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Evolutionary pattern and process within the *Vertigo gouldii* (Mollusca: Pulmonata, Pupillidae) group of minute North American land snails

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ABSTRACT

A phylogenetic analysis of 19 sibling taxa in the *Vertigo gouldii* group was conducted on 73 individuals sampled across North America using DNA sequence data of the mitochondrial genes *cytochrome oxidase subunit 1* (*CO1*) and *16S ribosomal RNA* (*16S*), and the *internal transcribed spacer-2* of the *nuclear ribosomal RNA* (*ITS-2*) gene. The results of these analyses were found incongruent with previous taxonomic concepts used to define the *V. gouldii* group and its composite taxa that were based entirely on conchological features. The mtDNA sequence data suggest that some previous members of the traditional *V. gouldii* group may be more closely related to *V. modesta*. They also suggest that *V. gouldii* may itself consist of seven species-level branches spread across two deeply rooted clades. Revision of geographical distributions on the basis of these analyses suggests that these *Vertigo* species may commonly possess continental-sized ranges in spite of their minute size and limited active dispersal ability. High levels of sympatry within the group are also confirmed, with up to four species being known to co-occur within single microsites. These data also suggest that rates of diversification have been non-constant. Assuming a 1%/my rate of base pair substitution, a 10-fold diversification pulse is indicated from 6.7–7.0 myBP, which would be co-incident with known mid-late Miocene global climate changes.

1. Introduction

Vertigo (Gastropoda, Stylommatophora, Pupillidae) is a genus of minute land snails with ovoid shells that generally range between 1.5 and 3 mm in length and possess a rounded aperture with 0–6 (sometimes more) apertural lamellae at maturity (Pilsbry, 1948). Prior to the Neogene (23 mya), *Vertigo* was a component of the sub-tropical northern hemisphere arcto-tertiary forest fauna (Pilsbry, 1948). This community fragmented following climatic cooling and drying from the Neocene onward (Stanley, 2004), causing a number of land snail genera (e.g., *Strobilops*, *Hendersonia*, and *Carychium*) to become restricted to highly disjunct distributions centered on the eastern and western North America, eastern Asia, western Europe, and/or the Caucasus (Pilsbry, 1948). Although *Vertigo* is currently distributed throughout the Holarctic, North America represents the global diversity center for the genus, with two thirds of known modern taxa being restricted to this region. The North American taxa also encompass the entire known global range of shell morphologies (Pilsbry, 1948) and habitat preferences (Nekola and Coles, in press), with population densities exceeding 2000/m^2 in favorable habitats (Coles and Nekola, 2007).

Because *Vertigo* demonstrate a high degree of aphallism and reduction in the male genitalia (Pokryszko, 1987), both species-level and supraspecific taxonomy has historically relied entirely upon shell characters such as overall shape, surface sculpture, aperture shape and lamellar configuration. Two subgenera, *Vertigo* (*Angustula*) and *Vertigo* (*Vertillaria*), constituting a total of perhaps only two species, have been given official taxonomic status (Pilsbry, 1948), while the genus *Nearctula* has been recently resurrected (Roth and Sadeghian, 2006) to encompass the *Vertigo californica* group of Pilsbry (1948). The remaining *Vertigo* have been traditionally assigned to a number of informal taxonomic groups that Pilsbry (1948) found quite "difficult to formulate".

The *Vertigo gouldii* group is the most diverse of these, containing up to 19 nominal taxa, or approximately 1/3 of the North American total. Pilsbry (1948) distinguished its members by their possession of strong and sharp shell striation in combination with intermediate apertural lamellae strength as compared to the strong lamellae of the *Vertigo ovata* group and weak lamellae of the *Vertigo modesta* group. The *Vertigo gouldii* group ranges across almost all of North America, with some of its constituent taxa appearing to have extremely wide distributions. For example, *V. gouldii* itself is considered to represent a single variable species ranging from British Columbia (Forsyth, 2004) to the 'sky islands' of the desert southwest (Bequaert and Miller, 1973), the southern Appalachians, and northern Maine (Hubricht, 1985). However, many *Vertigo gouldii*

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group members are also believed to possess limited geographic and ecological ranges (Fig. 1) with most of these range-restricted taxa having been assigned global conservation status rankings of 'vulnerable' or higher (NatureServe, 2009) and listed for threatened or endangered species protection within various U.S. states. Such local endemism might be expected for minute land snails, whereby low rates of active dispersal (1–100 m/yr; Schilthuizen and Lombaerts, 1994; Hausdorf and Hennig, 2003) coupled with their inability to actively cross barriers of only 100–1000 m (Baur, 1988; Schilthuizen and Lombaerts, 1994) might allow for easy development of isolated populations. However, small snails have also proven capable of extreme feats of passive dispersal, as has been shown for *Balea* which has been repeatedly carried across 9000 km of open eastern Atlantic Ocean (Gittenberger et al., 2006). Whether the extensive ranges of some *V. gouldii* group members are accurate, or actually represent the composite distribution of multiple smaller-ranged cryptic taxa, is an issue that remains unexplored.

On the basis of current taxonomy, members of the *Vertigo gouldii* group also appear to possess remarkable degrees of micro-scale sympatry. In the continental-wide land snail community database detailed in Nekola (2005), fully 48% of 1000 m^2 sites harboring members of the *Vertigo gouldii* group supported more than two taxa, with up to six being recorded from single sites. Nekola and Smith (1999) also reported up to four *Vertigo gouldii* group taxa co-occurring within single 400 cm^2 microsites. Such patterns stand in marked contrast to other land snails which often display strongly allopatric distributions. For example, in the North American southwest taxa in the genera *Ashmunella, Oreohelix, Holospira*, and *Sonorella* tend to represent single mountain endemics with only single representatives of each being found within a given site (Bequaert and Miller, 1973; Metcalf and Smartt, 1997). Similar strongly allopatric distributions appear common for helicids on Porto Santo (Cameron et al., 1996), camaenids in Western Australia (Solem, 1988; Cameron, 1992), clausiliids in the Aegean (Douris et al., 1998), and Gastrocoptinae from karst towers in southeastern Asia (Schilthuizen et al., 1999; Tongkerd et al., 2004). This raises the question of whether current taxonomic concepts within the *Vertigo gouldii* group are flawed, with multiple shell types actually representing the same species, making sympatry levels lower.

Lastly, because *Vertigo gouldii* group members are almost completely restricted to forest habitats, they likely have experienced variation in diversification rates since the Paleogene. Increased levels of allopatric speciation may have been caused not only by arcto-tertiary forest fragmentation, but also from more recent cyclical climatic changes associated with late-Pliocene and Pleistocene glaciations.

To address these issues, we present the results of a continental-wide phylogenetic analysis of the 19 nominal, sibling taxa of the *Vertigo gouldii* group of North American *Vertigo* based on DNA sequence of the mitochondrial genes: *cytochrome oxidase subunit 1* (*CO1*) and *16S ribosomal RNA* (*16S*); and the *internal transcribed spacer-2* (*ITS-2*) of the nuclear *ribosomal RNA* gene cluster and its flanking sequence. This analysis is used to consider phylogenetic relationships, the nature of actual taxon ranges, sympatry levels, and diversification rates within the *V. gouldii* group. Because molecular tools have not previously been used to address these issues within a wide-ranging group of minute land snails within a continental setting, this study may also provide novel insights into the evolutionary mechanisms for many small soil organisms.

2. Materials and methods

2.1. Selection of taxa

The taxa included in this study (Fig. 1, Table 1) comprise all but two of the 14 taxa assigned to the *Vertigo gouldii* group by Pilsbry (1948): *V. wheeleri* Pilsbry, 1928 was excluded because it appears synonymous with *V. rugulosa* Sterki, 1890 (Hubricht, 1974), and *V. hebardi* Vanatta, 1912 because extant populations are unknown and material suitable for DNA extraction does not exist. We also excluded *Vertigo hubrichti variabilis* Frest, 1991 because no individual we have seen out of ~2500 of *V. hubrichti* from across its entire range agrees with the description or line drawing provided in Frest (1991). However, we included *V. brierensis, V. iowaensis, V. nylanderi, V. meramecensis* and three apparently novel forms from Alaska (*Vertigo* AK 1–3 of Table 1) based on their shell morphologies (Pilsbry, 1948; VanDevender, 1979; Frest, 1991). *Vertigo modesta, V. ventricosa*, *V. hinkleyi* and *V. californica* (a.k.a. *Nearctula rowelli* of Roth and Sadeghian, 2006) were included as putative congeneric outgroups whereas *Gastrocopta tappaniana, Vallonia gracilicosta*, *Pupilla muscorum* and *P. hebes* were included as extra-generic outgroups within the Pupillidae.

2.2. Biogeographic range and sympatry data

Geographical distributions for all named *Vertigo gouldii* group members as outlined above were compiled from Pilsbry (1948), Oughton (1948), Frest and Fay (1981), Hubricht (1985), Frest (1991), and Nekola and Coles (in press). Estimates of within-site sympatry, using both initial taxonomic concepts and those informed by sequence analyses are based on the dataset outlined in Nekola (2005) consisting of 1177 sites, 274 molluscan taxa and 529,176 individuals. For purposes of this paper, analyses were limited to the 701 sites supporting at least one member of the *V. gouldii* group.

2.3. Specimen selection for DNA analysis

Specimens selected for DNA analysis were either live-collected during 2007 (44 individuals), preserved in absolute ethanol (13 individuals largely from Coles collection at the Florida Museum of Natural History), or mummified with an intact epiphragm along with visual evidence of dry tissue in the shell apex and no apparent tissue decomposition (17 individuals largely from the Nekola collection). Accession numbers for the lots from which these specimens were selected are provided in Table 1.

For the majority of taxa within the *Vertigo gouldii* group, individuals were selected from three populations representing the known geographic and ecological range of each (Table 1). Examples of both the large and small shell polymorphism noted by Nekola (2001) were included for *V. cristata*. Two individuals were also analyzed from each of two *V. hubrichti* populations (Potawatomie State Park and Blue Springs East) and one *V. meramecensis* population (Brush Creek Canyon). We endeavored to sequence topotype or near-topotype material when possible, including: *V. hinkleyi* – Miller Canyon, Arizona (specimen #NS53); *Vertigo gouldii basidens* – Bland, New Mexico (#17); *V. gouldii inserta* – Bear Wallow, Arizona (#NS30); *V. nylanderi* – McConnell Brook, Maine (#NS36); *V. paradoxa* – Caribou Stream, Maine (#NS39).

