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Species assignment in *Pupilla* (Gastropoda: Pulmonata: Pupillidae): integration of DNA-sequence data and conchology

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ABSTRACT

Using the *Pupilla* faunas of Europe, North America, the Altai region of central Asia and eastern Asia, we consider whether the existing taxonomy based primarily on shell apertural characteristics correlates with relationships established on the basis of mitochondrial and nuclear DNA-sequence data. We obtained DNA sequence from nuclear ITS1 and ITS2 and mitochondrial COI and CytB from 80 specimens across 22 putative *Pupilla* taxa. The sequence data were analysed using maximum likelihood, maximum parsimony, Bayesian and neighbour-joining phylogenetic tree reconstruction, as well as base-pair substitution and insertion-deletion analysis. Revised species-level concepts were generated by identifying reciprocally monophyletic clades that exhibited unique conchological features. These analyses document that, although many previously described taxa have biological merit, the highly plastic nature of shell apertural features makes them unreliable indicators of species identity in several independent lineages. However, shell surface sculpture and architecture appear to provide more reliable diagnoses. Because of the traditional reliance of species-level taxonomy in *Pupilla* on plastic apertural features, too many species-level entities have been described in Europe and the Altai. Also, because taxonomically useful shell sculpture features have tended to be ignored, too few species have been described in eastern Asia and North America. As a result, confusion exists about species ranges, ecological tolerances and interpretation of Quaternary fossils within the genus. Based on these analyses three new species are described: *P. alaskensis*, *P. hudsonianum* and *P. hokkaidoensis*.

INTRODUCTION

The use of protein markers and DNA sequence data has shown that conchological traits can be poor indicators of relatedness, for example in eastern North American Polygyridae (Emberton, 1995), Thailand Gastrocoptinae (Tongkerd *et al.*, 2004) and the clausiliid subfamily Alopiinae from Greece (Uit de Weerd *et al.*, 2004). Similarly, our analysis of DNA sequence data in the genus *Vertigo* (Pupilloidea) indicates that while shell features do generally provide accurate assignment of genetically validated species-level entities, they are too labile to resolve evolutionary relatedness (Nekola *et al.*, 2009).

We now extend our molecular studies to include the genus *Pupilla*, a close relative of *Vertigo*. Both genera are in the Orthurethra and have long been considered to be in the same family, the Pupillidae (Pilsbry, 1948; Hubricht, 1985). Like *Vertigo*, *Pupilla* is distributed across the entire Holarctic and members of both genera commonly co-occur within the same sites.

Pupilla species possess a minute shell (<5 mm in height) of conserved cylindrical-ovate form, although shell apertures show considerable variability, ranging from simple to callused and/or lamellate. Consequently, apertural features have been much used as diagnostic species-specific characters (Pilsbry, 1921, 1948). However, preliminary DNA sequence analysis of putative North American *P. muscorum* suggested that this emphasis on shell apertural characters for species assignment was unsatisfactory (Nekola *et al.*, 2009). We have thus undertaken the present study to examine whether the classically used apertural traits used to identify *Pupilla* species are able accurately to assess taxonomy supported by mitochondrial and nuclear DNA sequence data across the Holarctic range of the genus. Because genitalic structure has been found to be of only limited utility in making species-level taxonomic distinctions in this genus (Pokryszko *et al.*, 2009), DNA sequence data offer the only practical non-shell-based method for cross-validation of taxonomic concepts in *Pupilla*.

METHODS

Specimen selection and identification

Specimens used for analysis were primarily obtained from collections made in 2000–2012 (Nekola, 2005; Horsák *et al.*, 2010, 2012; Horsák, Chytrý & Axmanová, 2013; Nekola & Coles, 2010). These include most of the currently recognized *Pupilla* taxa from western and central Europe (east to the Ural Mountains), central Asia (Altai Republic), Japan (Hokkaido) and North America (Canada and the USA including Alaska), as established by original descriptions, authoritative accounts of regional molluscan faunas and monographs (Pilsbry, 1921, 1948; Kerney & Cameron, 1979; Schileyko, 1984; Meng & Hoffman, 2008; Pokryszko *et al.*, 2009; von Proschwitz *et al.*, 2009). We were unable to secure tissue samples from only two species within these target regions: *P. seminskii* Meng & Hoffman, 2009 (Altai Republic) and *P. sterkiana* Pilsbry, 1889 (North America). For each analysed taxon, an attempt was made to select multiple individuals from across their known geographic and ecological range. Six specimens also represent topotype or near-topotype material: AP2 (*P. altaica*), AP13 (*P. alluvionica*), ET7 (*P. muscorum xerobia*), P1 (*P. hebes kaibabensis*), P10 (*P. syngenes*) and P12 (*P. sonorana*). Archival museum material up to 65 years old up to was used to supplement the specimen set for *P. triplicata* (specimens H19-21; Table 1).

Each specimen was taxonomically assigned using currently recognized diagnostic conchological features (Table 2) as reported by Pilsbry (1921, 1948), Kerney & Cameron (1979), Schileyko (1984), Meng & Hoffman (2008), Pokryszko *et al.* (2009) and von Proschwitz *et al.* (2009). In these works, apertural lamellar architecture has been given particular weight, with little variation being reported in their number, shape or placement within a given taxonomic concept. Apertural crest size, callus development and colour are also frequently used as diagnostic features. Shell sculpture, suture depth and shell apex shape have been used less frequently to distinguish some entities.

Based on these diagnoses, shells from all analysed individuals and their respective populations were examined for nine conchological traits (see below).

DNA extraction, PCR amplification and sequence analysis

Live specimens of *Pupilla* were preserved in absolute ethanol, allowed to desiccate at ambient temperature and humidity, or in some cases were used before death. DNA was extracted using the Omega BioTek Mollusk DNA Extraction Kit. Because of the inability of water to displace air within these tightly coiled shells, shell destruction was required to allow access of proteinase to mummified tissue. Thus (with few exceptions) specimens were taken from lots containing multiple examples of each respective taxon, with the actual specimens used for DNA preparation being imaged at 15× magnification prior to shell destruction using methods described by Nekola, Coles & Bergthorsson (2009).

The internal transcribed spacers (plus flanking sequence) of the nuclear ribosomal RNA complex (ITS1 and ITS2), and mitochondrial cytochrome oxidase subunit I (COI) and cytochrome b (CytB) were amplified using published methods with modifications as listed in Table 3. PCR products were sequenced in both forward and reverse directions using Perkin Elmer ABI Big Dye termination and standard protocols. COI and CytB sequences were also obtained from the GenBank database for data analysed by von Proschwitz *et al.* (2009) that could be unambiguously assigned to a single individual (*P. muscorum*, Baden-Würtemberg, Germany; *P. pratensis*, Lagmansro, Östergötland, Sweden; *P. pratensis*, Mecklenburg-Vorpommern, Germany) and for two outgroups (*Vertigo pusilla* and *Gastrocopta cristata*) that were previously analysed by Nekola & Rosenberg (2013).

Phylogenetic analyses

Sequences (excluding primer regions) were aligned using ClustalX with adjustment by eye for ITS1 and ITS2. COI and CytB were concatenated, and ITS1 and ITS2 sequences were analysed as a single construct by omitting 81 invariant base pairs from the intervening 5.8S region. Mega v. 5.0 was used to conduct neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses separately for the concatenated nuclear and mitochondrial DNA sequences. NJ analysis was based on maximum composite distance including transitions and transversions with pairwise gap deletion. MP analysis used the close-neighbour interchange search option with the random addition of 10 replicate trees. ML analysis used all sites and was based on the Tamura-Nei substitution model, a five-category gamma distribution for substitution rates, and the nearest neighbour interchange ML heuristic method. In all cases support values were estimated from 1000 bootstrap replicates. Additionally, Bayesian trees were generated using MrBayes v. 3.1 (Huelsenbeck & 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. Because none of these methods makes full informatic use of insertions and deletions, we also constructed a matrix of all variable bases in both the ITS1 and ITS2 regions, including not only base-pair substitutions, but also insertions and deletions.

Post-hoc species delimitation and conchology

Identification of potential species-level (and higher) clades based on DNA sequence data was accomplished by examining the nDNA and mtDNA trees for highly supported, reciprocally-monophyletic clades. This approach was of limited value for the nDNA data, because of low node support due to limited variation of ~90 informative sites across ~1500 bp. To help resolve relationships using these data, we examined the matrix of variable sites by eye for base-pair substitutions, insertions and deletions held in common among groups of sequences. Apparent incongruencies in specimen placement between the nuclear *vs* mitochondrial sequences were identified as potential cases of interspecific mitochondrial introgression or incomplete lineage sorting.

We have not used any of the various methods for species demarcation using single-locus analyses of base-pair variation (e.g. generalized mixed Yule-coalescent functions). Although we have previously used these methods (Nekola *et al.*, 2009), they universally require generation of ultrametric trees, which assume constant evolutionary rates across all clades. As a result, these methods do not function well when base-pair substitution rates are clade-specific. Because assumption of rate homogeneity appeared unjustified within the current *Pupilla* dataset, we have instead opted for reciprocal-monophyly as our decision-rule to identify potential species-level clades based on genetic data.

We then attempted to verify the biological validity of these potential genetically-supported species concepts by reanalysing shell features from the imaged shells as well as additional shells within each analysed population. The range of expressed shell variation within each reciprocally-monophyletic species-level clade was documented for nine conchological traits: height (mm), width (mm), shell form, apex shape, shell sculpture, suture depth, aperture shape, apertural crest and callus strength, and apertural lamellae number and configuration. Potential species-level clades were considered taxonomically validated when some subset of the above shell features was found to be unique to and thus diagnostic of that entity. Based on this revised taxonomy, we then updated biogeographic and ecological information for each species based on our extensive community ecology datasets (e.g. Horsák *et al.*, 2010; Nekola, 2014) in combination with other published accounts.

Table 1. Information for specimens of *Pupilla* used in DNA sequence analysis.

