-bodied species and their characteristically high levels of genetic polymorphism make them excellent ecological and evolutionary models for studies on the phylogeography, phylogenetics, and conservation of organisms in fragmented populations. This study aims to elucidate the biodiversity, systematics, and distributions of genetic lineages within the genus *Oreohelix* at the northern and western periphery of their range.

**Results:** We found four mitochondrial clades, three of which are putative subspecies of *Oreohelix subrudis*. One clade was geographically widespread, occurring within numerous sites in Cypress Hills and in the Rocky Mountains, a second was geographically restricted to the Rocky Mountains in Alberta, and a third was restricted to the Cypress Hills region. A fourth clade was the small-bodied species, *O. cooperi*. ITS2 sequence and screening data revealed three haplotype clusters, of which one was *O. cooperi*. Cluster 1 was typical of the first clade and cluster 2 was typical of the second and third clades. ITS2 alleles were shared in a narrow contact zone between the first and second clades, suggestive of hybridization between the two.

**Conclusions:** A sky island known as Cypress Hills, in southeastern Alberta, Canada, is a biodiversity hotspot for terrestrial land snails in the genus *Oreohelix*. The observed
phylogeographic patterns likely reflect reproductive isolation during the Last Glacial Maximum,
followed by secondary contact due to passive, long-range dispersal resulting from low vagility,
local adaptation, and complex glacial history.

**Keywords:** Glaciation, Isolation, Secondary Contact, Sky Islands, Speciation, Hybridization
Background

The distribution of species is determined by their responses to localized conditions within a heterogeneous landscape, biological characteristics such as body size and life history, and vagility [1]. A species spatial distribution within a landscape determines population connectivity and is critical for population persistence and for maintaining genetic diversity. Typically, peripheral populations experience lower gene flow, reduced genetic diversity, and they tend to occur in marginal habitats [2]. Because core and peripheral populations are often locally-adapted to different environments, selection can occur and reduce gene flow between them as immigrants and any of their offspring will tend to have reduced fitness. In some cases, these marginal populations can become completely isolated from the core populations and give rise to new species [3]. Population connectivity is not the only challenge, population dynamics are also important. Peripheral population sizes are often small and survival is dependent on maintaining a critical population size to prevent the negative effects of genetic drift or demographic stochasticity. The impact of small population size is more pronounced in peripheral populations, especially in species with limited dispersal ability.

Species with low vagility, such as terrestrial gastropods, lack a reliable method of long-distance dispersal, and can persist in isolated, disconnected populations. Indeed, some land snails can limit their life-time home ranges to only a few meters [4]. As a result, land snails are often severely restricted in their ability to respond to rapid and localized anthropogenic changes such as habitat alteration, fragmentation, introduced species, and climate change [5]. These traits make land snails excellent models for conservation biologists seeking to understand and mitigate biodiversity loss and they also make them excellent models for phylogeographic and phylogenetic studies seeking to understand the roles of history and selection in the origin and
maintenance of genetic diversity [4, 6]. Studies completed over the past two decades document unusually high intraspecific sequence variation in mitochondrial DNA (10-30%) in some terrestrial snails providing an additional opportunity to understand how natural selection has shaped their history. Since land snails often exist within peripheral, unconnected populations that experience reduced gene flow, the importance of biogeographic and phylogenetic differences between core and peripheral populations can be straightforward to assess. However, extensive population fragmentation and limited dispersal also results in conservation concerns.

The dire conservation status of many of the estimated 50,000 to 200,000 described and undescribed species of molluscs is well-recognized by malacologists, systematists, and conservation biologists [7]. Of the ~5,000 mollusc species evaluated by the International Union for the Conservation of Nature in 2016, almost half are listed as extinct, critically endangered, threatened, or vulnerable [8] and 50% of those are terrestrial gastropods [9]. This is an important recognition from a conservation viewpoint because terrestrial snails play important roles in soil building, nutrient turnover, decomposition, and they are important determinants of plant community structure and terrestrial food web dynamics [5].

Terrestrial ‘mountain snails’ in the genus Oreohelix (Stylommatophora; Oreohelicidae) are large-bodied (up to 2 cm in shell diameter), air-breathing, and conspicuous components of the terrestrial mollusc community in high-elevation habitats in western North America [10, 11]. The 80 described species within the genus [11] are found in a broad range of habitats that extend north-south from Canada to northern Mexico, and east-west from the Black Hills in South Dakota to the Sierra Nevada Mountains in California. Within this continent-wide distribution, individual species vary extensively in the breadth of their habitat requirements and in their patterns of endemicity. Whereas species such as O. subrudis and O. strigosa occur throughout
much of the range of the genus, others are described from single limestone sky islands, mountaintops, or isolated mountain valleys [10]. Patterns of strong endemicity and concerns regarding declines in local population sizes have led to conservation concerns. Currently, of the 19 species of *Oreohelix* considered to occur in Montana, 42% are listed as ‘Species of Concern’ [12] and one of three Oreohelids in Wyoming is listed as vulnerable.