To allow for the highest probability of observing hybridization and introgression, when possible, specimens were selected from sites supporting multiple micro-sympatric *Vertigo gouldii* group taxa (Table 2). Sympatric individuals were sequenced from: Benderville Wayside (*V. hubrichti*, *V. iowaensis*); Blanco River (*V. gouldii basidens*, *V. gouldii coloradensis*); Bland (*V. gouldii arizonensis*, *V. gouldii basidens*); Brush Creek Canyon (*V. gouldii gouldii, V. meramecensis*); Nenana North (*V. hannai, V. paradoxa*, *Vertigo* AK 1, *Vertigo* AK 2, *Vertigo* AK 3); Neutriosa South (*V. concinnula*, *V. gouldii arizonensis, V. gouldii inserta*); Potawatomie State Park (*V. hubrichti*, *V. iowaensis*); and Russell Rock (*V. bollesiana*, *V. gouldii gouldii*).

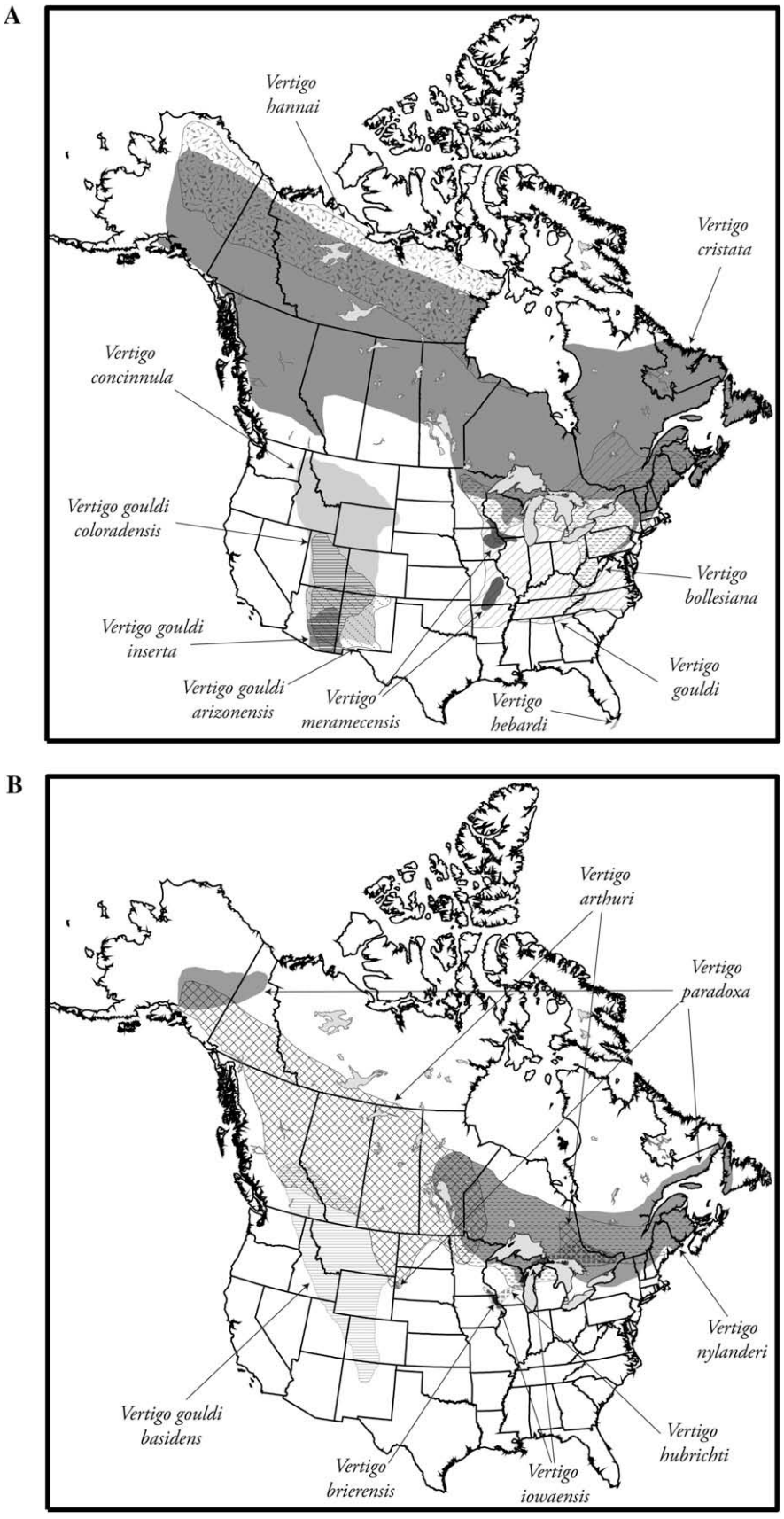

Fig. 1. Distribution maps for all putative members of the *Vertigo gouldii* group based on traditional taxonomic concepts.

Table 1
Location and habitat information, Accession number (numbers preceded by an "N" are from the Nekola collection, "C" from the Coles collection), and DNA sample number for each sequenced specimen.

Taxon State/Province	Site/County	Habitat	Lon./Lat.	Accession number	Sample number
Gastrocopta tappaniana C.B. Adams, 1916					
Maine	Wesley School, Washington County	White Cedar swamp forest	67.6590 W., 44.9274 N.	N 16345	NS50
Pupilla hebes Ancey, 1881					
Alaska	Happy Valley	Upland tundra	148.7302 W., 69.3355 N.	N 15142	NS48
Pupilla muscorum Linné, 1758					
Iowa	Crawford Quarry, Linn County	Calcareous roadside verge	91.7400 W., 41.9866 N.	N 14592	22
Minnesota	Lake Bemidji, Beltrami County	Sandy wooded lakeshore	94.8247 W., 47.5328 N.	N 9054	23
Vallonia gracilicosta Reinhardt, 1883					
Alaska	Nenana	Xeric S-facing Aspen forest	149.0979 W., 64.5698 N.	N 14928	NS49
Vertigo arthuri Von Martens, 1882					
Manitoba	Pisew Falls	Upland aspen-fir-spruce forest	98.4013 W., 55.1989 N.	C 10707/503s	15
Alaska	Chickaloon	Xeric S-facing Aspen forest	148.4752 W., 61.7788 N.	N 15401	NS4
Alaska	Falls Creek	Dry Aspen forest	149.5758 W., 60.9844 N.	N 15354	NS5
Manitoba	Devils Lake Wayside	Aspen-oak-birch forest	98.9119 W., 52.4035 N.	N 11289	NS6
North Dakota	Wessels WMA, Pembina County	Aspen forest	97.8878 W., 48.8147 N.	C 10722/518s	NS7
Vertigo bollesiana Morse, 1865					
Maine	Mt. Carmel, Aroostook County	Upland rock outcrop	68.1823 W., 47.3272 N.	N 15493	NS10
Maine	Russell Rock, Aroostook County	Xeric rock outcrop	67.8489 W., 46.3078 N.	N 15564	NS11
Maine	Collins Siding, Aroostook County	White Cedar swamp forest	68.1316 W., 47.1113 N.	N 16137	NS12
Vertigo brierensis Leonard, 1972					
Iowa	Williams Creek 5, Allamakee County	Open, mossy algific slope	91.4782 W., 43.1373 N.	N 5165	11
Vertigo californica Rowell, 1861 [*Nearctula rowelli* of Roth and Sadeghian, 2006]					
California	Moss Landing, Monterey County	Coastal scrub	121.7884 W., 36.8095 N.	N 13934	NS51
Vertigo concinnula Cockerell, 1897					
Arizona	Neutriosa South, Apache County	Aspen-Pine forest	109.1619 W., 33.9039 N.	N 14007	NS54
Vertigo cristata Sterki, 1919 [small morph of Nekola, 2001]					
Maine	Roque Bluffs Rd., Washington County	Acid coastal Spruce forest	67.4961 W., 44.6363 N.	C 11563/592	NS16
Wisconsin	Sugar Camp Bog, Oneida County	Acid peatland	89.2958 W., 45.8499 N.	C 11635/599	NS17
Maine	Blind Brook, Aroostook County	White Cedar swamp forest	68.9291 W., 46.5788 N.	N 15736	NS19
Vertigo cristata Sterki, 1919 [large morph of Nekola, 2001]					
Quebec	Sunny Mountain, Nunavik District	Upland tundra	67.2348 W., 55.0647 N.	N 13686	NS44
Maine	Jack Mountain, Aroostook County	Cool wooded rock outcrop	68.7330 W., 46.5741 N.	N 15724	NS45
Alaska	Earthquake Park, Anchorage	Aspen-spruce forest	149.9889 W., 61.1990 N.	N 15312	NS46
Vertigo gouldii arizonensis Pilsbry and Vanatta, 1900					
New Mexico	Bland, Sandoval County	Rich, mesic forest	106.4593 W., 35.7474 N.	N 14819	31
Arizona	Neutriosa South, Apache County	Aspen-Pine forest	109.1619 W., 33.9039 N.	N 14006	NS1
New Mexico	Nogal Canyon, Lincoln County	Oak-Ash forest	105.7839 W., 33.4987 N.	N 13092	NS2a
New Mexico	Emory Pass, Grant County	Rich, mesic forest	107.7936 W., 32.9094 N.	N 14217	NS2b
New Mexico	4th of July Canyon, Torrance County	Maple-oak forest	106.3812 W., 34.7837 N.	N 14741	NS3
Vertigo gouldii basidens Pilsbry and Vanatta, 1900					
New Mexico	Bland, Sandoval County	Rich mesic forest	106.4593 W., 35.7474 N.	N 14820	17
New Mexico	Tusas Ridge, Rio Arriba County	Open Aspen grove	106.0381 W., 36.6519 N.	N 13016	NS8
Colorado	Blanco River, Archuleta County	Rich, mesic mixed forest	106.8857 W., 37.1452 N.	N 13055	NS9
Vertigo gouldii coloradensis Cockerell, 1892					
Colorado	Blanco River, Archuleta County	Rich, mesic mixed forest	106.8857 W., 37.1452 N.	N 13056	NS13
Arizona	Mt. Lemmon, Pima County	Aspen-spruce forest	110.7848 W., 32.4413 N.	N 14044	NS14
Arizona	Buena Vista Peak, Cochise County	Aspen-fir-pine forest	109.2722 W., 31.9176 N.	C 10783/616s	NS15
Vertigo gouldii gouldii Binney, 1843					
Minnesota	Deer Creek, Fillmore County	Wooded limestone bluff	92.3443 W., 43.7322 N.	N 14646	26
Ohio	Cliffton Gorge, Greene County	Wooded limestone bluff	83.8366 W., 39.7955 N.	N 14775	27
New York	Syracuse, Onondaga County	Wooded limestone pavement	76.1105 W., 43.0074 N.	N 13961	28
Tennessee	Tellico Gorge, Monroe County	Wooded rocky slope	84.1831 W., 35.3303 N.	C 1332	38
Maine	Russell Rock, Aroostook County	Xeric rock outcrop	67.8489 W., 46.3078 N.	N 15566	NS20
Iowa	Brush Creek Canyon, Fayette County	Wooded limestone bluff	91.6890 W., 42.7796 N.	N 1554	NS21
Arkansas	Buffalo River, Searcy County	Wooded sandstone bluff	92.5649 W., 36.0858 N.	N 14342	NS22
Vertigo gouldii inserta Pilsbry, 1919					
Arizona	Neutriosa South, Apache County	Aspen-Pine forest'	109.1619 W., 33.9039 N.	N 14008	NS29
Arizona	Bear Wallow, Pima County	Pine forest	110.7302 W., 32.4211 N.	N 14062	NS30
Arizona	Bigelow Campground, Pima County	Rich, mesic mixed forest	110.7282 W., 32.4154 N.	N 14072	NS31
Vertigo hannai Pilsbry, 1919					
Manitoba	Launch Road, Churchill	Upland tundra	93.8716 W., 58.7447 N.	C 10712/508s	NS23
Alaska	Happy Valley	Upland tundra	148.7302 W., 69.3355 N.	N 15144	NS24
Alaska	Last Tree	Spruce-alder forest	149.7970 W., 67.9406 N.	N 15072	NS25
Alaska	Coldfoot North	Rich peatland	150.1359 W., 67.3512 N.	N 15040	NS26