Taxon/location	Latitude/longitude	Specimen code	Collection and lot no.	Name supported by DNA sequence data (when different)	GenBank accession no.			
					CO1	CytB	ITS1	ITS2
Pupilla alluvionica Meng & Hoffman, 2008								
Russia								
Belyashi, Altai	49.2691°N, 87.9838°E	AP13	JN		KM518545	KM518468	KM518390	KM518312
Pupilla alpicola (Charpentier, 1837)								
Russia								
Belyashi, Altai	49.5206°N, 88.0180°E	AP12	JN		KM518546	KM518469	KM518391	KM518313
Slovakia								
Rakša	48.8792°N, 18.8901°E	H6	MH		KM518547	KM518470	KM518392	KM518314
Važec	49.0685°N, 19.9994°E	H11	MH		KM518548	KM518471	KM518393	KM518315
Rakša	48.8792°N, 18.8901°E	H12	MH		KM518549	KM518472	KM518394	KM518316
Liptovská Teplička	48.9632°N, 20.1044°E	H13	MH		KM518550	KM518473	KM518395	KM518317
Pupilla altaica Meng & Hoffman, 2008								
Russia								
Usť-Muny, Altai	51.7297°N, 85.7382°E	AP1	JN	*P. turcmenica*	KM518551	KM518474	KM518396	KM518318
Kurai, Altai	50.3080°N, 87.6485°E	AP2	JN	*P. turcmenica*	KM518552	KM518475	KM518397	KM518319
Kurai, Altai	50.2334°N, 87.7894°E	AP17	MH	*P. turcmenica*	KM518553	KM518476	KM518398	KM518320
Pupilla bigranata (Rossmässler, 1839)								
France								
Pont, Calvados	48.9774°N, 0.0903°W	AP25	BC 2014.013.00081	*P. muscorum*	KM518554	KM518477	KM518399	KM518321
Pupilla blandi Morse, 1865								
Canada								
Irvine, Alberta	49.9595°N, 110.2511°W	AP34	BC 2014.013.00079		KM518555	KM518478	KM518400	KM518322
Moose Jaw, Saskatchewan	50.0434°N, 105.6246°W	AP35	BC 2014.013.00074		KM518556	KM518479	KM518401	KM518323
USA								
Logan Canyon, Utah	41.7426°N, 111.7603°W	P7	JN 18327	*P. hebes*	KM518557	KM518480	KM518402	KM518324
Las Vegas, New Mexico	35.6415°N, 105.1875°W	P15	JN 18417		KM518558		KM518403	KM518325
Ruby Mountains, Nevada	40.7751°N, 115.3364°W	P16	JN 18280	*P. hebes*	KM518559	KM518481	KM518404	KM518326
Wilson Arch, Utah	38.2745°N, 109.3724°W	P18	JN 18237	*P. hebes pithodes*	KM518560	KM518482	KM518405	KM518327
Pupilla blandi charlestonensis Pilsbry, 1948								
USA								
East Tintic Range, Utah	39.9660°N, 112.0484°W	P2	JN 18257	*P. hebes*	KM518561	KM518483	KM518406	KM518328
Bullion Canyon, Utah	38.4171°N, 112.3126°W	AP28	JN 17211	*P. hebes pithodes*	KM518562	KM518484	KM518407	KM518329
Pupilla blandi pithodes Pilsbry & Ferriss, 1917								
USA								
Bullion Canyon, Utah	38.4171°N, 112.3126°W	AP27	JN 17210		KM518563	KM518485	KM518408	KM518330
Tusas Ridge, New Mexico	36.6519°N, 106.0381°W	AP38	JN 13013		KM518564	KM518486	KM518409	KM518331
Bland, New Mexico	35.7504°N, 106.4569°W	P9	JN 14816		KM518565	KM518487	KM518410	KM518332

Continued

Table 1. *Continued*

Taxon/location	Latitude/longitude	Specimen code	Collection and lot no.	Name supported by DNA sequence data (when different)	GenBank accession no.			
					CO1	CytB	ITS1	ITS2
Pupilla hebes (Ancey, 1881)								
Japan								
Toyokoro, Nakagawa, Hokkaido	42.6050°N, 143.5564°E	VH29	JN	*P.hokkaidoensis*	KM518566	KM518488	KM518411	KM518333
USA								
Knik Island, Anchorage, Alaska	61.5084°N, 149.0343°W	AP29	JN 15390	*P. alaskensis*	KM518567	KM518489	KM518412	KM518334
Ute Creek Canyon, Colorado	38.5848°N, 105.9686°W	AP37	JN 12898	*P. blandi*	KM518568	KM518490	KM518413	KM518335
Happy Valley, Alaska	69.3355°N, 148.7302°W	NS48	JN 15142	*P. alaskensis*	GQ921663	KM518491	KM518414	KM518336
Jarbidge Mountains, Nevada	41.6867°N, 115.5061°W	P5	JN 18292	*P. hebes pithodes*	KM518569	KM518492	KM518415	KM518337
Loope East, California	38.6591°N, 119.7222°W	P14	JN 17254		KM518570	KM518493	KM518416	KM518338
Pupilla hebes kaibabensis Pilsbry & Ferriss, 1911								
U.S.A.								
Kaibab Plateau, Arizona	36.8299°N, 112.2542°W	P1	JN 18400	*P. hebes*	KM518571	KM518494	KM518417	KM518339
Pupilla hebes nefas Pilsbry & Ferriss, 1910								
USA								
Santa Catalina Mts., Arizona	32.4196°N, 110.7311°W	P6	JN 14052	*P. hebes pithodes*	KM518572	KM518495	KM518418	KM518340
Pupilla cf. *khunjerabica* Auffenberg & Pokryszko, 2009								
Russia								
Chagan-Uzun, Altai	50.0869°N, 88.3941°E	AP11	MH		KM518573	KM518496	KM518419	KM518341
Pupilla cf. *limata* Schileyko, 1989								
Russia								
Bestyakh, Yakutia	61.3624°N, 128.8433°E	AP20	MH		KM518574	KM518497	KM518420	KM518342
Kapitonovka, Yakutia	62.3292°N, 129.9282°E	AP39	MH		KM518575	KM518498	KM518421	KM518343
Pupilla loessica Ložek, 1954								
Russia								
Belyashi, Altai	49.4186°N, 87.5928°E	AP5	JN		KM518576	KM518499	KM518422	KM518344
Kosh-Agach, Altai	49.9929°N, 88.5496°E	AP6	JN		KM518577	KM518500	KM518423	KM518345
Belyashi, Altai	49.2804°N, 87.4955°E	AP7	JN		KM518578	KM518501	KM518424	KM518346
Kosh-Agach, Altai	49.6609°N, 88.2278°E	AP8	JN		KM518579	KM518502	KM518425	KM518347
Belyashi, Altai	49.2691°N, 87.9838°E	AP9	JN		KM518580	KM518503	KM518426	KM518348
Belyashi, Altai	49.5206°N, 88.0180°E	AP10	JN		KM518581	KM518504	KM518427	KM518349
Ulagan, Altai	50.4767°N, 87.6301°E	AP19	MH		KM518582	KM518505	KM518428	KM518350
Pupilla muscorum (Linnaeus, 1758)								
Europe								
Czech Republic								
Brno, Moravia	49.2509°N, 16.5738°E	mtG-Pup	MH		KM518583	KM518506	KM518429	KM518351
USA (naturalized)								
Cedar Rapids, Iowa	41.9866°N, 91.7400°W	22	JN 14592		GQ921664			
Syracuse, New York	43.0074°N, 76.1105°W	AP26	JN 13955		KM518584	KM518507	KM518430	KM518352

Canada								
Cochrane, Alberta	51.2642°N, 114.7326°W	AP36	BC 2014.013.00058	*P. hudsonianum*	KM518585	KM518508	KM518431	KM518353
Churchill, Manitoba	58.7086°N, 94.1230°W	P8	JN 11098	*P. hudsonianum*	KM518586	KM518509	KM518432	KM518354
La Grande Pointe, Quebec	50.2059°N, 63.3968°W	P13	BC 2014.013.00001	*P. hudsonianum*	KM518587	KM518510	KM518433	KM518355
USA								
Bullion Canyon, Utah	38.4171°N, 112.3303°W	P17	JN 17219	*P. hebes*	KM518588	KM518511	KM518434	KM518356
Lake Bemidji, Minnesota	47.5328°N, 94.8247°W	23	JN 9054	*P. hudsonianum*	GQ921662			KM518357
Lake Bemidji, Minnesota	47.5328°N, 94.8247°W	AP33	JN 9054	*P. hudsonianum*	KM518589	KM518512	KM518435	KM518358
Pupilla muscorum xerobia Pilsbry, 1914								
USA								
Bannon Ranch, New Mexico	36.9166°N, 103.7800°W	ET7	JN 16491	*P. blandi*	KM518590	KM518513	KM518436	KM518359
Lake Bemidji, Minnesota	47.5328°N, 94.8247°W	P4	JN 9054	*P. blandi*	KM518591	KM518514	KM518437	KM518360
Pupilla pratensis (Clessin, 1871)								
Russia								
Aktash, Altai	50.4472°N, 87.6078°E	H3	MH	*P. alpicola*	KM518592	KM518515	KM518438	KM518361
Ulagan, Atlai	50.4767°N, 87.6218°E	H4	MH	*P. alpicola*	KM518593	KM518516	KM518439	KM518362
Europe								
Czech Republic								
Pozděchov, Moravia	49.2339°N, 17.9864°E	H1	MH	*P. alpicola*	KM518594	KM518517	KM518440	KM518363
Vysoké Mýto, Bohemia	49.9611°N, 16.1892°E	H7	MH	*P. alpicola*		KM518518	KM518441	KM518364
Slovakia								
Závod	48.5331°N, 16.9963°E	H5	MH	*P. alpicola*	KM518595	KM518519	KM518442	KM518365
Pupilla sonorana (Sterki, 1899)								
USA								
Sacramento Mountains, New Mexico	32.7141°N, 105.7541°W	P12	BC 2005.011.03117		KM518596	KM518520	KM518443	KM518366
Pupilla sterrii (Forster, 1840)								
Albania								
Periferi Dibre	41.8172°N, 20.5003°E	AP16	MH		KM518597	KM518521	KM518444	KM518367
Czech Republic								
Pavlov, Moravia	48.8773°N, 16.6635°E	H8	MH		KM518598	KM518522	KM518445	KM518368
Praha-Hlubočepy, Bohemia	50.0419°N, 14.3761°E	H14	MH		KM518599	KM518523	KM518446	KM518369
Klentnice, Moravia	48.8467°N, 16.6405°E	AP22	MH		KM518600	KM518524	KM518447	KM518370
Russia								
Verkhne Bikberda, Bashkortostan	52.3207°N, 56.8032°E	AP15	MH		KM518601	KM518525	KM518448	KM518371
Slovakia								
Valaská Dubová	49.1511°N, 19.6441°E	AP21	MH		KM518602	KM518526	KM518449	KM518372
Pupilla syngenes (Pilsbry, 1890)								
USA								
Mogollon, New Mexico	33.3944°N, 108.8056°W	AP30	BC 2005.011.02961		KM518603	KM518527	KM518450	KM518373
Kaibab Plateau, Arizona	36.6918°N, 112.2989°W	P10	BC		KM518604	KM518528	KM518451	KM518374
Pupilla syngenes dextroversa Pilsbry & Vanatta, 1900								
USA								
Kaibab Plateau, Arizona	36.6918°N, 112.2989°W	P11	BC	*P. syngenes*	KM518605	KM518529	KM518452	KM518375