Oreohelid mountain snails form an important component of invertebrate communities, particularly in the Rocky Mountains, Intermountain West, and other sky islands of western North America. The northern part of their range extends into southwestern Canada, a region heavily impacted during the last glacial maximum [13, 14]. Cypress Hills Interprovincial Park (Cypress Hills) is an elevated plateau approximately 400 km² in southeastern Alberta and southwestern Saskatchewan surrounded by prairie and farmland. The plateau remained an unglaciated nunatak during the last glacial maximum [14] and is separated from the Rocky Mountains to the west by approximately 250 km. Despite the distance, the slopes of Cypress Hills contain many of the same flora and fauna characteristic of the Canadian Rocky Mountains [15]. While sky islands in the Intermountain West were once connected by habitat corridors that land snails were capable of traversing [10], the Cypress Hills and other adjacent sky islands do not appear to be connected.

The biodiversity, systematics, and distributions of Oreohelid mountain snails located at the northern and western range limits in western Canada are poorly known. In a recent study, we utilized molecular markers and traditional morphological assessments to show that the mountain snail, *O. cooperi*, previously considered to be endemic to the Black Hills region of South Dakota and Wyoming, was present in isolated, high-elevation sites in Cypress Hills Park in southern Alberta [16]. The results of that study further indicated that *O. cooperi* was sympatric with at least two other Oreohelid snails within the Cypress Hills sky island. The systematics,
biodiversity and distributions of the Oreohelids in this region, and in Canada in general, are unknown. More generally, phylogenetic relationships between Oreohelid lineages present in this sky island relative to those found in the Rocky Mountains to the west and to the south in the U.S. and Mexico are unknown.

The objective of this study is to characterize phylogeographic and phylogenetic patterns for *Oreohelix* spp. that occur at sites located along their western and northern range edge. One focus is to characterize regional and local phylogenetic patterns for Oreohelids in the Cypress Hills sky island where *O. cooperi* has recently been established as endemic in Canada [16]. A second focus is to utilize the results of previous phylogenetic studies involving Oreohelids sampled further south and west [10, 17] to evaluate the role of past glaciation and dispersal in determining the contemporary distributions of *Oreohelix* spp. in western Canada.

**Results**

**COI sequences**

COI sequencing revealed four genetically distinct and well-supported mitochondrial clades (Fig. 4). Automated selection of a phylogenetic tree model based on AICc showed the best score (−lnL = 1456) for a maximum likelihood model: Kimura 81 with gamma distribution (K81+G; gamma shape of 0.1882; Fig. 4). Sequence divergence between clades ranged from 3% to 26% with an average clade divergence of 15.2% ± 9.9%, while average sequence divergence within clades was less than 2%.

In samples collected from the Cypress Hills, all of the snails found on shrub-dominated slopes clustered into a single clade that matched *O. cooperi* mitochondrial DNA from [10] and [16]. The snails from habitats adjacent to Elkwater Lake in the western side of Cypress Hills
clustered into another clade that had 99.8% sequence similarity to *Oreohelix sp. B* [10], henceforth referred to as Clade B (Fig. 4). The remainder of snails found throughout eastern Cypress Hills clustered into a third clade with 96% sequence similarity with *Oreohelix sp. E* [10], hereafter referred to as Clade X. At sites adjacent to Highway 41 (CH04-07), there was a sharp gradient of mitochondrial groups (Fig. 2), where a distance of less than 400 m separated Clades B and X (Fig. 5). In that region, CH06 was the only site that contained both Clade B and X snails, whereas all the sites adjacent to CH06 contained a single mitochondrial clade (Fig. 2, 3). There were three instances when *O. cooperi* was found with another COI Clade at the same site in Cypress Hills (CH01, CHO, CHN, and CHT), indicating that these species can exist in sympatry in ecotones in Cypress Hills.

For the snails collected from the Rocky Mountains, two sister clades were identified: Clades B and B’. Clade B’ snails were confined to seven sites within a small geographic area in the Castle area and Crowsnest Pass (RM02, 04, 06–10). At four of those sites, all of the snails sampled contained B’ mitochondrial DNA. Overall, the Rocky Mountains snails had moderate genetic diversity (h = 0.54 compared to 0.75 in CH, and π = 0.0128 compared to 0.0805 in CH) at the COI locus, and low genetic diversity within sites (Table 1). If COI sequences from *O. cooperi* are removed from the analyses, CH snails had h = 0.63 ± 0.03 and π = 0.0278 ± 0.0006, which is still higher than the Rocky Mountains. Individually, each clade in Cypress Hills had a lower haplotype diversity than Cypress Hills as a whole (Clade B h = 0.17 ± 0.05, Clade X h = 0.33 ± 0.09). Only the CH04 site consisted of a single haplotype, whereas six Rocky Mountains sites consisted of a single haplotype (Table 1).

*ITS2 sequences*
Maximum likelihood haplotype analysis of ITS2 sequenced data revealed three distinct clusters including one matching the COI *O. cooperi* clade (Fig. 5). The most commonly sampled cluster (Cluster 1) was populated predominantly by COI Clade X snails, while the next most populous cluster (Cluster 2) was dominated primarily by COI Clades B and B’ (Table 3). ITS2 haplotypes were shared between COI Clades X, B, and B’, but no haplotypes were shared with *O. cooperi*. Mitochondrial Clade X contained only 11 individuals that had alleles from the Clade B ITS2 cluster, each of which was found close to the contact zone for B and X (Table 3). Forty four of the Clade B and X snails in Cypress Hills were heterozygous for both ITS2 haplotype clusters, 38 of which belonged to Clade B. All snails sequenced and screened with clade specific primers from the Rocky Mountains belonged to ITS2 cluster 2 (Table 3). All 41 individuals from the Rocky Mountains belonging to Clade B’ contained ITS2 cluster 2 alleles.