(*continued on next page*)

Table 1 (*continued*)

Taxon State/Province	Site/County	Habitat	Lon./Lat.	Accession number	Sample number
Alaska	Nenana North	Aspen-alder forest	149.0902 W., 64.6066 N.	N 14953	NS27
Vertigo hinkleyi Pilsbry, 1921					
Arizona	Miller Canyon, Cochise County	Rich, mesic forest	110.2824 W., 31.4105 N.	N 14091	NS53
Vertigo hubrichti Pilsbry, 1934					
Wisconsin	Potawatomie State Park, Door County	Wooded limestone bluff	87.4250 W., 44.8774 N.	N 185	9
Wisconsin	Potawatomie State Park, Door County	Wooded limestone bluff	87.4250 W., 44.8774 N.	N 185	10
Iowa	Blue Springs East, Winneshiek County	Wooded algific slope	91.9413 W., 43.4096 N.	N 8883	12
Iowa	Blue Springs East, Winneshiek County	Wooded algific slope	91.9413 W., 43.4096 N.	N 8883	13
Wisconsin	Benderville Wayside, Brown County	Wooded limestone bluff	87.8420 W., 44.6132 N.	C 11636/600	NS28
Vertigo iowaensis Frest, 1991					
Wisconsin	Potawatomie State Park, Door County	Wooded limestone bluff	87.4250 W., 44.8774 N.	N 186	6
Wisconsin	Benderville Wayside, Brown County	Wooded limestone bluff	87.8420 W., 44.6132 N.	C 11636/600	NS32
Vertigo meramecensis Van Devender, 1979					
Iowa	North Bear Creek, Winneshiek County	Wooded limestone bluff	91.6220 W., 43.4478 N.	N 5192	51
Iowa	Clark Cabin, Allamakee County	Wooded limestone bluff	91.5724 W., 43.4458 N.	N 5340	NS33
Iowa	Brush Creek Canyon, Fayette County	Wooded limestone bluff	91.6890 W., 42.7796 N.	N 1555	NS34
Iowa	Brush Creek Canyon, Fayette County	Wooded limestone bluff	91.6890 W., 42.7796 N.	N 1555	NS35
Vertigo modesta Say, 1824					
Alaska	S. Fork Koyukuk River	Riparian Alder scrub	150.2886 W., 67.0197 N.	N 15241	NS58
Vertigo nylanderi Sterki, 1909					
Maine	McConnell Brook, Aroostook County	White Cedar swamp forest	68.5953 W., 46.6120 N.	N 15709	NS36
Manitoba	Sturgeon Gill Road	Willow-Alder swamp forest	99.1653 W., 53.4731 N.	C 10708/504s	NS37
Wisconsin	Blueberry Marsh, Brown County	Acid Tamarack swamp forest	87.8924 W., 44.5323 N.	N 12266	NS38
Vertigo paradoxa Sterki, 1900					
Maine	Caribou Stream, Aroostook County	Wooded limestone bluff	68.0119 W., 46.8590 N.	N 9898	NS39
New York	Clark Reservation, Onondaga County	Wooded limestone bluff	76.0972 W., 43.0009 N.	N 13996	NS40
Quebec	La Grand Pointe, Duplessis District	Coastal limestone turf	63.4013 W., 50.2017 N.	N 13460	NS41
Alaska	Chugach State Park	Acid peatland	149.5387 W., 61.2964 N.	C 7166	32
Alaska	Nenana North	Aspen-Alder forest	149.0902 W., 64.6066 N.	N 14954	NS42
Vertigo ventricosa Morse, 1865					
Maine	Portage Lake, Aroostook County	Acid peatland	68.5408 W., 46.7850 N.	N 15915	NS52
Vertigo AK 1					
Alaska	Nenana North	Aspen-Alder forest	149.0902 W., 64.6066 N.	N 14949	NS43
Vertigo AK 2					
Alaska	Nenana North	Aspen-Alder forest	149.0902 W., 64.6066 N.	N 14950	NS47
Vertigo AK 3					
Alaska	Nenana North	Aspen-Alder forest	149.0902 W., 64.6066 N.	N 14951	NS56

2.4. DNA extraction

All specimens (live, ethanol-preserved, and mummified) were treated identically. Entire cleaned individuals were placed in a sterile 1.7 ml Eppendorf tube and rapidly ground using a clean, flame-polished ~3 mm diameter glass rod. It was found neither necessary nor advantageous to freeze samples prior to grinding. DNA was prepared using the DNeasy Tissue Kit (QIAGEN), whereby 200 μL of digestion buffer was immediately added to each ground specimen, followed by vigorous vortexing. Subsequent incubation and purification followed the manufacturer's instructions. Purified DNA samples were heat-treated at 95 °C for approximately 10 min prior to storage at −80 °C. Extraction yield was determined using a NanoDrop-1000 Spectrophotometer, and ranged from 1.2–4.0 μg DNA/specimen. DNA stored for >20 days tended to lose its ability to act as a template. In such cases sample DNA was re-purified using an Eppendorf PerfectPrep 96 PCR cleanup kit according to the manufacturer's instructions. Re-purified sample DNA was stored at −20 °C and used within 1 week.

2.5. PCR amplification and DNA sequencing

Selected *CO1*, *16S*, and *ITS-2* regions were amplified using published methods with modifications as follows: *CO1* was amplified following the method of Gittenberger et al. (2004) using their LCO1490-Alb and HCO2198-Alb primer sets. *16S* was amplified using the method of Tongkerd et al. (2004) using the 16Sar-L primer (forward) of Jorgensen et al. (2004) and the 16Sbr primer (reverse) of Palumbi (1996). *ITS-2* was amplified using the method of Wade and Mordan (2000).

PCR products were treated with 0.5 μL of ExoSAP-IT (USB). 3 μL of treated product (~50 ng) was used as template for dye termination using 0.5 μL of BigDye 2.0 dye-terminator with 0.2 μM reverse or forward primer and a 10 μL volume. Cycling parameters follow the STeP protocol of Platt et al. (2007). Dye-terminated products were precipitated by adding 2.5 of 125 mM EDTA and 30 μL of absolute ethanol. The DNA pellets were washed with 70% ethanol, air dried and dissolved in 10 μL of formamide for sequence analysis using an ABI 3130xl (Applied Biosystems).

2.6. Phylogenetic analyses

Sequences were aligned using CLUSTALX, and have been deposited in the NCBI GenBank GQ921483–GQ921664. The number of substitutions within and between taxa was calculated for each of the three gene segments using Mega 4.0 (Tamura et al., 2007). Genetic distances were calculated using Maximum Composite Likelihood in Mega 4.0, including both transitions and transversions, assuming homogeneous patterns among lineages, uniform rates among sites, and using pairwise gap deletion.

Table 2
Co-occurring *Vertigo gouldii* group taxa from those sample sites supporting multiple forms, using traditional taxonomic concepts.