Continued

Table 1. *Continued*

Taxon/location	Latitude/longitude	Specimen code	Collection and lot no.	Name supported by DNA sequence data (when different)	GenBank accession no.			
					CO1	CytB	ITS1	ITS2
Pupilla triplicata (Studer, 1820)								
Russia								
Ozero Kureevo, Altai	52.4811°N, 85.7605°E	AP32	MH		KM518606	KM518530	KM518453	KM518376
Czech Republic								
Točník, Bohemia	49.8905°N, 13.8866°E	H2	MH		KM518607	KM518531	KM518454	KM518377
Pavlov, Moravia	48.8773°N, 16.6635°E	H9	MH		KM518608	KM518532	KM518455	KM518378
Milešov, Bohemia	50.5344°N, 14.9437°E	H10	MH		KM518609	KM518533	KM518456	KM518379
Kamýk, Bohemia	50.5643°N, 14.0887°E	H15	MH		KM518610	KM518534	KM518457	KM518380
Chroustov, Moravia	49.1718°N, 16.0502°E	H16	MH		KM518611	KM518535	KM518458	KM518381
Hracholusky, Bohemia	49.9974°N, 13.7904°E	H17	MH		KM518612	KM518536	KM518459	KM518382
Louny, Bohemia (1948)		H19	VL		KM518613	KM518537	KM518460	KM518383
Kamýk, Bohemia (1970)		H20	VL		KM518614	KM518538	KM518461	KM518384
Pupilla triplicata (Studer, 1820)								
Czech Republic								
Srdov, Bohemia (1950)		H21	VL		KM518615	KM518539	KM518462	
France								
Cahors, Dordogne	44.4772°N, 1.4303°E	AP31	BC 2014.013.00082		KM518616	KM518540	KM518463	KM518385
Russia								
Nugush, Bashkortostan	53.0066°N, 56.5356°E	AP23	MH		KM518617	KM518541	KM518464	KM518386
Pupilla turcmenica (O. Boettger, 1889)								
Russia								
Kosh-Agach, Altai	50.0729°N, 88.7201°E	AP3	MH		KM518618	KM518542	KM518465	KM518387
Belyashi, Altai	49.2955°N, 87.7344°E	AP4	MH		KM518619	KM518543	KM518466	KM518388
Kurai, Atlai	50.2334°N, 87.7894°E	AP18	MH		KM518620	KM518544	KM518467	KM518389

All specimens from the Nekola collection (JN) are currently maintained at the University of New Mexico before ultimate deposition at the Academy of Natural Sciences at Drexel University (ANSP). All specimens from the Coles collection (BC) are housed at the National Museum of Wales; all accession numbers for this material are preceded by 'NMW.Z'. All specimens from the Horsák collection (MH) are housed at the Department of Botany and Zoology at Masaryk University, Brno. Material from the Vojen Ložek collection (VL) is housed at Charles University, Prague.

Table 2. Historical taxonomic and traditional conchological concepts for analysed *Pupilla*.

Taxon	Height (mm)	Width (mm)	Shell form	Apex shape	Shell sculpture	Suture depth	Apertural crest	Apertural callus	Apertural lamellae
alluvionica	3.3–4.3	2.2–2.4	Wide cylindrical	Tapered/ domed	Smooth/weak striae		Clearly evident	Hardly thickened, clearly white	0–1 (rare weak parietal)
alpicola	2.8–3.3	1.8–1.9	Wide cylindrical	Domed	Obvious	Deep	Weak - absent		0–1 (rare weak parietal)
altaica	2.5–3.2	1.6–1.8	Cylindrical ovoid	Domed	Fine ribs with dermal edges		Pronounced	Thick, white	2–3 (angular pad; palatal rare)
bigranata	3.0–3.6	1.6	Cylindrical ovoid	Tapered	Almost smooth; fine lines only	Shallow	Present	Thick, white	2–3 (rare columellar)
blandi	2.5–3.3	1.5–1.6	Cylindrical	Domed	Delicate striations	Deep	Present	Yellow/tan	3
blandi charlestonensis	3.0	1.4	Cylindrical	Domed	Slightly striate	Deep	Well developed	Brown	3 (palatal, parietal strong, long)
blandi pithodes	3.1–3.7	1.7–1.8	Wide cylindrical	Domed	Delicate striae	Deep	Low - strong	Weak; brown	3
hebes	3.1–4.0	1.6–1.9	Subcylindric	Domed	Minute striae	Deep	None - weak	None	0–1 (rare parietal)
hebes kaibabensis	2.7–2.8	1.5	Subcylindric	Domed	Minute striae	Deep	None - weak	None	0–1 (rare parietal)
hebes nefas	3.2–4.2	1.7–1.9	Subcylindric, sinistral	Domed	Minute striae	Deep	None - weak	None	1 (parietal)
cf. *khunjerabica*	3.2–4.0	1.7–1.9	Cylindrical ovoid	Tapered/ domed	Almost smooth, striae faint, irregular	Moderately deep	Weak	Thin - lacking	0
cf. *limata*	2.8–3.1	1.6–1.7	Cylindrical ovoid	Tapered/ domed	Delicate striae	Moderately deep	Absent - weak	None	0
loessica	3.0–3.4	1.7–1.75	Cylindrical ovoid	Tapered/ domed	Finely, irregularly ribbed	Deep	Absent - weak	None	0
muscorum	3.2–4.0	1.7	Cylindrical ovoid	Tapered	Almost smooth; fine lines only	Shallow	Strong	Thick, white	0–2
muscorum xerobia	2.3–2.8	1.4–1.5	Cylindrical ovoid	Domed	Almost smooth; fine lines only	Shallow	Strong	Thick, white	1 (parietal)
pratensis	3.5–4.5	1.9–2.1	Cylindrical ovoid	Domed	Fine striation	Deep	Weak	Weak, white	0–2 (weak if present); no depression over palatal
sonorana	2.5–3.3	1.3–1.4	Cylindrical	Domed	Fine striae	Deep	Strong	Thick, white	3 (all long)
sterrii	2.8–3.5	1.6	Cylindrical	Tapered/ domed	Coarse striae	Very deep	Weak - moderate	Moderately thick, white	2 (both peg-shaped)
syngenes	3.0–4.2	1.7–1.8	Biconic, widest at *c.* 4th whorl; 8 narrow whorls; sinistral	Tapered/ domed	Delicate striae	Shallow	Strong		3
syngenes dextroversa	3.0–4.5	1.6–1.8	Biconic, widest at *c.* 4th whorl; 8 narrow whorls; dextral	Tapered/ domed	Delicate striae	Shallow	Strong		3
triplicata	2.2–2.8 (4)	1.4	Cylindrical	Domed	Fine, close striation	Deep	Moderate	Distinct, white	3 (blade shaped)
turcmenica	3.0–3.2	1.1–1.4	Cylindrical; shell thinner	Tapered/ domed	Coarse striae	Very deep	Weak	Weak, white	0–2 (weak parietal and/or palatal)

Table 3. Primers used for genetic analysis.

Region	Direction	Anneal	Sequence	Source
COI	f	45°C	5′-ATTCAACGAATCATAAAGATATTGG-3′	Author Design
	r		5′-TATACTTCAGGATGACCAAAAAACCA-3′	Author Design
CytB	f	47°C	5′-TGAGGTGCAACAGTNATTAC-3′	Author Design
	r		5′-GCAAATAAAAAGTATCACTCTGG-3′	Author Design
ITS1	f	52°C	5′-TAACAAGGTTTCCGTATGTGAA-3′	Armbruster & Bernhard (2000)
	r		5′-TCACATTAATTCTCGCAGCTAG-3′	Author Design
ITS2	f	52°C	5′-CTAGCTGCGAGAATTAATGTGA-3′	Wade & Mordan (2000)
	r		5′-GGTTTCACGTACTCTTGAAC-3′	Author Design

RESULTS

DNA sequence data

A total of 80 specimens from 22 putative *Pupilla* taxa underwent DNA extraction (1–12 individuals/taxon, see Table 1). DNA sequences were obtained for 79 specimens for COI, 77 specimens for CytB and 77 specimens for ITS1 and ITS2. All COI and CytB amplicons consisted of 655 and 377 bp, respectively, and could be unambiguously aligned. The COI amplicon contained 240 and CytB 137 variable sites. The ITS1 amplicon length was 615–635 bp and the ITS2 amplicon length was 868–874 bp. All *Pupilla* ITS sequences could be unambiguously aligned, but those of the outgroup (*Vertigo pusilla* and *Gastrocopta cristata*) could not. The 5.8S region between the ITS amplicons (based on 23 *Pupilla* specimens for which it was determined) consisted of 81 invariant bases so that the entire contiguous sequence between the 5′-end of the ITS1 amplicon and the 3′-end of the ITS2 amplicon is 1569–1593 bp. The total informative sites in ITS consisted of 56 bp substitutions and 36 bp comprising 12 insertions/deletions.