*Morphological characteristics*

Oreohelid snails collected from shrub-dominated slopes were *O. cooperi* (CH09 and CH10), measuring 8.5 ± 0.1 mm (95% CI) in maximum shell length. Average shell length of mitochondrial Clade B (sites CH03 and CH04) was almost double the size of *O. cooperi* at 15.0 ± 0.3 mm, while snails belonging to mitochondrial Clade X (sites CH07 and CH08) measured 15.4 ± 0.4 mm in length. An unpaired *t*-test showed that mean shell sizes of *O. cooperi* were significantly smaller than either of the two large-bodied snails (B and *O. cooperi*: *t*_118 = 39.4; *p* < 0.0001; X and *O. cooperi*: *t*_118 = 32.8; *p* < 0.0001), but the two large morphs where not significantly different in shell size from each other (*t*_118 = 1.20; *p* = 0.23).

**Discussion**
We obtained Oreohelid snails from 58 sites in two areas of southern Alberta and Saskatchewan. Two closely-related mitochondrial DNA clades were found in the Rocky Mountains, and three clades were found in Cypress Hills. Of the four COI clades identified, only *Oreohelix cooperi* was morphologically distinguishable [13, 16, 17]. The larger snails have been identified as belonging to the species complex *Oreohelix subrudis*, and are widespread throughout the Rocky Mountains and sky islands in Montana and Wyoming [10]. Two of the lineages sampled in Canada had highly restricted ranges: Clades X and B’. Clade X has only been found in Cypress Hills and is most closely related to Clade E in Wyoming described by [10] indicating that Clade X is either a glacial relict in Cypress Hills or colonized Cypress Hills from the periglacial zone immediately south of Cypress Hills and the Laurentide ice sheet. Clade B’ is restricted to the northern end of the sampled range in the Rocky Mountains and is closely related to Clade B. This proximity suggests that Clade B’ recently diverged from Clade B in the Rocky Mountains. The presence of isolated lineages restricted to a small range as well as widespread lineages is concordant with findings from [10] on Oreohelid snails collected further to the south.

High levels of genetic divergence at the mitochondrial locus and cryptic speciation are commonly reported in studies of terrestrial snails [18]. [19] attributes the high divergence to one of four possibilities: (i) relatively rapid mitochondrial divergence, (ii) prolonged isolation followed by secondary contact, (iii) selection pressure to maintain multiple mitochondrial clades, or (iv) colonization patterns leading to the co-occurrence presence of many divergent mitochondrial clades. The COI patterns observed in the snails sampled are best described by a combination of (ii) and (iv). Secondary contact following prolonged isolation (ii) best describes the presence of the three divergent mitochondrial lineages in Cypress Hills, particularly Clades B
and X where nuclear ITS data show limited mixing. One possibility is that each of the clades of the *O. subrudis* species complex evolved in isolation on a sky island. Temperature and precipitation changes characterizing the Pleistocene glaciations allowed some of these clades to come into secondary contact. During the temperature and precipitation fluctuations in the early Holocene [20, 21], Cypress Hills and other sky islands such as the Black Hills or Bighorn Hills remained as relatively stable habitat for Oreohelids [22]. This effect may be especially pronounced in a few areas, such as Cypress Hills and the Bighorn Mountains (WY1, WY2, and WY3 from [10]). Secondary contact following prolonged isolation has been found in the dusky Arion slug (*Arion subfuscus*; [23]). [23] reported highly divergent mitochondrial sequence data with low nuclear ITS1 divergence, which matches the pattern we found in our Oreohelid snails. [23] suggests that hybridization of nuclear loci occurred in the slugs, leading to the maintenance of multiple divergent mitochondrial lineages.

Alternatively, colonization patterns leading to many divergent mitochondrial clades (iv) could account for the overall patterns of distribution of *O. cooperi* and *O. subrudis* in North America, including Cypress Hills. Under this model, an area is colonized either actively through a habitat corridor or through passive long-range dispersal [24-26]. The new population quickly expands in size, thereby reducing the likelihood of further invasion. Colonization patterns favouring multiple divergent mitochondrial clades were found to be the best explanation for the distribution of mitotypes in grove snails (*Cepea nemoralis*) across northwestern Europe [19] due to their high population sizes and distances between patches of suitable habitat. These authors argued that populations of grove snails are effectively arranged like stepping stones, and dispersal events are rare.
ITS2 and cytonuclear discordance

ITS2 data revealed three genetic clusters; one in the Rocky Mountains, and three in Cypress Hills. One cluster was exclusive to *O. cooperi*. These results confirm those from our earlier study showing *O. cooperi* is genetically distinct from other sympatric Oreohelids and it is endemic in Canada to the Cypress Hills [16]. Snails in Cluster 1 were typical of *O. sp. X* and cluster 2 was typical of *O. sp. B*, but the two divergent mitochondrial clades showed overlap at the ITS2 locus, particularly surrounding the contact zone in the Cypress Hills. There are two possibilities why Clades B and X exhibit mitochondrial and nuclear discordance. The first possibility is incomplete lineage sorting, and the second is introgression and hybridization [27]. While these are not mutually exclusive, hybridization and introgression better explain the ITS2 pattern observed in Cypress Hills. If incomplete lineage sorting was occurring, there would be no geographic pattern to the nuclear allele distribution in Cypress Hills [28]. However, there is a clear geographic pattern to both the mitochondrial COI and the nuclear ITS2. While both alleles are common in a small area surrounding the contact zone near Elkwater Lake, to the east of the contact zone near Highway 41 alleles belonging to Clade 1 become much more common.