State or Province/Site		Co-occurring *Vertigo gouldii* group taxa
Alaska	Chickaloon	*Vertigo arthuri*, *V. cristata*, *Vertigo* AK 3
	Chugach State Park	*Vertigo cristata*, *V. paradoxa*
	Falls Creek	*Vertigo arthuri*, *V. cristata*
	Nenana North	*Vertigo hannai*, *V. paradoxa*, *Vertigo* AK 1, *Vertigo* AK 2, *Vertigo* AK 3
Arizona	South Fork Koyukuk River	*Vertigo hannai*, *Vertigo* AK 1, *Vertigo* AK 2
	Bear Wallow	*Vertigo gouldii coloradensis*, *V. gouldii inserta*
	Buena Vista Peak	*Vertigo concinnula*, *V. gouldii coloradensis*
	Mt. Lemmon	*Vertigo concinnula*, *V. gouldii coloradensis*, *V. gouldii inserta*
Arkansas	Neutriosa South	*Vertigo concinnula*, *V. gouldii arizonensis*, *V. gouldii inserta*
	Buffalo River	*Vertigo gouldii gouldii*, *V. meramecensis*
Colorado	Blanco River	*Vertigo concinnula*, *V. gouldii basidens*, *V. gouldii coloradensis*
Iowa	Blue Springs East	*Vertigo bollesiana*, *V. gouldii gouldii*, *V. hubrichti*
	Brush Creek Canyon	*Vertigo bollesiana*, *V. gouldii gouldii*, *V. meramecensis*
	Clark Cabin	*Vertigo gouldii gouldii*, *V. meramecensis*
	North Bear Creek	*Vertigo gouldii gouldii*, *V. meramecensis*
	Williams Creek 5	*Vertigo bollesiana*, *V. brierensis*, *V. gouldii gouldii*, *V. hubrichti*, *V. iowaensis*
Maine	Blind Brook	*Vertigo bollesiana*, *V. cristata*, *V. nylanderi*, *V. paradoxa*
	Caribou Stream	*Vertigo gouldii gouldii*, *V. paradoxa*
	Collins Siding	*Vertigo bollesiana*, *V. cristata*, *V. gouldii gouldii*, *V. nylanderi*
	Jack Mountain	*Vertigo bollesiana*, *V. cristata*, *V. gouldii gouldii*, *V. paradoxa*
	McConnell Brook	*Vertigo bollesiana*, *V. cristata*, *V. nylanderi*, *V. paradoxa*
	Mt. Carmel Wayside	*Vertigo bollesiana*, *V. cristata*, *V. gouldii gouldii*
	Russell Rock	*Vertigo bollesiana*, *V. cristata*, *V. gouldii gouldii*, *V. paradoxa*
Manitoba	Pisew Falls	*Vertigo arthuri*, *V. cristata*, *V. paradoxa*, *Vertigo* AK 3
Minnesota	Deer Creek	*Vertigo gouldii gouldii*, *V. hubrichti*
New Mexico	Bland	*Vertigo concinnula*, *V. gouldii arizonensis*, *V. gouldii basidens*
New York	Emory Pass	*Vertigo concinnula*, *V. gouldii arizonensis*
	Tusas Ridge	*Vertigo gouldii basidens*, *V. gouldii coloradensis*
	Clark Reservation	*Vertigo gouldii gouldii*, *V. paradoxa*
	Syracuse University	*Vertigo bollesiana*, *V. gouldii gouldii*
Quebec	La Grande Pointe	*Vertigo cristata*, *V. paradoxa*
Wisconsin	Benderville	*Vertigo bollesiana*, *V. gouldii gouldii*, *V. hubrichti*, *V. iowaensis*
	Blueberry Marsh	*Vertigo cristata*, *V. nylanderi*
	Potawatomie State Park	*Vertigo bollesiana*, *V. gouldii gouldii*, *V. hubrichti*, *V. iowaensis*

Phylogenetic trees for each gene, as well as a concatenated sequence from the two mitochondrial genes, were constructed as follows: (1) nearest-neighbor-joining trees were generated in Mega 4.0 using pairwise gap deletion with support values being estimated from 1000 bootstrap replicates. (2) Maximum parsimony trees were generated in Mega 4.0 using close neighbor interchange search option (search level = 1) with an initial tree by random addition of 10 replicate trees. (3) Bayesian trees were generated using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) using a GTR substitution model assuming gamma-shaped rate variation over 1,000,000 generations with a sampling frequency of once each 1000 generations. (4) Maximum likelihood trees were generated using TreePuzzle 5.2 (Schmidt et al., 2002) using the HKY substitution model. For the concatenated trees, three samples were removed as they lacked either *CO1* or *16S* sequences: NS45 (*V. cristata* large morph) and NS51 (*V. californica*) require *16S* sequences, while NS7 (*V. arthuri*) requires the *CO1* sequence.

2.7. Species delimitation

Identification of provisional species-level branches was accomplished using the Generalized Mixed Yule-Coalescent (GMYC) function of Pons et al., (2006), which analyzes branch lengths to determine the temporal threshold(s) within which intra-species variation is supported. Because of the limited sequence variability noted in both the nuclear *ITS-2* and mitochondrial *16S* regions (see below), analyses were limited to the mtDNA *CO1* sequence as it provided the most resolution. The *CO1* nearest-neighbor-joining tree was used because its major modes were supported by all of the other phylogenetic reconstruction methods. This tree was converted to an ultrametric format using PATHd8 (Britton et al., 2007) in combination with estimated temporal constraints (see below), with polytomies being converted to dichotomies with zero branch lengths using the APE library of R. Two GMYC analyses were accomplished: GMYC with a single threshold was first conducted following removal of all zero-length branch tips. Multiple-threshold GMYC was then repeated on the full dataset. The range of supported threshold dates from these two methods was plotted on a log-number-of-lineages by time graph.

2.8. Diversification rates

We explored diversification patterns using the LASER package (Rabosky, 2006a). This approach generates Akaike Information Criterion (AIC) values for best-fit parameterizations of a constant Yule branching (e.g. pure birth) model, plus Yule models with 2, 3, 4, and 5 segments of differing branching rates. *p*-Values were calculated for the observed AIC difference between a given variable-rate model and the constant-rate model using 5000 randomized constant-rate null tree comparisons. A variable-rate birth-death model (Rabosky, 2006b) was not used as LASER was unable to locate a valid optimum in the likelihood surface.

Because this method assumes no intra-species diversification, a pruned *CO1* nearest-neighbor-joining tree was generated by analyzing only a single representative for each putative species-level taxa indicated by GMYC analysis in addition to three supplemen-

tary taxa supported by their highly distinctive and unique shell features (see below). Specimens included in this pruned tree were: 22, 23, 26, 28, 51, NS1, NS4, NS12, NS13, NS15, NS16, NS23, NS29, NS36, NS43, NS47, NS48, NS49, NS50, NS51, NS52, NS53, NS54, and NS58. This tree was then converted to an ultrametric format using PATHd8 (Britton et al., 2007) in combination with estimated temporal constraints (see below) with polytomies being converted to dichotomies with zero-length branches using the APE library of R. The pruned ultrametric tree was plotted with supported thresholds of rate diversification change.

3. Results

3.1. DNA sequence data

DNA sequences were obtained for 72 specimens for *CO1*, 71 specimens for *16S*, and 42 specimens for the *ITS-2* region. All *CO1* fragments were 655 bases in length and could be unambiguously aligned. The *16S* fragment length was 443–446 bases for *Vertigo* individuals, 450–454 for *Pupilla* individuals, and 455 for *Gastrocopta tappaniana*. Sequences were unambiguously aligned within all genera. *ITS-2* and flanking regions was 629 bases for *Vertigo ventricosa*, 763 for *V. californica*, 702 for the remainder of *Vertigo*, and 907 for *Pupilla muscorum*. While the flanking regions were highly conserved across genera and could be unambiguously aligned, *ITS-2* itself could only be reliably aligned within the genus *Vertigo*. Patterns of DNA sequence divergence within and between groups are described in Table 3.

3.2. Phylogenetic analyses

The four methods of phylogenetic reconstruction resulted in essentially identical trees for *CO1*, *16S*, and concatenated *CO1* + *16S* (Figs. 2 and 3). The limited amount of sequence variability observed in the *ITS-2* region prevented clear resolution of its tree, although it is heuristically similar to the mtDNA trees as it identifies the same deeply rooted clades. Uit deWeerd et al. (2004) also noted that the nuclear *ITS-1* and *ITS-2* regions were only useful in demarcation of deeply rooted Stylommatophoran gastropod clades due to their very low base pair substitution rates. The concatenated mtDNA tree is not presented because *CO1* and *16S* sequences were not available for all specimens and because its topology is essentially identical to the *CO1* tree.

These trees indicate that the genus *Vertigo* represents a monophyletic clade. Even though sometimes considered a member of a different genus (*Nearctula* of Roth and Sadeghian, 2006), *Vertigo californica/Nearctula rowelli* is in fact more similar in *CO1* to other *Vertigo* species (64–88 bp differences) than it is to *Vallonia, Gastrocopta* or *Pupilla* (91–137 bp differences; Table 3). While *Pupilla* itself appears monophyletic, specimens of *P. muscorum* occur in two branches with the Minnesota individual actually being more similar to *P. hebes*. Sequence analysis generates a *Vertigo gouldii* group phylogeny which is incongruent with the traditional taxonomy, however. The clade containing all purported members of the group includes not only *V. nylanderi* (which Pilsbry, 1948 suggested might be included within it) but also *V. modesta*. *Vertigo cristata* and most *V. gouldii coloradensis* are in fact much more closely related to *V. modesta*. Moreover, the named subspecies of *V. gouldii* are spread across a number of deeply rooted clades.

The focal specimens fell into two well-supported clades: the *Vertigo modesta* clade which consists of *V. modesta, V. concinnula, V. cristata, V. gouldii coloradensis* (in part, see below), *Vertigo* AK 2 and 3; and the *Vertigo gouldii* clade which consists of all the remaining taxa plus *Vertigo* AK 1. Both demonstrate a striking parallelism in terms of an east–west phylogeographic division: Within the *Vertigo modesta* clade the division consisting of *V. cristata*, *V. gouldii coloradensis* (in part, see below), *Vertigo* AK 2 and *Vertigo* AK 3 are split into two well-supported subclades, with one centered on the western mountains, and the other on the north-east. The *Vertigo gouldii* clade is also demarcated into two well-supported geographically distinct subclades, one being centered on the midwest and southwest (including *V. gouldii arizonensis, V. gouldii inserta, V. meramecensis* and the Chiricahua Mountains form of *V. gouldii coloradensis*) and the other on the east and north (including *V. arthuri, V. bollesiana, V. brierensis, V. gouldii basidens, V. gouldii gouldi, V. hannai, V. iowaensis, V. nylanderi*, and *V. paradoxa*) (see Fig. 4).