Phylogenetic reconstructions and supported taxonomic entities

Phylogenetic tree reconstruction and base-pair variation maps based on concatenated COI + CytB mtDNA and ITS1 + ITS2 nDNA sequences support the presence of 17 putative reciprocally-monophyletic species or subspecies-level taxa (Figs 1 and 2, Table 4): *P. alluvionica, P. alpicola, P. blandi, P.* cf. *khunjerabica, P.* cf. *limata, P. hebes, P. hebes pithodes, P. loessica, P. muscorum, Pupilla* n. sp. (Alaska), *Pupilla* n. sp. (Hokkaido), *Pupilla* n. sp. (Hudsonian), *P. sonorana, P. sterrii, P. syngenes, P. triplicata* and *P. turcmenica*. The three new species identified by these analyses are formally described below and will be referred to hereafter as *P. alaskensis, P. hokkaidoensis* and *P. hudsonianum*, respectively.

Pupilla alluvionica is a xeric rock outcrop and steppe species that differs from all other analysed *Pupilla* by possessing adult shells >2.1 mm in diameter with a smooth or very weakly striate shell, a crest and a white callus. The single individual analysed for DNA sequence possessed an ITS1 + ITS2 sequence with four bases different from all other *Pupilla* (111C and 577A in ITS1; 333G and 490C in ITS2). However, its COI + CytB sequence was part of the same highly supported clade defining *P. turcmenica*, which consistently co-occurs with *P. alluvionica* in the Altai.

Pupilla alpicola is a wetland species whose shells are up to 2.1 mm wide with a shallow suture and a body whorl often slightly narrower than the penultimate whorl. This species is defined by 92C and usually 340C in ITS2. Two subpopulations are noted, one with AC at 171–172 in ITS1 and the other maintaining the consensus GT at these positions. While the former subpopulation is more prevalent in Europe and the latter in central Asia, individuals characteristic of either occur throughout its known range. *Pupilla pratensis* has been traditionally differentiated from *P. alpicola* by lacking a depression or flattening on the palatal wall of the aperture and having a rather pronounced shell apex (von Poschwitz *et al.*, 2009). However, *P. pratensis* shares the same unique ITS2 bases as *P. alpicola,* with individuals referable to *P. pratensis* occurring in both of the unique ITS1 subpopulations. Additionally, *P. pratensis* mtDNA occurs throughout the same highly supported clade that contains all analysed *P. alpicola*. If *P. pratensis* is considered a shell form of *P. alpicola*, then *P. alpicola* is monophyletic for both nDNA and mtDNA.

According to Pilsbry (1948), *P. blandi* is characterized by a cylindrical shell with a prominent crest, a yellow to tan callus and three apertural lamellae. Using these criteria, individuals conchologically assignable to *P. blandi* demonstrate polyphyly both in ITS1 + ITS2 and COI + CytB, occurring within three well-supported species-level clades. However, if these traditional conchological characteristics are abandoned in favour of surface sculpture, with *P. blandi* being differentiated by its irregular, very weak striae, shiny shell surface and shallow suture, this species becomes a strongly supported monophyletic entity for COI + CytB that uniquely possesses a GAC insertion at 181–183 in ITS2. Because this entity varies in apertural lamella number from 0 to 3, and has a crest and callus ranging from weak to strong, it includes a number of shell forms that were previously assigned to other taxa including *P. hebes* and *P. muscorum xerobia*.

Pilsbry (1948) characterized *P. hebes* as possessing a minutely striate, subcylindric shell with no apertural callus, an absent to rarely weak apertural crest and absent to rarely weak parietal lamella. Shells displaying these traits demonstrate polyphyly among three different species-level clades. However, as with *P. blandi*, monophyletic grouping is apparent when a different suite of shell features is used to diagnose *P. hebes*, including a cylindrical-ovoid shell tapered for the upper 1/3-1/4 of the shell height, a normal to deep suture and possession of numerous sharp thread-like striae. Shells possessing these features all uniquely possess 508T in ITS1, while sharing 495A in ITS1 with *P. blandi* and *P. hebes pithodes*. Using the shell characters of Pilsbry (1948) for identification causes some individuals within this group to be incorrectly assigned to *P. blandi*, *P. blandi charlestonensis* and *P. muscorum*. It should be noted that COI + CytB suggests that *P. hebes* exists as two discrete subpopulations, one ranging throughout the Great Basin from California to north-central Utah (samples P5, P7, P14 and P16) and the other being restricted to the canyonlands region of the Colorado Plateau (samples P1, P2 and P17). This latter subpopulation would equate to *P. hebes kaibabensis* of Pilsbry (1948). However, as the shells of this clade completely overlap with typical material as well as possessing identical ITS1 + ITS2 sequence, it seemed best not to formally recognize this subpopulation at this time.

Pilsbry (1948) characterized *P. blandi pithodes* as being wider than *P. blandi*, with a weak to absent crest and callus. He hypothesized that it was intermediate between *P. blandi* and *P. hebes*. ITS1 + ITS2 indicate that in fact this entity is more

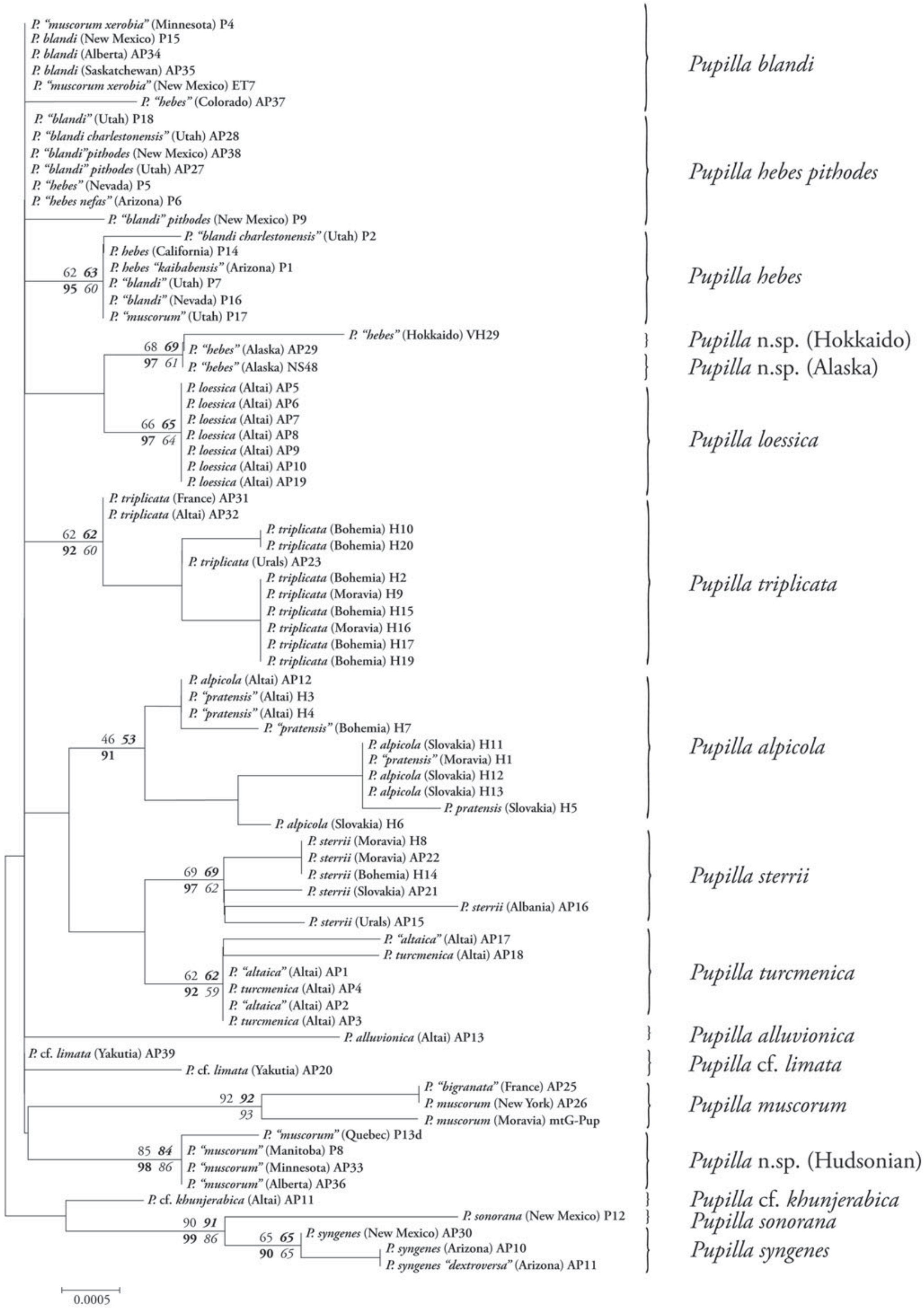

Figure 1. Maximum-likelihood phylogenetic tree reconstruction for *Pupilla* based on concatenated ITS1 + ITS2 data. Nodes with strong to moderate support across all four phylogenetic reconstruction methods have been labelled to the left of that node by four support values: upper left (normal font) is for NJ; upper right (bold italic font) is for MP; lower left (bold font) is for Bayesian; lower right (italic font) is for ML. Branch tip labels represent initial identifications based on traditional conchological features, whereas labels to the right of brackets represent valid names supported by both nDNA and mtDNA sequence analysis.