Glacial history

Oreohelids have a fossil record in North America dating back to the Cretaceous period [29], but the current distribution of Oreohelids in southern Alberta is a consequence of the Quaternary glacial history of the region. During the LGM, Cypress Hills was inaccessible to terrestrial snails and surrounded by ice. As the ice sheets receded, the southern slopes were the first to become ice free and allow access to the hills, while the northern slopes retained ice for much longer [30]. The earliest indication of vegetation in Cypress Hills is approximately 9000
years before present [22] based on pollen data from Harris Lake (49.6663 °N 109.9044 °W) on the northern side of Cypress Hills. Literature is sparse regarding the vegetation on the southern slopes of Cypress Hills because many lakes immediately south of Cypress Hills are anthropogenic. More recent work based on zonal reconstruction of vegetation from sites across in North America suggests that 13 000 to 14 000 years ago, before the start of the late glacial warming period, Cypress Hills and the Sweet Grass Hills demarcated the meeting point between the western Cordilleran forest and the eastern boreal forest in a thin zonal band adjacent to the Laurentide ice sheet [31]. According to this model, the band of Cordilleran forest extended from west of Cypress Hills to the Rocky Mountains. While it did not extend far into the ice-free corridor of the Rocky Mountains until much later [32], the forest did remain adjacent to the Rocky Mountains in a narrow band following the Rocky Mountains south into Montana. As such, the Cordilleran forest was continuous with site MT1, the only location in the U.S. where Clade B has been found [10]. The band of boreal forest that extended from Cypress Hills is also thought to have been connected to the Black Hills, a site where *O. cooperi* is common. These bands of forest were transient and replaced by prairie within 2 000 years [33]. While the most recent glacial maximum reached the farthest south, previous glacial maximums could have resulted in forests connecting to Cypress Hills as well, providing earlier corridors [34]. Although sky islands such as Cypress Hills and the Black Hills were once connected, the extent to which Oreohelids used these corridors is uncertain. The vegetation would have been capable of supporting Oreohelids, however, they also require the presence of other abiotic factors, such as calcareous deposits [13]. Limited fossil evidence exists of Oreohelids east of Montana during the most recent forest expansion [35, 36], so they were either present in low densities or absent.
Glacial refugia and colonization

Cypress Hills may have acted as a glacial refugium for at least some Oreohelids during the LGM. It is uncertain if Cypress Hills was habitable during the LGM and there is a lack of consensus in the literature [15, 37]. Some studies suggest it may have been habitable for some species such as lodgepole pine (*Pinus contorta*) [38, 39], but not other species such as jack pine (*Pinus banksiana*) [40]. The absence of fossil evidence of Oreohelids in the Great Plains is consistent with Oreohelids already being present in Cypress Hills or the Sweet Grass Hills during the LGM [35, 36]. This does not solve the problem of how the snails colonized Cypress Hills in the first place. Including COI data from [10], the snails of Cypress Hills are paraphyletic and could not have evolved *in situ*.

Clade X is the only clade in Cypress Hills that is not present on other sky islands to the south or elsewhere in their range and is therefore likely a glacial relict. It is disjunct from its closest relative, Clade E, in northern Wyoming (WY2) over 500 km to the south. Further evidence that Clade X is a glacial relict is the pattern of the haplotype network and distribution of Clades X and B (Fig. 5). The starburst pattern in each is typical of recent population expansions (For more examples, see: [41, 42]). Clade B in Cypress Hills occupies a much smaller proportion of available habitat than Clade X. This could be evidence of Clade B being a later introduction to Cypress Hills. It is likely that Clade B was present in the Rocky Mountains prior to the LGM. Furthermore, the sharing of ITS2 alleles between the mitochondrial groups is indicative of introgression between the two clades. It also shows asymmetry with Cluster 1 containing most of Clade X snails and the presence of two distinct genetic clusters. A second, less likely, possibility is that Clade B was present in Cypress Hills during the LGM and the Rocky Mountains were colonized from the east rather than from the south. The presence of
Clade B’ in the Rocky Mountains indicates that Clade B was present in the Rocky Mountains long enough for Clade B’ to diverge. Clade B contains several shared haplotypes in Cypress Hills and the Rocky Mountains, suggesting recent connectivity.

Clades B, C, D, E, and X from our study and [10] are relatively closely related. This species complex likely originated in Montana or Wyoming as most of the diversity occurs in that area, and Oreohelids have been present in Yellowstone Park area for more than 5 million years [29]. Temperature and precipitation fluctuations of the Pleistocene and early Holocene may have allowed colonization of the sky islands via habitat corridors and then isolated each sky island. Ice caps covered many of the sky islands that are currently occupied by Oreohelid land snails, but the distances between them are relatively small and sky islands were connected by suitable habitat for longer periods of time [10, 43]. Similar patterns have been found in glacial relict montane grasshoppers (Melanoplus sp.) in the same area [44, 45].