3.3. Species delimitation

PATHd8 indicated that all lineages except for *Pupilla* ($p = 0.026$), *Vertigo meramecensis* ($p = 0.036$), and *Vertigo* AK 2 ($p = 0.011$) passed a rate homogeneity test, with the former two having too great a substitution rate and the latter being too slow. Single-threshold GMYC analysis of the unique sequence *CO1* ultrametric tree identified the threshold between within and between-species variation to have occurred at 2.7 myBP, assuming a 1%/my bp substitution rate (Fig. 5). This analysis identified 21 provisional species, including among the outgroup taxa: *Pupilla hebes*, 2 species inside of *Pupilla muscorum*, *Gastrocopta tappaniana*, *Vallonia gracilicosta, Vertigo californica, Vertigo hinkleyi, Vertigo ventricosa*; and within the focal taxa: *Vertigo arthuri, Vertigo bollesiana, Vertigo cristata, Vertigo gouldii arizonensis* (hereafter referred to as *V. arizonensis*), *Vertigo gouldii coloradensis* (hereafter referred to as *V. coloradensis*), *Vertigo gouldii inserta* (hereafter referred to as *V. inserta*), *Vertigo gouldii* along with a conchologically indistinguishable cryptic species, *Vertigo hannai, Vertigo meramecensis, Vertigo modesta*, *Vertigo* AK 1, and *Vertigo* AK 2. However, this assessment appears too conservative, as three taxa that were lumped appear to be justifiable at the species level: *Vertigo concinnula, Vertigo nylanderi*, and the Chiricahua Mountains form of *V. coloradensis*. First, each was identified as a unique species in the multiple-threshold GMYC

Table 3
Genetic differentiation ranges for *CO1*, *16S*, and the *ITS-2* region. 'Number' represents the average number of base pair differences observed across that given comparison for the gene fragment in question. 'Percent' represents the average percent distance in sequences based on maximum composite likelihood with pairwise gap deletion.

Comparison	*CO1*		*16S*		*ITS-2*	
	#	%	#	%	#	%
With outgroups outside *Vertigo*	91–137	16.0–25.9	90–121	32.3–62.9	–	–
With outgroups within *Vertigo*	64–88	10.7–15.3	22–46	5.6–12.6	6–17	1.0–2.5
Between *V. gouldii* and *V. modesta* clades	55–77	9.1–13.0	22–30	5.6–7.6	5–13	0.7–1.9
Between main *V. gouldii* clade branches	11–68	1.6–11.2	3–26	0.7–6.5	1–8	0.1–1.2
Between main *V. modesta* clade branches	13–60	2.0–9.9	3–16	0.7–3.8	2–4	0.2–0.6
Between *V. arthuri* forms	0–4	0.0–0.6	0.3–1.9	0.1–0.2	0	0.0
Within species	0–12.4	0.0–2.0	0–3.3	0.0–0.7	0–3	0.0–0.5
Within population	0–3	0.0–0.5	0	0.0	0	0.0

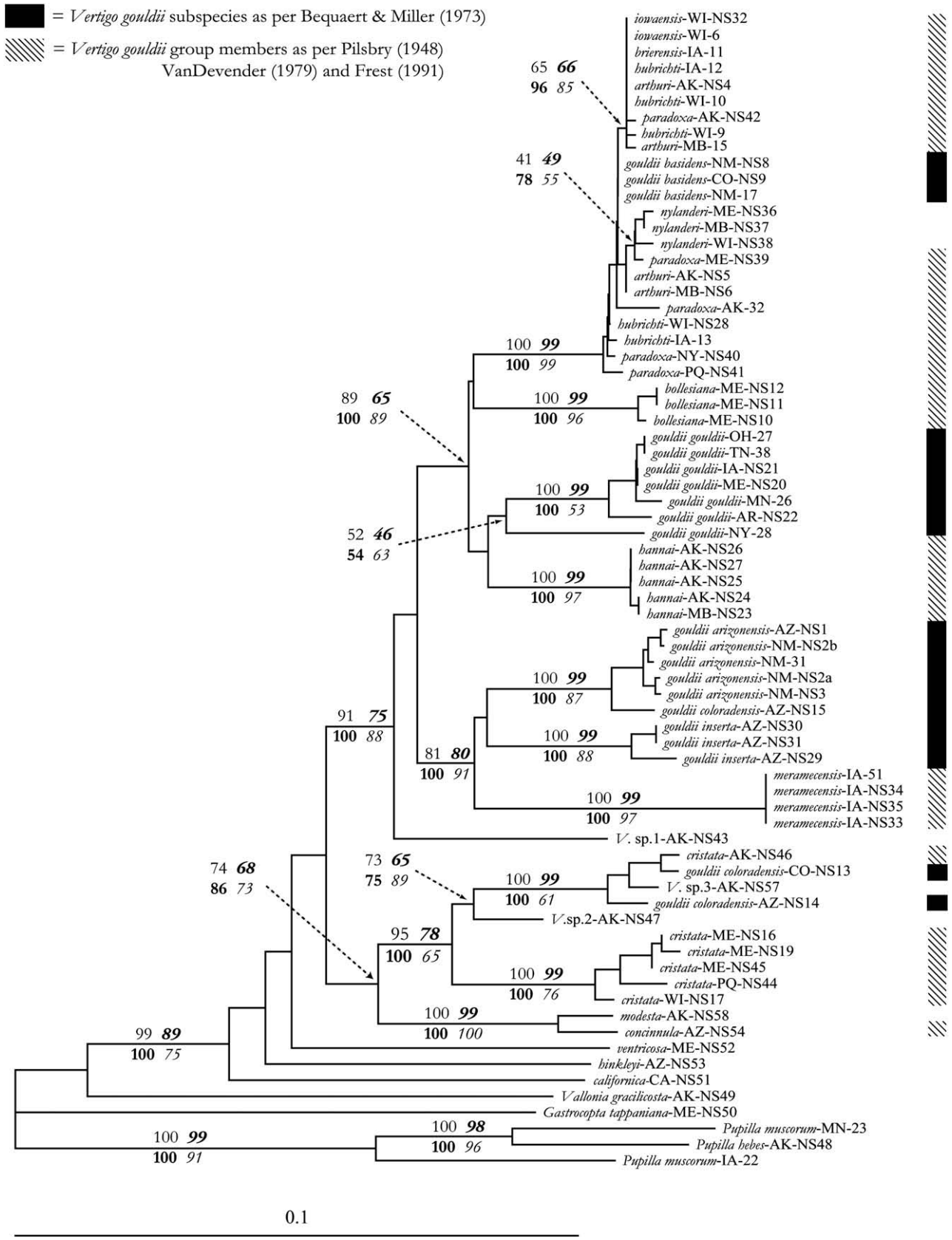

Fig. 2. *CO1* nearest neighbor-joining tree, showing the distribution of *Vertigo gouldii* group individuals (hatched bar) and putative *Vertigo gouldii* subspecies (black bar). Nodes with strong to moderate support across all four phylogenetic reconstructions have been labeled by four numbers to the left of each node. The upper left (normal font) represents the support values for the nearest-neighbor-joining tree. The upper right (***bold italic font***) represents support values for the maximum parsimony tree. The lower left (**bold font**) represents support values for the Bayesian tree. The lower right (*italic font*) represents support values for the maximum likelihood tree.

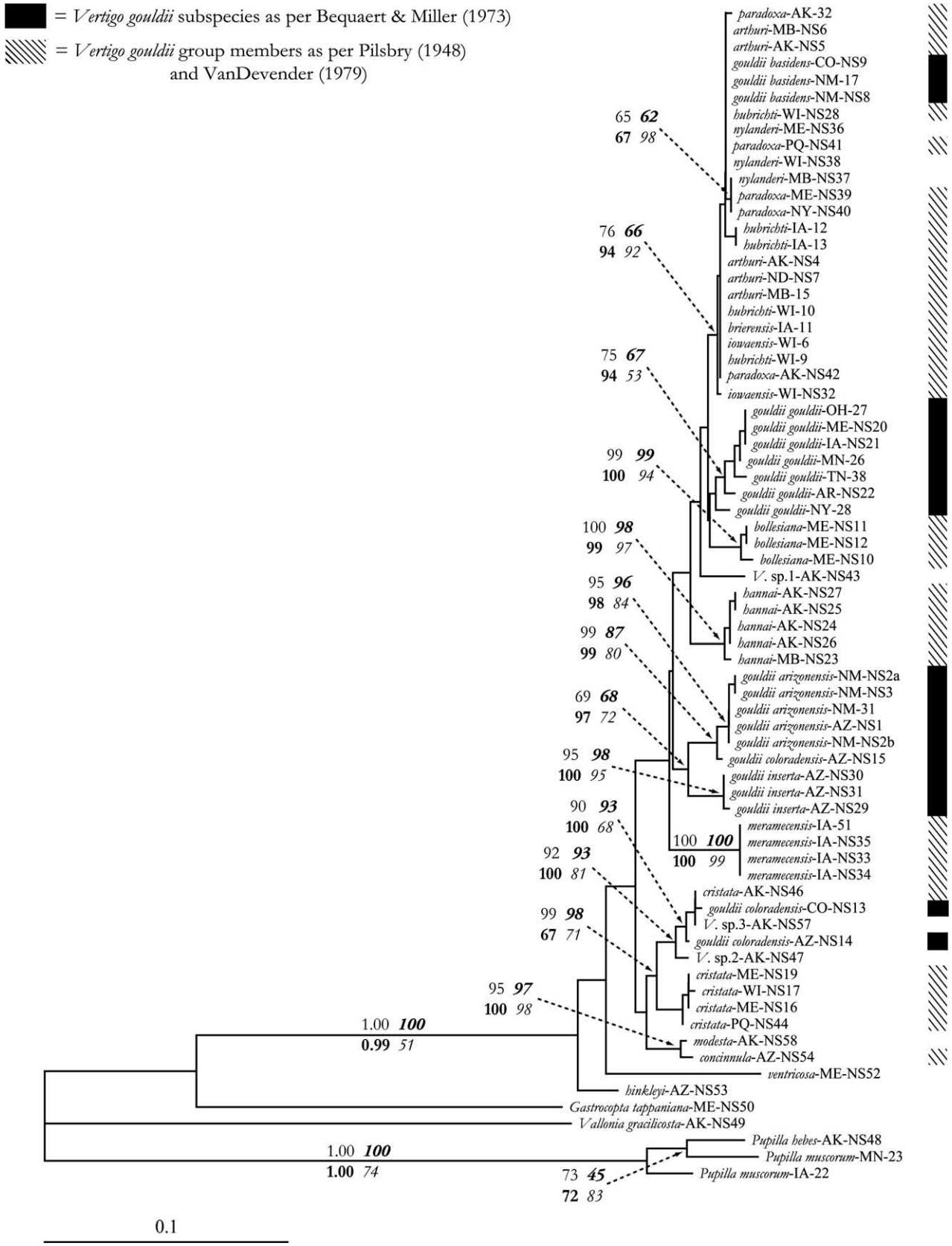

Fig. 3. *16S* nearest neighbor-joining tree, showing the distribution of *Vertigo gouldii* group individuals (hatched bar) and putative *Vertigo gouldii* subspecies (black bar). Nodes with strong to moderate support across all four phylogenetic reconstructions have been labeled by four numbers to the left of each node. The upper left (normal font) represents the support values for the nearest-neighbor-joining tree. The upper right (***bold italic font***) represents support values for the maximum parsimony tree. The lower left (**bold font**) represents support values for the Bayesian tree. The lower right (*italic font*) represents support values for the maximum likelihood tree.