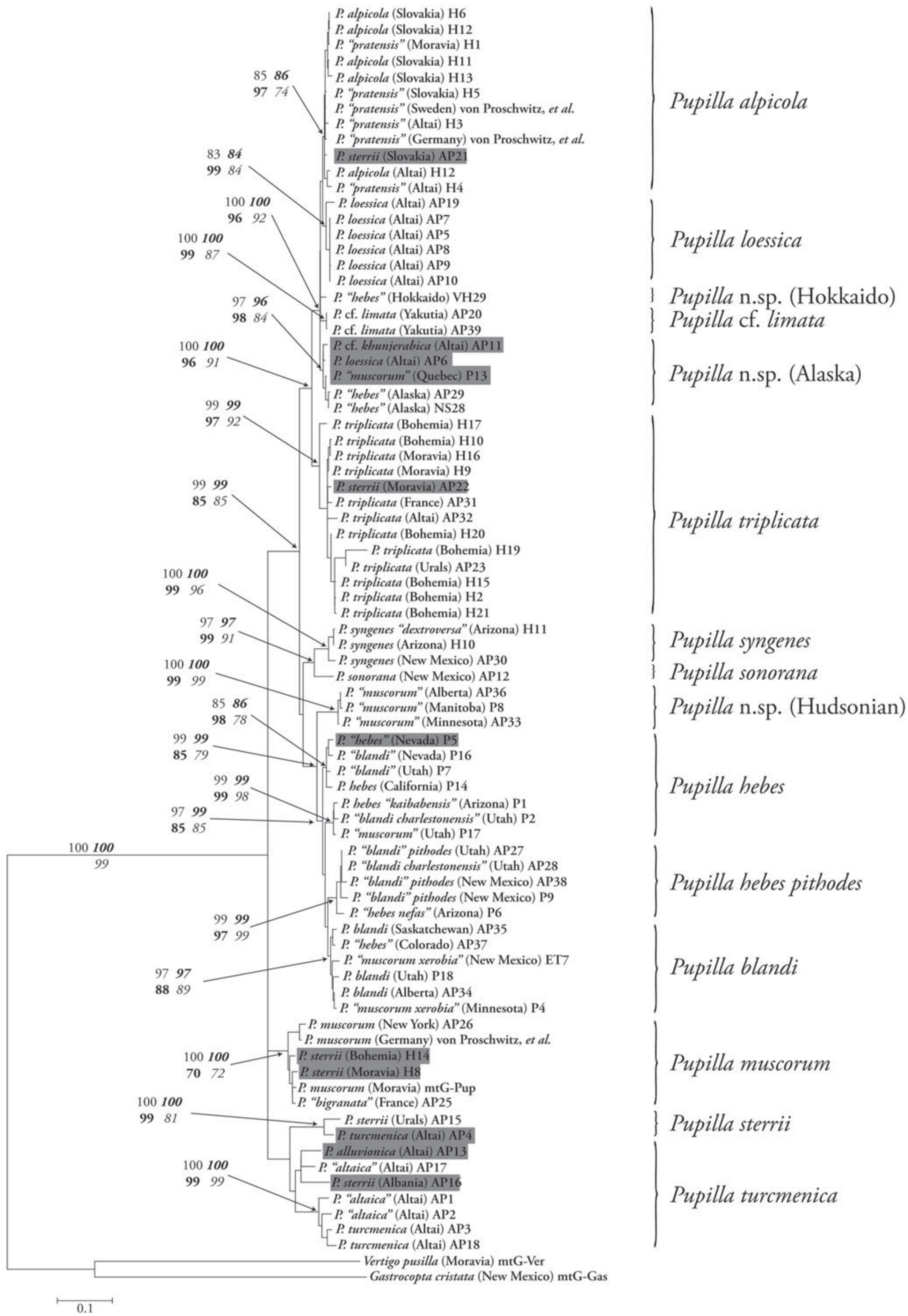

Figure 2. Maximum-likelihood phylogenetic tree reconstruction for *Pupilla* based on concatenated COI + CytB. Labelling conventions as in Figure 1. Specimens that have a significantly different topological location as compared with the nDNA tree are highlighted in gray.

Table 4. Matrix of variable bases in ITS1 and ITS2.

Species / Sample information	ITS1	ITS2	SG
	1 1 1 2 2 2 2 2 3 3 3 3 3 3 4 4 4 4 4 5 5 5 5 5 5 6 6 6 6 3 3 4 4 4 4 4 4 4 4 4 6 6 7 7 7 7 7 7 9 1 7 7 0 0 1 6 6 1 1 3 5 5 7 3 4 5 8 9 0 0 3 5 6 7 0 1 2 2 8 9 0 1 2 3 4 5 6 7 8 8 9 0 1 2 3 4 5 5 1 1 2 2 7 8 6 7 1 2 9 5 8 5 7 4 3 8 5 8 9 8 9 1 7 9 7 5 9	1 1 1 1 1 1 1 2 2 2 2 2 2 3 3 3 3 3 3 4 4 4 4 5 5 5 5 5 5 6 7 7 5 7 7 8 9 3 4 5 7 8 8 8 0 0 0 3 7 9 1 3 3 4 5 5 1 1 1 9 9 0 0 0 2 4 5 8 8 1 4 6 6 9 3 2 7 3 6 7 1 2 3 0 1 7 3 8 5 4 3 6 0 6 8 4 5 6 0 1 4 5 6 9 1 1 3 8 7 0	
Pupilla blandi			
P. blandi (New Mexico); P15	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATATGAC--TGATGCTTC----TA---AATTACA	1
P. blandi (Alberta); AP34	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATATGAC--TGATGCTTC----TA---AATTACA	1
P. blandi (Saskatchewan); AP35	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATATGAC--TGATGCTTC----TA---AATTACA	1
P. m. xerobia (New Mexico); ET7	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATATGAC--TGATGCTTC----TA---AATTACA	1
P. m. xerobia (Minnesota); P4	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATATGAC--TGATGCTTC-GCCTA---AATTACA	1
P. hebes (Colorado); AP37b	GCGGCCCAGGC--------TTGATATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATATGAC--TGATGCTTC----TA---AATTACA	1
Pupilla hebes			
P. h. kaibabensis (Arizona); P1	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-ATAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. blandi (Utah); P7	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-ATAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. hebes (California); P14	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-ATAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. blandi (Nevada); P16	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-ATAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. muscorum (Utah); P17	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-ATAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. b. charlestonensis (Utah); P2	GCGGCCCAGGC--------TTGTTATGTGTAC-TGTC-ATAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
Pupilla hebes pithodes			
P. b. pithodes (New Mexico); AP38	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. b. pithodes (Utah); AP27	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. b. charlestonensis (Utah);AP28	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. h. nefas (Arizona); P6	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. blandi (Utah); P18	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. b. pithodes (New Mexico); P9	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AACTACA	1
Pupilla cf. limata			
P. cf. *limata* (Yakutia); AP39	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTACA	1
P. cf. *limata* (Yakutia); AP20	GCGGCCCAGGC--------TTGTTATGTGTAC-TTCC-AGAT-TGGTAT	-GTGTATAT-----TGGTGCTTC----TA---AATTACA	1
Pupilla hudsonianum			
P. muscorum (Minnesota); AP33	NCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	AGTGTATAT-----TGATGCTTC----TA---AGTCACA	2
P. muscorum (Alberta); AP36	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	AGTGTATAT-----TGATGCTTC----TA---AGTCACA	2
P. muscorum (Manitoba); P8	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	AGTGTATAT-----TGATGCTTC----TA---AGTCACA	2
P. muscorum (Quebec); P13	GCGGCCCAGGCGGCA----TTGTTATGTGTAC-TTTC--GAT-TGGTAT	AGTGTATAT-----TGATGCTTC----TA---TGTCACA	2
Pupilla syngenes			
P. syngenes (Arizona); P10	GCGGCCCAGGC--------CTGTTATGTGTAC-TTTT--GAT-TGGTAT	-GTATATGT-----TAATGCTTC----TA---AATTACA	3
P. s. dextroversa (Arizona); P11	GCGGCCCAGGC--------CTGTTATGTGTAC-TTTT--GAT-TGGTAT	-GTATATGT-----TAATGCTTC----TA---AATTACA	3
P. syngenes (New Mexico); AP30	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTT--GAT-TGGTAT	-GTATATGT-----TAATGCTTC----TA---AATTACA	3
Pupilla sonorana			
P. sonorana (New Mexico); P12	GCGGCCCAGGCGGCA----TTGTTAAGTGTAC-TTTT--GAC-TGGTAT	-GTGTATGT-----TAATGCTTA----TA---AATTACA	3
Pupilla cf. khunjerabica			
P. cf. *khunjerabica* (Altai);AP11	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	-GTGTCTGT-----TGATGCTTC----TA---AATTACA	3
Pupilla loessica			
P. loessica (Altai); AP5	GCGGCCCAGGC--------TTGTTATCTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----AGATGCTTC----TA---AATTACA	4
P. loessica (Altai); AP6	GCGGCCCAGGC--------TTGTTATCTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----AGATGCTTC----TA---AATTACA	4
P. loessica (Altai); AP7	GCGGCCCAGGC--------TTGTTATCTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----AGATGCTTC----TA---AATTACA	4
P. loessica (Altai); AP8	GCGGCCCAGGC--------TTGTTATCTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----AGATGCTTC----TA---AATTACA	4
P. loessica (Altai); AP9	GCGGCCCAGGC--------TTGTTATCTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----AGATGCTTC----TA---AATTACA	4
P. loessica (Altai); AP10	GCGGCCCAGGC--------TTGTTATCTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----AGATGCTTC----TA---AATTACA	4
P. loessica (Altai); AP19	GCGGCCCAGGC--------TTGTTATCTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----AGATGCTTC----TA---AATTACA	4
Pupilla alaskensis			
P. hebes (Alaska); NS48	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	-GTGTATAG-----AGATGCTTC----TA---AATTACA	4
P. hebes (Alaska); AP29	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	-GTGTATAG-----AGATGCTTC----TA---AATTACA	4
Pupilla hokkaidoensis			
P. hebes (Hokkaido); VH29	-------------------TTGTTATGAGTAC-TTTC--GAT-TGGTAT	-GCGTATAG-----AGATGCTTC----TA---AATTACA	4
Pupilla alluvionica			
P. alluvionica (Altai); AP13	GCGGCCCAGGC--------TCGTTATGTGTAC-TTTC--GAT-TAGTAT	-GTGTATAT-----TGATGGTTC----CA---AATTACA	5