The restricted distribution of Clade B’ in the Rocky Mountains suggest more recent separation and divergence in situ from Clade B. The divergence between Clades B and B’ is much less than the divergence between any of the other subspecies of O. subrudis [10]. Clade B’ also has reduced ITS2 diversity. The Rocky Mountains foothills contained tundra-like vegetation throughout most of the Wisconsinan and an ice-free corridor throughout the last glacial maximum [32]. Trees did not colonize the majority of the ice-free corridor until approximately 8000 years before present, which is roughly concordant with pollen core data from Harris Lake to the east [22, 32]. Despite extensive surveys, we have not found Oreohelids above 1600 m in the Rocky Mountains. This is the elevation at which snow often remains year-round in southern Alberta and tundra-like, glacial relict vegetation is common.
The ITS2 haplotypes of *O. cooperi* in Cypress Hills are much more divergent than those found in either Clade B or X, suggesting that *O. cooperi* may have been in Cypress Hills for a longer period of time. Evidence counter to an early colonization is that the COI haplotypes found in Cypress Hills are shared with snails from both the Judith Mountains and Black Hills. The original source of *O. cooperi* is likely somewhere within or near Wyoming, which contains *O. cooperi*, *O. pygmaea*, and Clade A. These clades form a monophyletic group and likely diverged from the same source population.

*Contemporary patterns*

The divide between Clades B and X in Cypress Hills is associated with Highway 41. Highways have been demonstrated to be substantial barriers to terrestrial snails [46]. Highway 41 was constructed in the early 1900’s and does not explain the presence of two distinct lineages on either side. The highway may currently reduce gene flow between Clades B and X, but the two clades have been maintained in Cypress Hills since at least the LGM. The niches occupied by both Clades B and X are indistinguishable in Cypress Hills, and these snails have high population densities which may resist invasion from other taxa. Terrestrial snails are known for their ability to rapidly colonize or invade novel ecosystems due to their high reproductive capacity [47]. The spread of the colonizing snails would only stop at the edge of suitable habitat or an occupied niche. Previous work in pipits (*Anthus trivialis* and *A. spinoletta*) and buntings (*Emberiza citronella* and *E. hortulana*) has shown that long-term species segregation is possible through the combination of factors including habitat differences and interspecific competition [48].
While hybridization between the two mitochondrial clades is evident, it is limited to a narrow area and the introgression of ITS2 alleles is asymmetrical. Many more Clade B snails share ITS2 alleles with Clade X than *vice versa*. Most hybrids are limited to the contact zone. Oreohelids are simultaneous hermaphrodites, yet the pattern is similar to male-biased dispersal in dioecious organisms [27] suggesting unequal movement between male and female gametes in the contact zone. It could be that these snails seek out mates, but return to their usual resting place to give birth. This would mimic male-biased dispersal, and reduce inbreeding. Another possibility is asymmetrical mating, where snails may behave as males for some mates but females for others. Asymmetrical mating has been demonstrated in land snails including Oreohelids [49, 50] and has been observed in Oreohelids from Cypress Hills (Z. Dempsey, personal observations).

**Conclusions**

Using molecular techniques, three mitochondrial clades of Oreohelids have been identified in Cypress Hills, and two in the Rocky Mountains. Two of these lineages have not been found previously despite extensive genetic surveys in the U.S. The small morph of Oreohelid snail in Cypress Hills, identified as *O. cooperi*, has been demonstrated to be genetically distinct from large bodied snails of Cypress Hills and the Rocky Mountains. We propose that the Oreohelids of Cypress Hills and the Rocky Mountains are glacial relicts and restricted to niches that were much more widespread during the glacial maximum.

**Methods**

*Study areas and sampling*
Cypress Hills Interprovincial Park (CHP) is situated on the southern Canadian plains (49°30’N, 110° W) in the provinces of Alberta and Saskatchewan covering an area of approximately 2,590 km² [15]. The hills (maximum elevation of 1,420 m a.s.l.) comprise a plateau of pre-glacial bedrock that rises 430 m above the surrounding prairie [51]. The hills were surrounded by glaciers twice during the Quaternary period, but the plateau above approximately 1,350 m remained unglaciated [52]. Highland forest and grassland communities found in CHP are most similar to those of the Rocky Mountains located 300 km to the west and to the aspen parkland regions characteristic of the central areas of Canada’s three prairie provinces [15]). Forested areas comprise approximately 20% of CHP, with the remained comprised of fescue grassland and wetland habitats [15]. *Pinus contorta* (lodgepole pine), *Populus tremuloides* (trembling aspen) and *Picea glauca* (white spruce), *Populus balsamifera* (balsam poplar) are the dominant tree species [15]. Grassland communities are dominated by *Festuca campestris* (rough fescue) and other grasses that characterize the mixed-grass natural sub-region [53]. Other habitat characteristics that are relevant to the occurrence of Oreohelid snails in CHP are described in [16, 54].

The forested slopes of CHP provide ideal habitat for Oreohelid snails [13, 15, 16]. Similarly, the Rocky Mountains located to the west contain a patchwork of suitable conditions for Oreohelid snails that are interconnected with habitat corridors [55]. Contained in the Rocky Mountains of southwestern Alberta is Waterton Lakes National Park (WLNP). This park comprises a wide range of montane habitats including mountains, prairie, lakes, and wetlands. Vegetation consist primarily of fescue grasslands and deciduous forests including aspen forests at lower elevations and a transition to alpine meadows and coniferous forests at higher elevations.
Sites within the Castle Wildlands Area and Crowsnest Pass have similar habitats to those in Waterton Lakes National Park (Fig. 1).