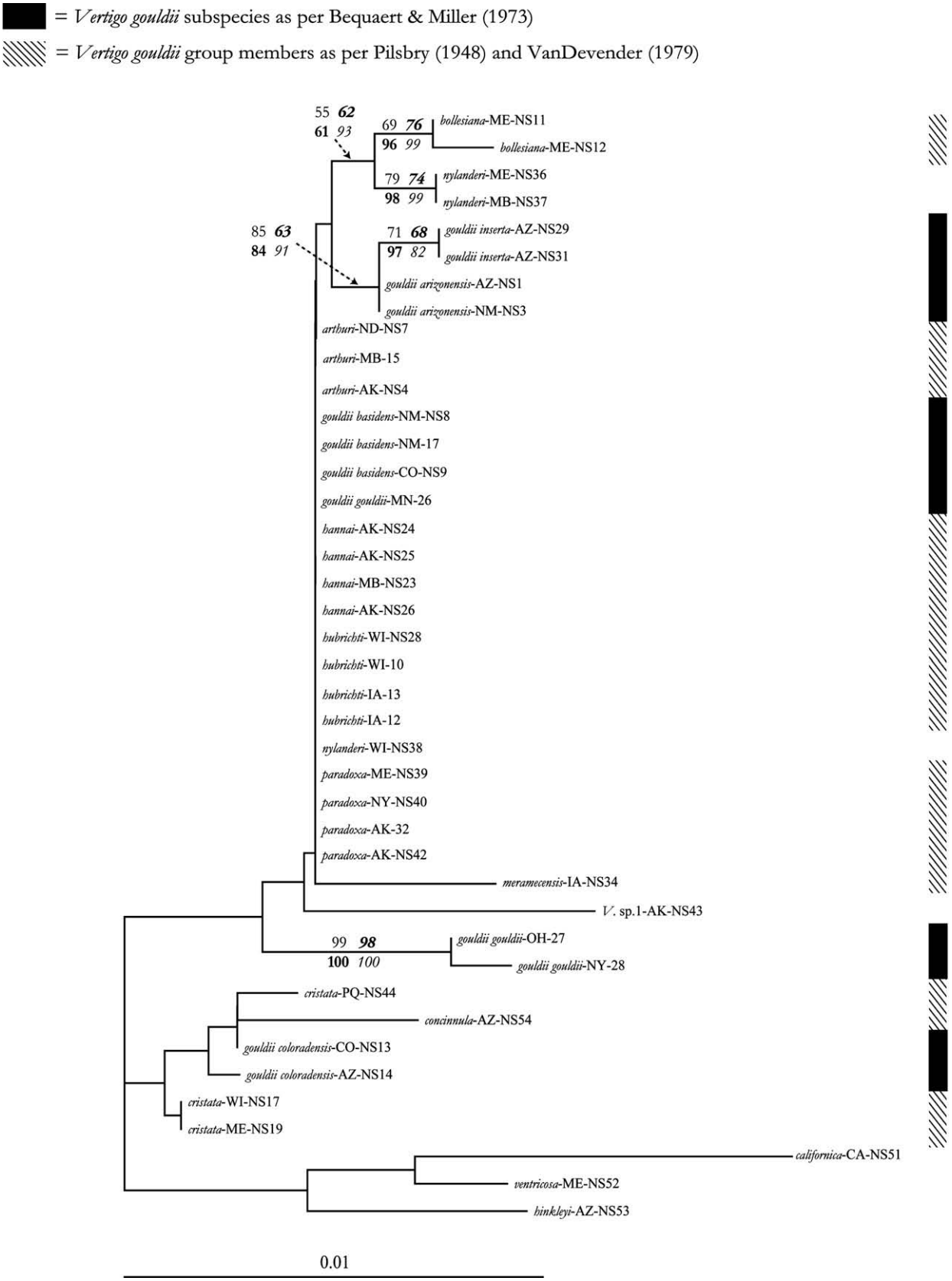

Fig. 4. The *ITS-2* region nearest neighbor-joining tree, showing the distribution of *Vertigo gouldii* group individuals (hatched bar) and putative *Vertigo gouldii* subspecies (black bar). Nodes with strong to moderate support across all four phylogenetic reconstructions have been labeled by four numbers to the left of each node. The upper left (normal font) represents the support values for the nearest-neighbor-joining tree. The upper right (***bold italic font***) represents support values for the maximum parsimony tree. The lower left (**bold font**) represents support values for the Bayesian tree. The lower right (*italic font*) represents support values for the maximum likelihood tree.

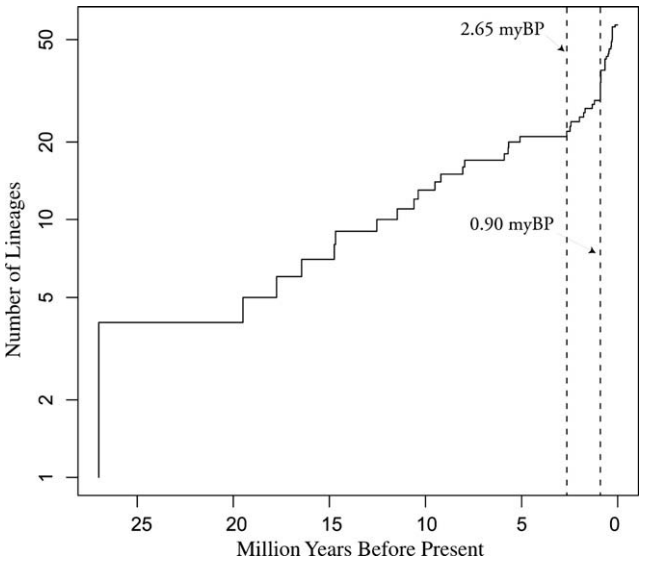

Fig. 5. Log number of lineages vs. time graph from GMYC analysis based on an ultrametric nearest-neighbor-joining *CO1* tree assuming a 1% substitution rate per million years. The threshold between intra- vs. inter-species variation is indicated by the vertical hatched line. The left line (2.65 myBP) reflects the results of single-threshold GMYC subjected to a dataset pruned of all zero-length branches. The right line (0.9 myBP) represents the most ancient threshold produced by multiple-threshold GMYC subjected to the entire unpruned dataset.

(see below). Second, each also possess unique shell characteristics which have never been found to intergrade with their nearest siblings (*V. modesta, V. arthuri*, and *V. arizonensis*, respectively), even within sites of co-occurrence. Additionally, in the case of *V. nylanderi* two individuals were found to be contained within a moderately supported *ITS-2* clade which was most closely related to *V. bollesiana*. As a result, we consider these three to be unique species for the following analyses.

The multiple threshold GMYC on the entire dataset identified thresholds at 0, 0.4, and 0.9 myBP. The latest of these provides an estimate of 36 unique species, many of which possess identical shell morphologies. In conjunction with the fact that the suggested ~1% bp difference threshold is much lower than that usually considered for species-level distinctions (Hebert et al., 2003), we feel that this result is too liberal. We thus suspect that the actual threshold demarcating species occur somewhere between 0.9% and 2.7% *CO1* base pair differences at a 1%/my bp substitution rate (Fig. 5).

3.4. Redefined geographic ranges and sympatry levels

The ranges of traditional members of the *Vertigo gouldii* are shown in Fig. 1. *Vertigo gouldii* and *V. cristata* were thought to possess a continental distribution, with the former occurring from southern Quebec and eastern Maine south to Georgia and Alabama and west to Arizona and southeastern British Columbia, while the latter was distributed across the extent of the North American taiga. *Vertigo concinnula* was considered limited to the western mountains, while *V. bollesiana*, *V. nylanderi*, and *V. paradoxa* were thought confined to the northeastern USA and adjacent Canada. *Vertigo arthuri* was believed confined to the western taiga, while *V. hannai* was limited to the arctic west of Hudson's Bay. *Vertigo brierensis*, *V. hubrichti*, *V. iowaensis*, and *V. meramecensis* were thought local endemics of the Midwestern USA, while *V. hebardi* was limited to the Florida Keys.

Provided the results of the above phylogenetic analyses, the inferred ranges of *Vertigo bollesiana, V. concinnula, V. hannai, V. meramecensis*, and *V. nylanderi* remained unchanged as the species-level concepts of these forms were supported. However, putative *V. cristata* is shown to actually comprise two distinct species, one limited to the eastern taiga (*V. cristata* s.str.) and the other to spruce-fir forest in the western cordillera from central Alaska to southern Arizona (*V. coloradensis*). Because no populations were sequenced from the taiga of central Canada, it is unknown how far west *V. cristata* or how far east *V. coloradensis* extends, or if their ranges overlap. The reinterpreted range of *V. arthuri* is greatly expanded to include the entire North American boreal zone from Newfoundland in the east (former *V. paradoxa*) to Alaska in the north-west (including both *V. arthuri* s.str. and former *V. paradoxa*) south to algific talus slopes (Nekola, 1999) of the upper Midwest (former *V. brierensis, V. iowaensis, V. hubrichti*) and the Rocky Mountains of northern New Mexico (former *V. gouldii basidens*). Notably, all of the taxa previously thought to represent upper Midwest local endemics are included in this single wide-ranging species. Lastly, *V. gouldii* is shown to not represent a single species with continental extent, but rather is limited to eastern North America. Each of the former western subspecies actually represent full species, with *V. arizonensis, V. inserta*, and the Chiricahua Mountains form of *V. coloradensis* being restricted to sky islands in the desert southwest. Based upon specimens held at the Academy of Natural Sciences of Philadelphia, shells consistent with the latter extend south in the montane forests of northern Mexico. Specimens from various other collections also suggest that the two undescribed Alaskan species display apparent Beringian affinities with *Vertigo* AK 1 perhaps extending from central Alaska as far west as the Kuril Islands, and *Vertigo* AK 2 perhaps extending from the Altai in Siberia to the western shore of Hudson's Bay.