Continued

Table 4. *Continued*

Species / Sample information	ITS1	ITS2	SG
	1 1 1 2 2 2 2 2 3 3 3 3 3 3 4 4 4 4 4 5 5 5 5 5 5 6 6 6 6 3 3 4 4 4 4 4 4 4 4 4 6 6 7 7 7 7 7 7 9 1 7 7 0 0 1 6 6 1 1 3 5 5 7 3 4 5 8 9 0 0 3 5 6 7 0 1 2 2 8 9 0 1 2 3 4 5 6 7 8 8 9 0 1 2 3 4 5 5 1 1 2 2 7 8 6 7 1 2 9 5 8 5 7 4 3 8 5 8 9 8 9 1 7 9 7 5 9	1 1 1 1 1 1 1 2 2 2 2 2 2 3 3 3 3 3 3 3 4 4 4 4 5 5 5 5 5 5 5 6 7 7 5 7 7 8 9 3 4 5 7 8 8 8 0 0 0 3 7 9 1 3 3 4 5 5 1 1 1 9 9 0 0 0 2 4 5 8 8 1 4 6 6 9 3 2 7 3 6 7 1 2 3 0 1 7 3 8 5 4 3 6 0 6 8 4 5 6 0 1 4 5 6 9 1 1 3 8 7 0	
Pupilla alpicola			
P. alpicola (Slovakia); H6	GCGGCCCAGGC--------TTACTATGTGTAC-TTTC--GAT-TGGTAT	-GTGYATAT-----TGATGCTTC----TA---AATTATA	6
P. alpicola (Slovakia); H11	GCGGCCCAGGC--------TTACTATGTGTAC-TTTC--GAT-TGGTAT	-GTGCATAT-----TGATGCTCC----TA---AATTACA	6
P. alpicola (Slovakia); H12	GCGGCCCAGGC--------TTACTATGTGTAC-TTTC--GAT-TGGTAT	-GTGCATAT-----TGATGCTCC----TA---AATTACA	6
P. alpicola (Slovakia); H13	GCGGCCCAGGC--------TTACTATGTGTAC-TTTC--GAT-TGGTAT	-GTGCATAT-----TGATGCTCC----TA---AATTACA	6
P. pratensis (Moravia); H1	GCGGCCCAGGC--------TTACTATGTGTAC-TTTC--GAT-TGGTAT	-GTGCATAT-----TGATGCTCC----TA---AATTACA	6
P. pratensis (Slovakia); H5	GCGGCCCAGGC--------TTACTATGTGTAC-TTTC--GAT-TGGTAT	-GTGCATAT-----TGATTCTCC----TA---AATTACA	6
P. pratensis (Bohemia); H7	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	-GTGCATAT-----TGATTCTCC----TA---AATTACA	6
P. alpicola (Altai); AP12	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	-GTGCATAT-----TGATGCTCC----TA---AATTACA	6
P. pratensis (Altai); H3	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	-GTGCATAT-----TGATGCTCC----TA---AATTACA	6
P. pratensis (Altai); H4	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	-GTGCATAT-----TGATGCTCC----TA---AATTACA	6
Pupilla muscorum			
P. bigranata (France); AP25	GCGGCCCAGGC--------TTGTTATGTGTAC-CTTC--GAT-TGATAC	-GTGTAGAT-----TGATGCCTC----TA---AATTACA	7
P. muscorum (New York); AP26	GCGGCCCAGGC--------TTGTTATGTGTAC-CTTC--GAT-TGATAC	-GTGTAGAT-----TGATGCCTC----TA---AATTACA	7
P. muscorum (Moravia); mtG-Pup	GCGGCCCAGGC--------TTGTCATGTGTAC-CTTC--GAT-TGATAT	-GTGTATAT-----TGATGCCTC----TA---GATTACA	7
Pupilla triplicata			
P. triplicata (France); AP31	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACA	8
P. triplicata (Altai); AP32	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GATATGGTAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACA	8
P. triplicata (Urals); AP23	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGCAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACA	8
P. triplicata (Bohemia); H10	GCGGCCCAGGC--------TTGTTATGTTTAC-TTTC--GAT-TGGCAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACA	8
P. triplicata (Bohemia); H20	GCGGCCCAGGC--------TTGTTATGTTTAC-TTTC--GAT-TGGCAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACA	8
P. triplicata (Bohemia); H2	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGCAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACG	8
P. triplicata (Moravia); H9	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGCAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACG	8
P. triplicata (Bohemia); H15	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGCAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACG	8
P. triplicata (Bohemia); H16	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGCAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACG	8
P. triplicata (Bohemia); H17	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGCAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACG	8
P. triplicata (Bohemia); H19	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GAT-TGGCAT	-GTGTATAT-----TGAAGCTTC----TA---AATTACG	8
Pupilla sterrii			
P. sterrii (Moravia); H8	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GTT-TGGTGT	-GTGTATAT-----TGATGCTTC----TA---AATTATA	9
P. sterrii (Moravia); AP22	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GTT-TGGTGT	-GTGTATAT-----TGATGCTTC----TA---AATTATA	9
P. sterrii (Bohemia); H14	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTC--GTT-TGGTGT	-GTGTATAT-----TGATGCTTC----TA---AATTATA	9
P. sterrii (Slovakia); AP21	GCGGCCCAGGCGGCAGGCATTGTTATGTGTAC-TTTC--GTT-TGGTAT	-GTGTATAT-----TGATGCTTC----TAGGTAATTATA	9
P. sterrii (Albania); AP16	GCGGCCCAGGC--------TTGTTATGTGTTTGTTTC--GTT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTGTA	9
P. sterrii (Urals); AP15	GCGGCCCAGGC--------TTGTTATGTGTAC-TTTCT-GTT-TGGTAT	-ATGTATAT-----TGATGCTTC----TA---AATTATA	9
Pupilla turcmenica			
P. altaica (Altai); AP1	GCGGCCCAGGC--------TTGTTGTGTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTATA	9
P. altaica (Altai); AP2	GCGGCCCAGGC--------TTGTTGTGTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTATA	9
P. turcmenica (Altai); AP3	GCGGCCCAGGC--------TTGTTGTGTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTATA	9
P. turcmenica (Altai); AP4	GCGGCCCAGGC--------TTGTTGTGTGTAC-TTTC-AGAT-TGGTAT	-GTGTATAT-----TGATGCTTCT---TA---AATTATA	9
P. turcmenica (Altai); AP18	GCGGCCCAGGC--------TTGTTGTGTGCAC-TTTC--GAT-AGGTAT	-GTGTATAT-----TGATGCTTC----TA---AATTATA	9
P. altaica (Altai); AP17	GCGACCCAGGC--------TTGTTGTGTGTAC-TTTC--GAT-TGGTAT	-GTGTATAT---CTTGATGCTTC----TG---AATTATA	9

For both amplicons, numbers refer to the base pairs downstream of the termination of the forward primer. Base pairs invariant across all samples are omitted, while base pairs that diverge from the genus consensus are highlighted. Dash indicates base-pair deletion. Samples are sorted by post-hoc supported species names (in bold italic), while initial names used prior to DNA analysis, and based on traditional conchological features, are listed in the Sample information. Potential groupings of related species are noted in the farthest-right column, labelled SG. Note that the insertions: ITS1 68–71 and 72–75; and ITS2 181–183 and 504–506, are microsatellite repeats.

closely related to *P. hebes*, lacking the GAC insertion at 181–183 in ITS2. However, it is distinguished from this species by possessing 508G in ITS1. The COI + CytB of *P. hebes pithodes* exists as a highly supported clade. Because we observed a gradation from barrel-shaped *P. pithodes* to more cylindrical *P. hebes* shells, we have chosen to consider *P. pithodes* as a subpopulation that occurs to the east of the main range of *P. hebes*, ranging from eastern Arizona and central Utah into Colorado, New Mexico and Texas. Use of traditional shell characteristics (Pilsbry, 1948) results in some *P. hebes pithodes* being incorrectly assigned to *P. blandi charlestonensis*. ITS1 + ITS1 and COI + CytB both indicate that the sinistral *P. hebes nefas* of southeastern Arizona should be regarded as a shell form of *P. hebes pithodes*.

DNA analysis also demonstrates that *P. alaskensis* and *P. hokkaidoensis*, which have previously been confused with *P. hebes*, actually represent undescribed species related to the central Asian *P. loessica*, as all share 207A in ITS2. Both of these new species uniquely share 177G in ITS2, with *P. hokkaidoensis* differing from *P. alaskensis* based on an 11-bp deletion at 38–48 in ITS1, 111C in ITS1 and 79C in ITS2. *Pupilla alaskensis* shells differ from *P. hebes* in their widely spaced, coarse, somewhat anastomosing striae. *Pupilla hokkaidoensis* shells differ from *P. hebes* in their rotund cylindrical shape with shallow suture and anastomosing coarse striae (see taxonomic descriptions below).

Pupilla cf. *khunjerabica* is represented in the sample by a single population from a riparian forest corridor in the Altai. This specimen possesses a cylindrical-ovoid shell tapered for the upper half of the shell height, very weak irregular thread-like striae, a moderately deep suture, a weak to absent callus or crest, and no apertural lamellae. Although its COI + CytB sequence fell within the highly supported clade defining *P. alaskensis*, it uniquely possessed 137C in ITS2, and was the only taxon outside the southwestern North America *P. syngenes/sonorana* group to possess 156G in ITS2.

Pupilla cf. *limata* was sampled from riparian forest in the Yakutia region of eastern Siberia and is characterized by a cylindrical-ovoid shell with numerous, sharp, somewhat anastomosing striae, a weak crest and no callus or apertural lamellae. While this taxon is highly supported as a monophyletic entity in COI + CytB, one of the two analysed individuals possessed ITS1 + ITS2 identical with *P. hebes pithodes* from western North America. The other individual uniquely possessed 444C in ITS1.

Pupilla loessica is a steppe-tundra species that in modern times is restricted to central Asia, although Pleistocene fossils extend west into central Europe (e.g. Horsák *et al*., 2010). It is distinguished by its numerous strong rounded anastomosing striae and lack of callus and apertural lamellae. It uniquely possessed 266C in ITS1 and shares 207A in ITS2 with *P. alaskensis* and *P. hokkaidoensis*. *Pupilla loessica* exists as a strongly supported clade in COI + CytB. While one individual (AP6) possessed COI + CytB characteristic of *P. alaskensis*, this individual had ITS1 + ITS2 characteristic of *P. loessica*.