Individual snails (n=474) were obtained from 58 sites in southern Alberta and Saskatchewan throughout the snow-free months of 2013-2016 (Fig. 2, 3). During visits to each site, plots approximately 10 m² were demarcated with flagging tape. A maximum of 30 Oreohelid snails were collected as they were encountered and preserved in 90% ethanol following methods in [54]. All sample sites were searched by two people for at least 30 minutes. Snails were stored in ethanol at -80 °C. Data on slope, aspect, and elevation were recorded for each site.

Assessments of shell characteristics including banding pattern and maximum shell width were determined for 30 adult snails per site. For morphological assessments, snails from six sites (CH03, CH04, CH07, CH08, CH09, and CH10) were selected based on preliminary genetic data, representing each of the three main mtDNA clades [16].

**DNA extraction, amplification, and sequencing**

Approximately 2 mg of foot tissue from each snail was used for a modified chelex DNA extraction following [16, 56]. Extracted DNA was amplified with COI barcoding primers LCO1490 and HCO2198 [57] and ITS2 primers LSU1F2990 and ITS4R3908 [58]. A 585 bp fragment of the COI locus was amplified under the following conditions: 1 cycle of 94 °C for 2 minutes, 50 °C for 45 seconds, and 72 °C for 1 minute; 37 cycles of 94 °C for 30 seconds, 50 °C for 45 seconds, and 72 °C for 1 minute, with a final cycle at 72 °C for 5 minutes. The PCR mix contained: 1 Unit Flexi DNA polymerase, 1x Flexi buffer, 0.2 mM dNTP, 0.4 µM of each primer, and 3 mM MgCl₂. The ITS2 amplification of a 472 bp region followed the same
protocol, with the exception of a 48 °C annealing temperature and 1 mM MgCl₂. Amplified DNA was sent to McGill University for sequencing.

**Sequencing data analyses**

Sequences were aligned, trimmed and overall sequence divergence at each locus was calculated in MEGA 6.06 [59]. DnaSP 5.1 was used to calculate haplotype (h) and nucleotide (π) diversities for both loci [60]. A phylogenetic tree using Jukes-Cantor model was constructed for COI using PAUP* 4.0a151 [61] for model selection. Maximum likelihood models of phylogenetic tree construction were ranked based on Akaike Information Criterion corrected (AICc) for finite sample sizes [61]. The model with the lowest AICc score was used to construct a phylogenetic tree that was validated with 1000 bootstrap replicates with 50% consensus. TreeGraph 2.9.2 [62] was used to create the final tree. Sequence data from the ITS2 locus were used to construct a maximum likelihood haplotype network with 300 iterations in PopART 1.7 [63].

**Screening primers**

Both COI and ITS2 data showed fixed differences between clades such that screening primers were developed targeting fixed nucleotide differences to type the remaining samples. The COI locus used a common primer HCOIcommO (5’ – TAA ACT TCA GGG TGA CCA AAA – 3’) with three clade specific primers in a multiplex PCR: LCOIspecOc635 (5’ – TGC TCT TTC ACT TTT AAT TCG AC – 3’) only amplified *Oreohelix cooperi*, LCOIspecOs563 (5’ – ATT GTT ACA GCC TAT GCC – 3’) only amplified *Oreohelix sp. B*, and LCOIspecOx217 (5’ – GTG CCC CAG GAA TAA ATT TG – 3’) only amplified *Oreohelix sp.*
X. The COI screening primer used a 48 °C annealing temperature and 1 mM MgCl₂. The ITS2 locus used a common reverse primer ITS4R3908 with three different forward primers in a multiplex PCR: ITS2FspecOc256 (5’ – CCG TGG TCT TAA GTT CAA A – 3’) amplified *O. cooperi*, ITS2FspecOs112 (5’ – TTA ACG AAA AGT GGA TGC T – 3’) preferentially amplified *O. sp. B*, and ITS2specOx356 (5’ – CTG CTG TGC TCT AGC ATT TAT – 3’) preferentially amplified *O. sp. X*. The ITS2 screening PCR had a 54 °C annealing temperature and 1.5 mM MgCl₂. Amplified DNA was visualized and scored on 3% agarose gel stained with ethidium bromide.

**Abbreviations**

CHP; Cypress Hills Park; COI: Cytochrome oxidase I; °C: Degrees Celsius; DNA: Deoxyribonucleic acid; ITS2: Internal transcribed spacer 2; mtDNA: Mitochondrial DNA; PCR: Polymerase chain reaction

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**Author’s Contributions**

**Authors’ Information**
Zach Dempsey completed his M.Sc. at the University of Lethbridge on phylogeography and parasite-host interactions in *Oreohelix* land snails. His research interests include molecular ecology, parasitology, and invertebrate zoology. Theresa Burg studies evolutionary and ecological aspects of natural populations and how they relate to physical and non-physical barriers. Cam Goater investigates the ecology and evolution of host-parasite interactions.

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**Availability of the data and materials**

DNA sequences will be available on GenBank under accession numbers …

**Ethics approval and consent to participate**

**Competing interests**

The authors declare that they have no competing interests.