Previous analyses have documented a remarkable degree of micro-scale sympatry among taxa in the *Vertigo gouldii* group (Nekola and Smith, 1999; Nekola, 2005). While the present analysis suggests that the number of *Vertigo gouldii* clade species should be reduced by almost half (from 19 to 11), the consequences on local sympatry levels were minimal. Use of the provisional taxonomy suggested here led to only a 13% reduction in the number of sites supporting at least two co-occurring *V. gouldii* clade species (from 48% to 42%), a 14% reduction in the average number of *V. gouldii* clade species per site (from 1.83 to 1.59), and a 33% reduction in the maximum number of co-occurring taxa (from 6 to 4).

3.5. Diversification rates

PATHd8 indicated that all lineages except for *Pupilla* clade ($p = 0.024$) and *Vertigo* AK 2 ($p = 0.021$) passed a rate homogeneity test, with the former having too great a substitution rate and the latter being too low. Comparison of AIC values from LASER analysis (Table 4) indicates that diversification rate has not been constant, with a three-rate Yule model best fitting the data ($p = 0.0012$). In this case, diversification begins at a moderate rate, increases by almost 10-fold, and then drops to a rate almost 75% less than initial. The ultrametric tree and temporal location of these rate-breaks are presented in Fig. 6.

4. Discussion

The results from phylogenetic analysis of mitochondrial genes indicate that the informal groupings of Pilsbry (1948) are largely supported, with 14 of the 19 taxa initially considered members of the *Vertigo gouldii* group being found to reside within the strongly supported *Vertigo gouldii* clade. However, this traditional taxonomy based solely on shell characteristics was not congruent with the mtDNA sequence analysis in the case of the nominal taxa

Table 4
Results from Maximum Likelihood analysis of evolutionary rates for an ultrametric tree pruned to single examples of the 21 taxa recommended by single-threshold GMYC analysis in addition to the three additional species supported multiple-threshold GMYC analysis as well as by conchological evidence (*Vertigo concinnula*, *V. nylanderi* and the Chiricahua Mountains form of *Vertigo coloradensis*), based on statistics generated from the LASER toolkit. The initial four rows represents best-fit parameterizations of Yule (pure birth) models assuming 1–5 different evolutionary rates (per million years) with 0–4 break points (scaled in million years before present) separating them. Both rate and break point parameterization is based on a 1% change in *CO1* sequence per million years. The *p*-value is based on the likelihood that the observed AIC differences between the constant and a given variable-rate model are due to random chance.

	Rate1	Rate2	Rate3	Rate4	Rate5	Break1	Break2	Break3	Break4	AIC	*p*-value
Constant Yule	0.0951									45.3369	–
2-Rate Yule	0.1904	0.0317				6.48				35.9291	0.0044
3-Rate Yule	0.1439	1.1634	0.0384			6.97	6.72			33.2868	0.0012
4-Rate Yule	0.1439	1.1634	0.2406	0.0317		6.97	6.72	6.48		35.1402	0.0024
5-Rate Yule	0.1268	0.5921	0.1214	1.1634	0.0384	12.93	12.34	6.97	6.72	38.4342	0.0122

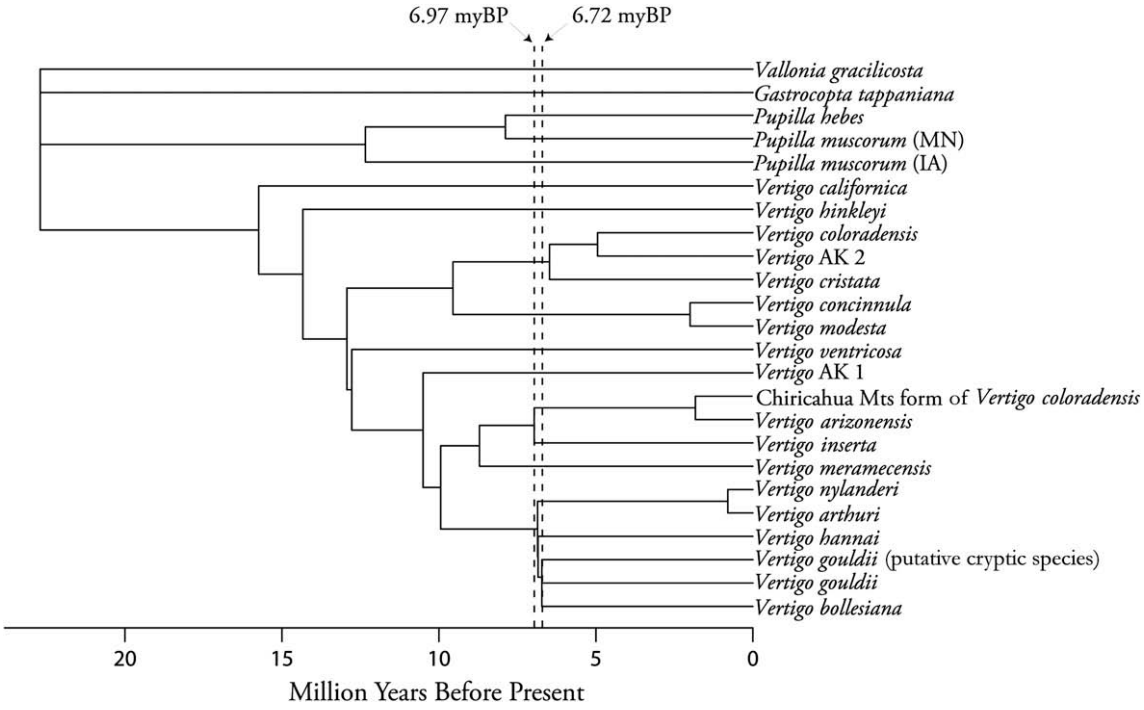

Fig. 6. Ultrametric tree generated by PATHd8 upon a nearest-neighbor-joining *CO1* tree pruned to single representatives of each of the species supported by GMYC or morphological data, assuming a 1% bp substitution rate per million years. The vertical dashed lines represent the two diversification rate-breaks identified via LASER analysis, with diversification rate increasing by ~10× at 6.97 myBP, and then falling by ~30× at 6.72 myBP.

Vertigo concinnula, *Vertigo cristata*, *Vertigo coloradensis*, *Vertigo* AK 2 and *Vertigo* AK 3 which appear to be members of the *Vertigo modesta* clade, with *V. concinnula* actually being sister to *V. modesta*. The close relationship between these latter two species was previously suggested by Bequaert and Miller (1973).

These analyses suggest instances of both oversplitting and over-lumping in the traditional taxonomy. Over-splitting is indicated because five putative taxa (*Vertigo arthuri, V. brierensis, V. gouldii basidens, V. hubrichti, V. iowaensis*, and *V. paradoxa*) appear to be members of the same species-level branch. Because its name has taxonomic priority, these all have been lumped into *V. arthuri*. While mtDNA indicates that *V. nylanderi* also resides in this same species-level branch, we are reluctant to accept this conclusion due to several unique morphological shell features which never intergrade with the various *V. arthuri* forms even in sites of co-occurrence. Over-lumping is also indicated because individuals considered part of *V. gouldii* occur in seven different species-level branches within the mtDNA tree, with some of these actually residing within the *Vertigo modesta* clade.

In addition, preliminary analysis of outgroup taxa also appears to undermine the validity of *Nearctula* as proposed by Roth and Sadeghian (2006). While the type member of this putative genus (*Vertigo californica*/*Nearctula rowelli*) is a member of a well-supported clade in *CO1* analyses that includes all other *Vertigo*, the nodes separating it from *V. ventricosa* and other taxa do not have high support. This specimen is also more similar in *CO1* to other *Vertigo* than it is to *Vallonia, Gastrocopta* or *Pupilla*. Its *ITS-2* region is also sufficiently similar to other *Vertigo* to allow alignment, while it could not be aligned with other genera. Additionally, incongruence was noted within *Pupilla muscorum*, with the individual sourced from an Iowa roadside containing a *CO1* sequence that is most similar to known European *P. muscorum* haplotypes (GenBank Accessions EF457915–EF457920). In contrast, putative *P. muscorum* from a native Minnesota habitat clusters strongly with western North American *P. hebes*.

Without a well resolved nuclear DNA tree, we cannot conclusively state whether such incongruencies between mtDNA and traditional taxonomy are due to hybridization, introgression, or congruent evolution in evolutionary labile shell features. Shimizu and Ueshima (2000) suggest that the mismatch between *CO1* sequences and morphology in certain individuals of the land snails *Euhadra peliomphala* and *E. grandtii* were due to past hybridization events. There is little evidence for this in our data, however, with only *V. coloradensis* appearing in more than one species-level

branch. Even in this lone case, introgression and hybridization appear unlikely as shells from the main *V. coloradensis* branch (part of the *Vertigo modesta* clade) and from the Chiricahua Mountains form (part of the *Vertigo gouldii* clade) are so readily differentiated that we strongly suspected them to be separate species prior to sequence analysis.

These phylogenetic analyses suggest that conchology alone is incapable of deciphering evolutionary relationships because shell features appear highly mutable over evolutionary time. For instance, degree of shell striation is not indicative of close evolutionary relatedness, with strongly and weakly striate taxa occurring in both the *Vertigo gouldii* and *Vertigo modesta* clades. The number, placement and size of apertural lamellae also do not indicate close association, with distantly related taxa such as *Vertigo inserta* and *V. hannai*, or *V. cristata* and *V. meramecensis* possessing identical lamellae configurations. *Vertigo* thus appears to follow other land snail groups such the clausiliid subfamily Alopiinae from Greece (Uit deWeerd et al., 2004), Thailand Gastrocoptinae (Tongkerd et al., 2004), and eastern North American Polygyridae (Emberton, 1995) in which shell features have proven unreliable indicators of phylogenetic relationships. The prediction of Pilsbry (1948) that analysis of non-conchological features would "repay cultivation" in the field of *Vertigo* taxonomy is thus vindicated.