Pupilla muscorum has traditionally been characterized by its cylindrical shell with smooth sculpture, shallow suture, strong crest, thick white callus and from zero to two parietal lamellae (Pilsbry, 1948; von Proschwitz *et al*., 2009). This species exists as a well defined clade in ITS1 + ITS2 by uniquely possessing 375C and 609A in ITS1 and 336C in ITS2. It also exists as a strongly supported clade in COI + CytB. *Pupilla bigranata*, which is distinguished from *P. muscorum* by its three strong apertural lamellae, was found to have COI + CytB sequence identical with Moravian *P. muscorum* and ITS1 + ITS2 sequence identical with New York *P. muscorum*. Individuals from the High Plains and Rocky Mountains of the western USA, which have been previously identified as *P. muscorum* using the above conchological characteristics, were shown by DNA sequence analysis actually to represent *P. blandi* or *P. hebes*.

In North America *P. muscorum* was thought to exist in two disjunct populations, one ranging from the northeastern Atlantic seaboard west through the Great Lakes to Iowa, and the other ranging across the northern taiga (Oughton, 1948; Hubricht, 1985). This latter entity (*P. hudsonianum*) exists as a well defined monophyletic group uniquely possessing 56A, 541G and 583C in ITS2. It also exists as a strongly supported clade in COI + CytB, although one individual (P13) possessed sequence characteristic of *P. alaskensis*. It is easily differentiated from *P. muscorum* by the strong taper over the top half of its shell height and its sculpture of dense thread-like striae (see taxonomic description below).

Pupilla syngenes possesses a distinctive shell that is widest in the top half and has three apertural lamellae including a long, curved blade-like parietal lamella. This species of wooded, xeric rock outcrops in the southwestern USA uniquely possessed 83A in ITS2 and also exists as a strongly supported clade in COI + CytB. The dextral individual of this typically sinistral species, termed *P. syngenes dextroversa*, had ITS1 + ITS2 and COI + CytB sequences identical to a typical individual within the same population.

Pupilla sonorana was compared by Pilsbry (1948) with *P. triplicata* and distinguished by its small size, columnar shell, strong crest and three apertural lamellae including a curved blade-like parietal lamella. It appears closely related to *P. syngenes* by sharing 453T in ITS1 and 156G and 233A in ITS2. However, it differed from that species in possessing 218A and 538C and a GGCA insertion from 68 to 71 in ITS1 and 83G and 356A in ITS2. As only one individual was analysed, no species-level clade can be assigned in COI + CytB. However, it differed from *P. syngenes* by 54 bp at these loci.

Pupilla triplicata of rock outcrops from western Europe to central Asia has shells that differ from *P. sonorana* only by their weak, rounded (rather than sharp) striae. This species uniquely possesses 295A in ITS2. Most individuals also exhibit 617C in ITS1. COI + CytB of this species form a highly supported clade. While two distinct subpopulations are suggested by 740A *vs* 740G in ITS2, there is no correspondence in COI + CytB or in any noted conchological features. As such, this grouping appears to have no taxonomic merit.

Pupilla sterrii of dry calcareous grasslands from central Europe to the Urals is characterized by its very deep suture and sharp, coarse, anastomosing striae. It is defined by uniquely possessing 509T in ITS1. The mtDNA of this species is highly variable, with individuals variously possessing COI + CytB characteristic of *P. alpicola, P. muscorum, P. triplicata* or *P. turcmenica*. No analysed individual from Europe was found to possess mtDNA sequence with the expected topological position as sister to *P. turcmenica*. However the Urals specimen did and it may represent the only individual with both nDNA and mtDNA characteristic of *P. sterrii*.

Pupilla turcmenica is a species of xeric calcareous grasslands that ranges across Asia Minor and central Asia. It is conchologically distinguished from the similar *P. sterrii* by its less deep sinus and more widely spaced striae. It uniquely displays 207G in ITS1, with its COI + CytB forming a highly supported clade. *Pupilla altaica* has been recently differentiated from this species by its larger crest, more massive white callus and the presence of an angular pad on the parietal wall of the aperture (Meng & Hoffman, 2008). However, this entity did not possess any unique ITS1 + ITS2 distinctions from *P. turcmenica*, being distributed throughout the same highly supported COI + CytB clade encompassing that species. As such it appears to simply represent the high-calcificiation endpoint within the normal conchological range of *P. turcmenica*.

These supported entities could be further associated into nine groups using ITS1 + ITS2 data (used to order Table 4). Group

1 consists of *P. blandi*, *P. hebes*, *P. hebes pithodes* and *P.* cf. *limata* and is characterized by insertion 495A in ITS1. Group 2 is represented only by *P. hudsonianum* and is characterized by the 56A insertion, 541G and 583C in ITS2. Group 3 consists of *P. syngenes*, *P. sonorana* and *P.* cf. *khunjerabica* and is characterized by 156G in ITS2. Group 4 consists *of P. loessica*, *P. alaskensis* and *P. hokkaidoenis* and is characterized by 207A in ITS2. Group 5 includes only *P. alluvionica* and is characterized by 111C and 577A in *ITS1* and 333G and 490C in ITS2. Group 6 includes only *P. alpicola* and is characterized by 92C and typically 340C in *ITS2*. Group 7 consists only of *P. muscorum* and is characterized by 375C and 609A in ITS1 and 336C in ITS2. Group 8 is represented only by *P. triplicata* and is characterized by 295A in ITS2. Group 9 consists of *P. sterrii* and *P. turcmenica* and is characterized by 717T in ITS2. Because no variable bases are shared between groups, however, possible relationships between them cannot be inferred.

The greater amount of variation within COI + CytB allows resolution of deeper relationships. The nine interspecific groups suggested by ITS1 + ITS2 are generally validated with high support by the mtDNA tree topology. The major exception is Group 1, whose members are spread across two major mtDNA clades: *P. blandi*, *P. hebes hebes* and *P. hebes pithodes* belong to one strongly supported clade, while *P.* cf. *limata* appears more related to *P. loessica* and *P. alpicola* in the mtDNA tree.

Topological incongruence between mitochondrial and nuclear DNA phylogenies

Comparison of the mtDNA tree with nDNA tree and base-pair variation matrix reveals topological incongruence in eleven specimens, or almost 15% of the total. These are largely limited to two groups: *P. alaskensis* and the *P. sterrii*/*turcmenica* clade. The highly supported mtDNA species-level clade containing both *P. alaskensis* specimens also harbours individuals with nDNA characteristic of *P. loessica*, *P.* cf. *khunjerabica* or *P. hudsonianum*. Individuals harbouring mtDNA characteristic of *P. turcmenica* may possess nDNA characteristic of either *P. alluvionica* or *P. sterrii*. In *P. sterrii*, specimens possessing nDNA characteristic of that species may harbour mtDNA characteristic of *P. alpicola*, *P. triplicata*, *P. muscorum* or *P. turcmenica*. While the current analysis is not capable of resolving the cause of these incongruencies, it does seem likely that mitochondrial introgression is responsible in the case of *P. sterrii* as European individuals variously possess mtDNA from all other known European species.

Conchological variation: traditional vs *other traits*

Comparison of conchological features among genetically-identified individuals demonstrates that the size of the apertural crest, degree of callus deposition, callus colour and number, shape and placement of apertural lamellae are of little taxonomic value (Table 5). For instance, in Europe *P. alpicola*, *P. muscorum* and *P. triplicata* all show variation ranging from zero to multiple apertural lamellae, and from absent/weak to strong crest and callus (Fig. 3). This same pattern is repeated in central Asia with *P. turcmenica* (Fig. 4) and in North America with *P. blandi* and *P. hebes* (Fig. 5). The reliance of traditional taxonomy on these traits is thus responsible for oversplitting in some regions (e.g. *P. bigranata* and *P. pratensis* in Europe and *P. altaica* in central Asia; Figs 3, 4) and for the abysmal initial sorting of western North American material (Fig. 5).

However, other conchological traits do accurately reflect genetic relationships and are capable of accurately sorting individuals into species-level groups (Table 5; Figs 3–5). The most important of these are shell sculpture, including not only shape, strength, density and complexity of shell striae, but also the lustre of the underlying shell surface. Suture depth and apex architecture were also found to be valuable for species identification, as was shell width, which appeared to be relatively independent of shell height.

SYSTEMATIC DESCRIPTIONS

Pupillidae
Pupilla Leach, in Fleming, 1828

Pupilla alaskensis Nekola & Coles, n. sp.

(Fig. 6A–H, K)

Types: Holotype (Fig. 6A–D, K): ANSP 458632, Happy Valley, North Slope Borough, Alaska, USA (69°20′7″N, 148°43′48″W). Paratypes: 10 shells, ANSP 458633, collected with holotype; ~100 shells, NMW.Z.2014.013.00013, collected with holotype; 5 shells ANSP 458634, Sukakpak Mountain, Yukon-Koyukuk Census Area, Alaska, USA (67°35′55″N, 149°47′4″W); ~60 shells NMW.Z.2014.013.00011, same loc. as preceding; 5 shells ANSP 458635, Livengood East, Yukon-Koyukuk Census Area, Alaska, USA (65°27′55″N, 148°20′40″W); 3 shells ANSP 458636, Knik I., Matanuska-Susitna Borough, Alaska, USA (61°30′30″N, 149°2′3″W).

Zoobank registration: urn:lsid:zoobank.org:act:D98DD52C-BFAE-4752-A9D4-18F116CE8957

Other material examined: NMW.Z.2005.011.01468, NMW.Z.2014.013.00002 – 00023 *c.* 1000 shells from Alaska, USA; 30 lots (3739 individuals) from Alaska, USA in Nekola collection.

Etymology: Specific name *alaskensis* refers to region in which species is known to occur.

Diagnosis: Shell small, cylindrical-ovoid, similar to *P. hebes* but differing by its deeper suture and shell sculpture of widely-spaced, anastomosing radial striae.