**Author details**

¹University of Lethbridge, Department of Biological Sciences, 4401 University Drive, Lethbridge, AB, T1K 3M4, Canada

²Corresponding author: Theresa Burg; Email: theresa.burg@uleth.ca; Phone: 403 332 5299
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Table 1. Summary of genetic polymorphism statistics for COI sequence data for *Oreohelix* spp. of snails. Each locus was analyzed by site, clade, and region including number of individuals (n), haplotypes (Hap), number of variable sites (V sites), and haplotype (h) and nucleotide (π) diversities. Site locations are described in Figure 1.

| Site  | Clade | n  | Hap | V sites | $h \pm SD$ | $\pi \pm SD$ |
|-------|-------|----|-----|---------|------------|--------------|
| CH01  | B/Co  | 11 | 2   | 95      | 0.18 ± 0.14| 0.0282 ± 0.0223|
| CH02  | B     | 15 | 3   | 2       | 0.26 ± 0.14| 0.0004 ± 0.0003|
| CH03  | B     | 8  | 3   | 2       | 0.46 ± 0.20| 0.0008 ± 0.0004|
| CH04  | B     | 9  | 1   | 0       | 0.00 ± 0.00| 0.0000 ± 0.0000|
| CH05  | B     | 7  | 3   | 2       | 0.52 ± 0.21| 0.0010 ± 0.0005|
| CH06  | X/B   | 8  | 7   | 38      | 0.96 ± 0.08| 0.0306 ± 0.0064|
| CH07  | X     | 8  | 3   | 2       | 0.68 ± 0.12| 0.0013 ± 0.0003|
| CH08  | X     | 12 | 2   | 1       | 0.17 ± 0.13| 0.0003 ± 0.0002|
| CH09  | Co    | 7  | 2   | 1       | 0.29 ± 0.20| 0.0005 ± 0.0004|
| CH10  | Co    | 8  | 4   | 4       | 0.79 ± 0.11| 0.0026 ± 0.0005|
| CH11  | X     | 10 | 2   | 1       | 0.20 ± 0.15| 0.0003 ± 0.0003|
| CH12  | X     | 5  | 2   | 2       | 0.40 ± 0.24| 0.0014 ± 0.0008|
| CH13  | Co    | 8  | 4   | 2       | 0.75 ± 0.14| 0.0015 ± 0.0004|
| CH14  | Co    | 8  | 7   | 6       | 0.96 ± 0.08| 0.0032 ± 0.0007|
| CH15  | X     | 5  | 3   | 3       | 0.70 ± 0.22| 0.0020 ± 0.0008|
| RM01  | B     | 8  | 2   | 1       | 0.25 ± 0.18| 0.0004 ± 0.0003|
| RM02  | B/B’ | 8  | 3   | 19      | 0.68 ± 0.12| 0.0078 ± 0.0050|
| RM03  | B     | 7  | 1   | 0       | 0.00 ± 0.00| 0.0000 ± 0.0000|
| RM04  | B/B’ | 8  | 4   | 19      | 0.75 ± 0.14| 0.0157 ± 0.0035|
| RM05  | B     | 8  | 2   | 1       | 0.54 ± 0.12| 0.0008 ± 0.0002|
| RM06  | B’    | 8  | 1   | 0       | 0.00 ± 0.00| 0.0000 ± 0.0000|
| RM07  | B/B’ | 7  | 6   | 22      | 0.95 ± 0.10| 0.0187 ± 0.0035|
| RM08  | B’    | 8  | 2   | 1       | 0.43 ± 0.17| 0.0007 ± 0.0003|
| RM09  | B’    | 7  | 3   | 2       | 0.67 ± 0.16| 0.0012 ± 0.0004|
| RM10  | B’    | 8  | 3   | 2       | 0.68 ± 0.12| 0.0013 ± 0.0003|
| RM11  | B     | 7  | 2   | 1       | 0.29 ± 0.20| 0.0004 ± 0.0003|
| RM12  | B     | 7  | 3   | 2       | 0.67 ± 0.16| 0.0012 ± 0.0004|
| RM13  | B     | 4  | 1   | 0       | 0.00 ± 0.00| 0.0000 ± 0.0000|
| RM14  | B     | 3  | 1   | 0       | 0.00 ± 0.00| 0.0000 ± 0.0000|
| RM15  | B     | 8  | 1   | 0       | 0.00 ± 0.00| 0.0000 ± 0.0000|
| RM16  | B     | 8  | 3   | 2       | 0.46 ± 0.20| 0.0008 ± 0.0004|
| RM17  | B     | 4  | 1   | 0       | 0.00 ± 0.00| 0.0000 ± 0.0000|

CH  | B/Co/X | 129 | 19 | 102 | 0.75 ± 0.02 | 0.0805 ± 0.0032 |
RM  | B/B’   | 118 | 9  | 20  | 0.54 ± 0.04 | 0.0128 ± 0.0008 |

*O. cooperi*  | Co  | 41  | 7  | 7   | 0.27 ± 0.09 | 0.0006 ± 0.0002 |
*O. sp. B*    | B   | 121 | 10 | 10  | 0.17 ± 0.05 | 0.0004 ± 0.0001 |
*O. sp. B’*   | B’  | 40  | 9  | 7   | 0.59 ± 0.09 | 0.0014 ± 0.0003 |
*O. sp. X*    | X   | 45  | 9  | 11  | 0.33 ± 0.09 | 0.0008 ± 0.0003 |
**Total**     | B/B’/Co/X | 247 | 25 | 108 | 0.74 ± 0.02 | 0.0598 ± 0.0040 |
Table 2. Summary of genetic polymorphism statistics for ITS2 sequence data for *Oreohelix* spp. Each locus was analyzed by site and region including number of individuals (n), haplotypes (Hap), number of variable sites (V sites), and haplotype (h) and nucleotide (π) diversities. Site locations are described in Figure 1.