However, it is also important to note that shell features do generally provide sufficient information for species-level identifications and can thus provide accurate documentation of species diversity and biogeography. Species-level identifications based on shells alone were found essentially identical to species concepts based on sequence analysis. For instance, we found all four southwestern *V. gouldii* 'subspecies', which these analyses indicate are full species, to always possess unique shell features even from sites of co-occurrence. Additionally, we noted complete blending of shell traits between all of the various putative "species" shown by these analyses to simply represent *V. arthuri* forms. Only two exceptions were noted: In the first, *V. cristata* and *V. coloradensis* were found to possess essentially identical shells, yet diverged over 7% in their *CO1* sequences. Because our *a priori* expectation was that *V. cristata* would represent a taiga species, while *V. coloradensis* would be restricted to the southwest mountains, we incorrectly initially assigned Alaskan specimen NS46 to *V. cristata*, when in fact it represents *V. coloradensis*. Second, these data also suggest the presence of an apparent cryptic species of unknown biogeographic range with a shell identical to *V. gouldii*.

4.1. Vertigo range size

The provisional taxonomic concepts based on sequence analysis alters our biogeographic understanding of this group, and document that *Vertigo* species commonly possess continental-sized ranges much larger than the 100 km median maximum extent suggested by Solem (1984). For example, all nominal upper Midwest local endemics simply represent forms within a single broadly distributed species (*V. arthuri*) which ranges across boreal North America from the Alaskan interior to western Newfoundland (~5200 km extent) and south in the Rockies to northern New Mexico (~4400 km), giving it one of the most extensive ranges of any western Hemisphere land snail. Other species with extensive ranges include *V. modesta* (~5300 km in North America, with named subspecies extending across the entire Holarctic), *V. coloradensis* (~4800 km), *V. hannai* (~3800 km), *V. cristata* (~3000 km), *V. gouldii* (~2700 km), *V. bollesiana* (~2000 km) and *V. concinnula* (~2000 km). Given that a number of these ranges entirely fall within areas covered by continental ice as recently as 12 kaBP, North American *Vertigo* thus appear to be as subject to long-range passive dispersal as is *Balea* in the eastern Atlantic (Gittenberger et al., 2006).

Members of the *Vertigo gouldii* clade limited to the southwest and midwest tended to possess more limited distributions, however: *Vertigo meramecensis* is restricted to mesic, wooded calcareous cliffs in two disjunct centers of distribution along a 900 km extent focused on the Upper Mississippi River valley and the Ozark Plateau, while *V. arizonensis* (~800 km), *V. inserta* (~250 km), and the Chiricahua Mountains form of *V. coloradensis* (~200 km) are limited to mesic 'sky island' forests in the desert Southwest. As all these taxa are restricted to isolated mesic habitats within a grassland or desert matrix, *Vertigo* would appear to experience a greater degree of habitat isolation per unit distance when their habitats are highly fragmented.

4.2. Sympatry levels

These analyses confirm that the members of the *Vertigo gouldii* clade possess remarkable degrees of micro-scale sympatry. Fully 42% of the 1000 m^2 sites from Nekola (2005) which harbor *V. gouldii* clade species supported more than two, with up to four species being found to co-occur within individual 400 cm^2 microsites of Nekola and Smith (1999). *Vertigo* thus clearly do not share allopatric distribution patterns exhibited by many land snail genera. These results also indicate that substrate differences are not required to maintain reproductive isolation within minute snail species, contrary to the suggestions of Tongkerd et al. (2004).

4.3. Diversification and global change

Identifying potential environmental catalysts underlying the 10-fold diversification pulse in the middle of the phylogram requires the estimation of evolutionary rates. Besides the normal cautions that should be applied towards any molecular clock analyses (Arbogast et al., 2002; Wilke, 2003; Gittenberger et al., 2004; Heads, 2005), *Vertigo* provides an additional challenge because fossil material is generally lacking. The only pre-Neogene North American *Vertigo* fossils are two Eocene taxa from Wyoming (Yen, 1946) which are unlike any modern species. Most Neogene fossil *Vertigo* material in North America is limited to Pleistocene-age lacustrian, loess, and cave-fill deposits. Essentially modern *V. gouldii, V. hannai, V. modesta, V. nylanderi*, and various *V. arthuri* forms have been reported from 10 to 20 kaBP sediments across eastern North America (Hubricht, 1985; Frest, 1991; Frest and Johannes, 1993). *Vertigo cristata, V. nylanderi*, and *V. paradoxa* are reported from 830 kaBP sediments from southern Illinois (Miller et al., 1994). While the average divergence between the latter two taxa is 0.9% in *CO1*, suggesting an approximate upper bound of 1%/my for the *CO1* substitution rate, the limited mitochondrial resolution between these taxa sorely undermines the utility of this report for rate estimation.

Substitution rates ranging from 0.7% to 2.4%/my have been suggested for bivalves and marine gastropods separated by the Isthmus of Panama (Marko, 2002). This estimate has been used in recent terrestrial gastropod phylogenetic studies, including the New Zealand Paryphantinae (Spencer et al., 2006). However, other researchers have claimed that much higher substitution rates exist within land snails, ranging up to 10–25% per million years (Chiba, 1999; Hayashi and Chiba, 2000; Thacker and Hadfield, 2000; Watanabe and Chiba, 2001; Haase et al., 2003; Gittenberger et al., 2004; Van Riel et al., 2005). While critical analysis of these claims is well beyond the scope of this contribution, we do note that rate estimation in all of these papers is not based on the fossil record but rather on inferred formation times of given biogeographic barriers. As these papers all assume that all observed divergence across a given barrier post-dates barrier generation, they also assume that a given dated isolation barrier is absolute with no potential for long-range dispersal being allowed. Given

the known extreme passive long-range capacity of land snails (Gittenberger et al., 2006), such assumptions of absolute vicariance, and consequent substitution rates, must be considered suspect.

Because the *Vertigo arthuri* forms and *V. nylanderi* possessed distinct shells at least 830 kaBP, and because their *CO1* sequences are only ~0.9% different, we follow Spencer et al. (2006) and estimate a 1%/my bp substitution rate. From this, LASER identifies the diversification pulse as occurring over a ~250 ka period ranging from 6.7 to 7.0 myBP. If an 0.5%/my rate is assumed, the resultant 500 ka period would have occurred from 13.4 to 14 myBP, while if a 2.5%/my rate is assumed, the resultant 100 ka period would have occurred from 2.7 to 2.8 myBP. During this time, seven main species-level branches were created, including *V. arthuri*/*nylanderi*, *V. bollesiana, V. hannai, V. gouldii*, its cryptic sister taxon, *V. arizonensis*/Chiricahua Mountains *V. coloradensis*, and *V. inserta*. Additionally, the split between *V. cristata* and *V. coloradensis* occurred within a few 100 ka of this same period. Thus, 53% of the recognized species-level branches owe their origin to the same relatively short temporal window. Additionally, the split between the *Vertigo gouldii* and *Vertigo modesta* clades may extend back 13 myBP (26 myBP at 0.5%/my, 5.2 myBP at 2.5%/my), while the split between eastern and western *Vertigo gouldii* subclades and between *V. modesta/concinnula* and *V. cristata/coloradensis*/AK 2 may extend back 10 myBP (20 myBP at 0.5%/my, 4 myBP at 2.5%/my).

Estimated dates for these deeper nodes provide additional circumstantial evidence to reject rapid (e.g. >10%/my) substitution rates for these *Vertigo*. At such levels, the *Vertigo gouldii* and *Vertigo modesta* clade split would have occurred ~1.3 myBP, with the major east–west split between *Vertigo gouldii* subclades and between *V. modesta/concinnula* and *V. cristata/coloradensis* happening ~1 myBP. Unfortunately, there are no known environmental drivers to explain such results. In fact, the principle impact of Pleistocene glaciations has been the repeated removal of the Great Plains grassland barrier allowing the mixing of eastern and western faunas during full glacial events (Frest and Rhodes, 1981). However, a ~1%/my substitution rate would suggest that diversification within and between the *Vertigo gouldii* and *Vertigo modesta* clades would extend back to the mid-late Miocene where a clear potential environmental trigger can be identified: the segregation of eastern and western North American mesic forests by Great Plains grasslands temporally overlaps with the above 4–20 myBP estimates for divergence between eastern and western clades (Stanley, 2004). It is also tempting to speculate that the identified diversification pulse may be more specifically related to the rapid global shift from C_3 to C_4-dominated grasslands which occurred from 6 to 8 myBP (Cerling et al., 1997). Such a change, presumably driven by additional climatic warming and/or drying, would have further isolated remaining pockets of arcto-tertiary forest, decreasing passive migration rates between them, and providing an impetus for allopatric speciation. This same general period has also been identified as a time of rapid diversification within Proboscideans (Rohland et al., 2007) and ground squirrels (Harrison et al., 2003).

As most species divisions within *Vertigo* appear to have been established before the onset of Pleistocene glaciations, it is unlikely that current distributions (especially for eastern and boreal taxa) provide any useful information regarding sites of origin. Rather, current ranges almost certainly reflect multiple colonization events from either southern or northern refugia (Soltis et al., 2006) across the two-dozen or more glacial cycles. It is also probable that the numerous *V. arthuri* forms represent much more recent diversifications related to cyclical Plio-Pleistocene climatic change. While *V. nylanderi* alone appears to have achieved some degree of reproductive isolation within this group during this time, the remaining forms still appear to frequently exchange genetic information when they come into contact.

A final question is what factors may be responsible for the recent slow diversification rates suggested in the LASER analysis. While we have no definitive answers, we do suggest two possible areas for future investigation. First, it is possible that LASER analysis may prejudice against identification of recent evolutionary events because it requires trees to be pruned to one individual of each recognized species. As a result, there is no way for incipient speciation events to be recognized. It is perhaps useful to note here that LASER analysis of all unique sequences suggests a very rapid rate of evolution over the last ~2 myBP, corresponding to the intra-species thresholds identified by GMYC. Also, it seems possible that elevated recent extinction rates (perhaps caused by cyclic global climate change during the Pleistocene) might lead to a random pruning of branch tips, leading to the appearance of recent evolutionary stasis.

This study thus provides important insights into the phylogeny and evolution of the *Vertigo gouldii* and *V. modesta* clades. Species in these clades appear to have largely diversified during a punctuated burst which may correlate with mid-late Miocene global climate change and forest fragmentation. This round of allopatric speciation did not lead to a change in preferred ecological niche space, however, which allowed newly generated species to later come back into contact at sub-meter scales following habitat coalescence. These now sympatric species remain reproductively isolated across continental extents.

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