| Site  | Cluster | n  | Hap | V sites | h ± SD     | π ± SD     |
|-------|---------|----|-----|---------|------------|------------|
| CH01  | Co      | 8  | 3   | 32      | 0.34 ± 0.14| 0.0151 ± 0.0074|
| CH02  | 1/2     | 13 | 10  | 33      | 0.88 ± 0.03 | 0.0166 ± 0.0032|
| CH03  | 1/2     | 5  | 4   | 10      | 0.60 ± 0.13 | 0.0064 ± 0.0023|
| CH05  | 1/2     | 8  | 5   | 12      | 0.61 ± 0.13 | 0.0069 ± 0.0022|
| CH06  | 1/2     | 8  | 10  | 17      | 0.87 ± 0.08 | 0.0116 ± 0.0026|
| CH07  | 1/2     | 8  | 3   | 2       | 0.51 ± 0.13 | 0.0014 ± 0.0004|
| CH09  | Co      | 5  | 5   | 70      | 0.67 ± 0.16 | 0.0410 ± 0.0147|
| CH10  | Co      | 8  | 2   | 1       | 0.23 ± 0.13 | 0.0006 ± 0.0003|
| CH11  | 1/2     | 8  | 5   | 14      | 0.61 ± 0.13 | 0.0093 ± 0.0028|
| CH12  | 1       | 4  | 2   | 1       | 0.43 ± 0.17 | 0.0010 ± 0.0004|
| CH15  | 1       | 8  | 2   | 1       | 0.40 ± 0.11 | 0.0009 ± 0.0003|
| RM03  | 2       | 1  | 1   | 0       | NA         | NA         |
| RM04  | 2       | 3  | 1   | 0       | NA         | NA         |
| RM06  | 2       | 4  | 1   | 0       | NA         | NA         |
| RM11  | 2       | 4  | 1   | 0       | NA         | NA         |
| RM15  | 2       | 8  | 1   | 0       | NA         | NA         |
| RM16  | 2       | 8  | 1   | 0       | NA         | NA         |

**CH** 1/2/Co, **RM** 2, **Total** 1/2/Co

| CH    | 1/2/Co | 83  | 24  | 84      | 0.73 ± 0.03 | 0.0303 ± 0.0025|
| RM    | 2       | 28  | 1   | 0       | NA         | NA         |
| Total | 1/2/Co | 114 | 23  | 82      | 0.75 ± 0.02 | 0.0259 ± 0.0021|
Table 3. Number of individuals belonging to each COI clade and ITS2 cluster for 474 samples of *Oreohelix* snails.

| ITS2 Cluster | COI Clade |   |   |   |
|--------------|-----------|---|---|---|
|              | B         | B' | X | Co|
| 1            | 56        |   |   | 128|
| 2            | 111       | 41 | 5 |   |
| 1/2          | 38        |   |   | 6 |
| Co           |           |   |   | 89|
| Total        | 205       | 41 | 139 | 89|


Figure 1. Satellite image of all *Oreohelix* sampling sites including [10]. Sites are coded based on COI clade present. Samples include *O. cooperi* (Co), *O. pygmaea* (Py), and *O. subrudis* (Clades B-E). The scale bar represents 20 km. Map Data: Google Earth (2018).

Figure 2. Relief image of *Oreohelix* COI sequencing (numbers) and screening (letters) results in Cypress Hills (CH) and the Rocky Mountains (RM). The scale bar in each inset represents 20 km. Map Data: Google Earth (2018).

Figure 3. Relief image of *Oreohelix* ITS2 sequencing (numbers) and screening (letters) results in Cypress Hills (CH) and the Rocky Mountains (RM). The scale bar in each inset represents 20 km. Map Data: Google Earth (2018).

Figure 4. Phylogenetic tree of COI haplotype data including sequences from [10]. Branch lengths and scale are proportional to average substitutions per site above branches. Bootstraps values below nodes are based on 1000 replicates.

Figure 5. Minimum spanning haplotype network constructed for the ITS2 locus. Dashes and numbers in brackets represent number of base pair differences between haplotypes. Size of the circle is proportional to the number of individuals sharing that haplotype. Shading corresponds to mitochondrial clade.
Figures

Figure 1

Satellite image of all Oreohelix sampling sites including [10]. Sites are coded based on COI clade present. Samples include O. cooperi (Co), O. pygmaea (Py), and O. subrudis (Clades B-E). The scale bar represents 20 km. Map Data: Google Earth (2018).

Figure 2

Relief image of Oreohelix COI sequencing (numbers) and screening (letters) results in Cypress Hills (CH) and the Rocky Mountains (RM). The scale bar in each inset represents 20 km. Map Data: Google Earth (2018).

Figure 3

Relief image of Oreohelix ITS2 sequencing (numbers) and screening (letters) results in Cypress Hills (CH) and the Rocky Mountains (RM). The scale bar in each inset represents 20 km. Map Data: Google Earth (2018).
**Figure 4**

Phylogenetic tree of COI haplotype data including sequences from [10]. Branch lengths and scale are proportional to average substitutions per site above branches. Bootstraps values below nodes are based on 1000 replicates.
Figure 5

Minimum spanning haplotype network constructed for the ITS2 locus. Dashes and numbers in brackets represent number of base pair differences between haplotypes. Size of the circle is proportional to the number of individuals sharing that haplotype. Shading corresponds to mitochondrial clade.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.jpg
- supplement2.jpg
- supplement3.jpg