GenBank: GQ921663, KM518334, KM518336, KM518412, KM518414, KM518489, KM518491, KM518567.

Description: Shell 2.6–3.3 mm tall × 1.6–1.8 mm wide, opaque to translucent, yellowish-brown to cinnamon-brown; ~6–6.5 whorls; apical whorls rounded-conical, remainder ovate-cylindrical to cylindrical; suture typically deep though sometimes of only normal depth with whorls consequently appearing swollen; shell surface silky in general appearance, the post-neanic whorls bearing sharp, irregular, often widely spaced, anastomosing radial striae occasionally developed into fine lamellae superimposed on a minutely and irregularly papillate surface (Fig. 6K); aperture ~1/4 of shell height, ranging from slightly wider than tall (Fig. 6A, E, G) through circular (Fig. 6F) to slightly taller than wide (Fig. 6H), in profile ascending onto body whorl (Fig. 6B); umbilicus closed by preceding whorls (Fig. 6C); peristome interrupted by body whorl, apertural lip flared (Fig. 6B, D), shell slightly contracted behind (Fig. 6D); crest absent or weakly developed but not thickened or callused (Fig. 6A–H); apertural lamellae generally absent, though a vestigial, plate-shaped columellar is occasionally present (Fig. 6E, G).

Geographical distribution: Currently known from just south of Arctic Ocean coastal plain in far northern Alaska to Pacific Coast near Anchorage, Alaska. It seems likely that this species will be found in adjacent areas of the Yukon and northwestern British Columbia, Canada. The published records for *P. hebes*

Table 5. *Pupilla* shell features for those taxonomic units validated by DNA sequence data.

Taxon	Height (mm)	Width (mm)	Shell form	Apex shape	Shell sculpture	Suture depth	Aperture shape	Apertural crest and callus	Apertural lamellae
alaskensis	2.6–3.3	1.6–1.8	Cylindrical ovoid to cylindrical	Tapered	Widely spaced, sharp, somewhat anastomosing coarse striae; dull shell	Normal to deep	Slightly wider than tall to slightly taller than wide	Absent to weak crest; no callus	0–1 (vestigal, plate-like columellar only)
alluvionica	3.3–4.3	2.0–2.4	Cylindrical ovoid	Tapered/domed	Low, rounded, irregular, wide spaced striae; shell shiny	Shallow/ normal	Taller than wide	Weak to very strong crest and white callus	0–1 (occasional weak parietal)
alpicola	2.8–4.2	1.6–2.1	Cylindrical ovoid; body whorl often narrower than penultimate	Tapered	Fine, rounded, close, irregular striae; shell shiny/ satiny	Shallow	Round to slightly wider than tall; small in proportion to shell size	Weak to strong crest; absent to moderate white callus	0–2 (occasional weak parietal and palatal) mostly palatal depression
blandi	2.3–3.2	1.2–1.6	Cylindrical	Domed	Irregular, very weak striae; shell shiny	Shallow	Round to slightly wider than tall	Weak to strong crest, absent to strong, brown to white callus	0–3
hebes	2.6–3.5	1.4–1.7	Cylindrical ovoid	Tapered for upper ⅓–¼ of shell height	Sharp, numerous thread striae; shell silky	Normal/deep	Round to taller than wide	Weak to strong crest; absent to strong, brown to white callus	0–3
hebes pithodes	2.9–3.3	1.6–1.8	Cylindrical ovoid; body whorl often narrower than penultimate	Tapered for upper ⅓–¼ of shell height	Sharp, numerous thread striae; shell silky	Normal/deep	Round to wider than tall	Weak to very strong crest; absent to strong brown to white callus	1–3
hokkaidoensis	3.0–3.1	1.7–1.8	Ovoid cylindrical	Tapered	Anastomosing coarse striae; shell silky to dull	Shallow	Round to wider than tall	Weak absent crest; no callus	0
hudsonianum	3.3–3.6	1.7–1.8	Cylindrical ovoid	Strongly tapered for upper ½	Dense thread striae; shell silky to dull	Normal to deep	Taller than wide (rarely) to wider than tall	Weak to strong; white callus	0–2 (weak parietal and vestigal columellar occasionally present)
cf. *khunjerabica*	2.9–3.5	1.7–1.8	Cylindrical ovoid	Tapered for upper ½ of shell height	Very weak, irregular thread striae; dull shell	Moderately deep	Taller than wide	Thin - lacking	0
cf. *limata*	2.7–3.1	1.6–1.7	Cylindrical ovoid	Tapered	Sharp, numerous, somewhat anastamosing striae; dull shell	Shallow	Taller than wide	Weak crest; no callus	0
loessica	2.6–3.6	1.6–2.0	Cylindrical ovoid	Tapered	Strong but rounded numerous, anastamosing striae; dull shell	Normal	Round	Weak to moderate crest; no callus	0
muscorum	2.7–4.0	1.6–1.8	Cylindrical ovoid	Tapered	Low, rounded, somewhat irregular striae; shell shiny to silky	Shallow	Round	Strong to very strong crest and white callus	1–2 (columellar absent)
sonorana	2.5–3.3	1.3–1.4	Ovoid cylindrical, widest in upper ½	Domed	Weak, irregular sharp thread striae; shell silky	Normal	Round	Strong crest; strong to very strong white callus	3 (palatal ranging from peg to long blade)

Continued

Table 5. *Continued*

Taxon	Height (mm)	Width (mm)	Shell form	Apex shape	Shell sculpture	Suture depth	Aperture shape	Apertural crest and callus	Apertural lamellae
sterrii	2.6–3.5	1.5–1.8	Cylindrical ovoid	Tapered/domed	Sharp, anastomosing coarse striae; shell silky to dull	Very deep	Round	Weak to strong crest; absent to strong white callus	1–2 (peg shaped palatal)
syngenes	3.0–4.5	1.6–1.8	Biconic, widest in upper ⅓ of shell; 8 + whorls	Tapered/domed	Weak thread striae; shell dull	Shallow	Taller than wide	Very strong crest; weak to moderate brown callus	3
triplicata	2.2–3.1 (rarely to 4)	1.3–1.6	Cylindrical to cylindrical ovoid	Tapered	Low, rounded, irregular striae; shell shiny to silky	Normal to deep	Round	Moderate to strong crest; absent to strong white callus	0–3 (columellar often absent or weak)
turcmenica	2.7–3.2	1.5–1.6	Cylindrical ovoid	Tapered/domed	Regular remote, somewhat anastomising coarse striae; shell dull	Deep	Round	Weak to strong crest; absent to very strong white callus	0–3 (columellar absent; angular pad sometimes present)

from Anchorage, Alaska in Forsyth (2004) refer to material of the present authors and represent *P. alaskensis*.

Habitat: This species has been found in upland and lowland tundra, taiga, fens, herb-rich meadows, coastal grasslands and riparian forest.

Remarks: Shell reminiscent of *P. loessica*, but differs from that species by its widely-spaced, sharp and only slightly anastomosing striae, deeper suture and weaker (or absent) crest. It differs from *P. hokkaidoensis* in its deep suture, darker shell colour and more regular striae (Table 5).

Pupilla hudsonianum **Nekola & Coles, n. sp.**

(Fig. 7A–H, K)

Types: holotype (Fig. 7A–D, K): ANSP 458637, Lake Bemidji State Park, Beltrami County, Minnesota, USA (47°31′58″N, 94°49′28″W). Paratypes: 10 shells, ANSP 458638, collected with holotype; ~50 shells, NMW.Z.2005.011.00835, collected with holotype; 2 shells, ANSP 458639, highway 40 at Rabbit Hill Road, east of Benchlands (Calgary), Bighorn #8 Municipal District, Alberta, Canada (51°15′51″N, 114°43′57″W); ~100 shells, NMW.Z.2014.013.00058, same loc. as preceding; 5 shells, ANSP 458640, Goose Creek Road, Churchill, Manitoba, Canada (58°42′30″N, 94°7′22″W); 1 shell, ANSP 458641, La Grande Pointe, Duplessis District, Quebec, Canada (50°12′21″N, 63°23′48″W); ~30 shells, NMW.Z.2014.013.00001, same loc. as preceding.

Zoobank registration: urn:lsid:zoobank.org:act:B9E21337-42C7-4BFD-87B9-EFE157D2A5A2.

Other material examined: NMW.Z.2014.01300054-00060, 00066-00071, 00080; *c.* 500 shells. Ten lots from Nekola collection (including one of Pleistocene fossil material; 651 individuals).

Etymology: The specific name *hudsonianum* refers to Hudson Bay and to the Hudsonian life zone, which has been used to refer to the North American taiga, and which defines much of this species' range.

Diagnosis: Shell ovoid-cylindrical, similar to *P. hebes*, but differentiated by its more ovate shell shape with a surface sculpture consisting of densely packed radial thread-like striae, giving shell a silky lustre.

GenBank: GQ921662, KM518353, KM518354, KM518355, KM518357, KM518358, KM518431, KM518432, KM518433, KM518435, KM518508, KM518509, KM518510, KM518512, KM518585, KM518586, KM518587, KM518589.

Description: shell 3.3–3.6 mm tall × 1.7–1.8 mm wide; opaque to translucent yellowish-brown; *c.* 6.5–7 whorls; apical whorls rounded-conical in outline, remainder cylindrical; suture moderately deep; shell surface silky in general appearance, postneanic whorls bearing irregular, dense, closely-spaced, weakly anastomosing radial thread-like striae superimposed on a minutely scaly surface, with minute papillae present between the scales (Fig. 7K); aperture *c.* $\frac{1}{4}$ of shell height, approximately circular (Fig. 7F) to wider than tall (Fig. 7A, E, G), rarely taller than wide (Fig. 7H), in profile ascending onto body whorl (Fig. 7B); umbilicus closed by preceding whorls (Fig. 7C); peristome interrupted by body whorl; apertural lip expanded, shell slightly contracted behind; lip thickened by a weakly to strongly developed pale callus of shallow depth corresponding to a weakly to strongly developed crest (Fig. 7A, B, D, E); apertural

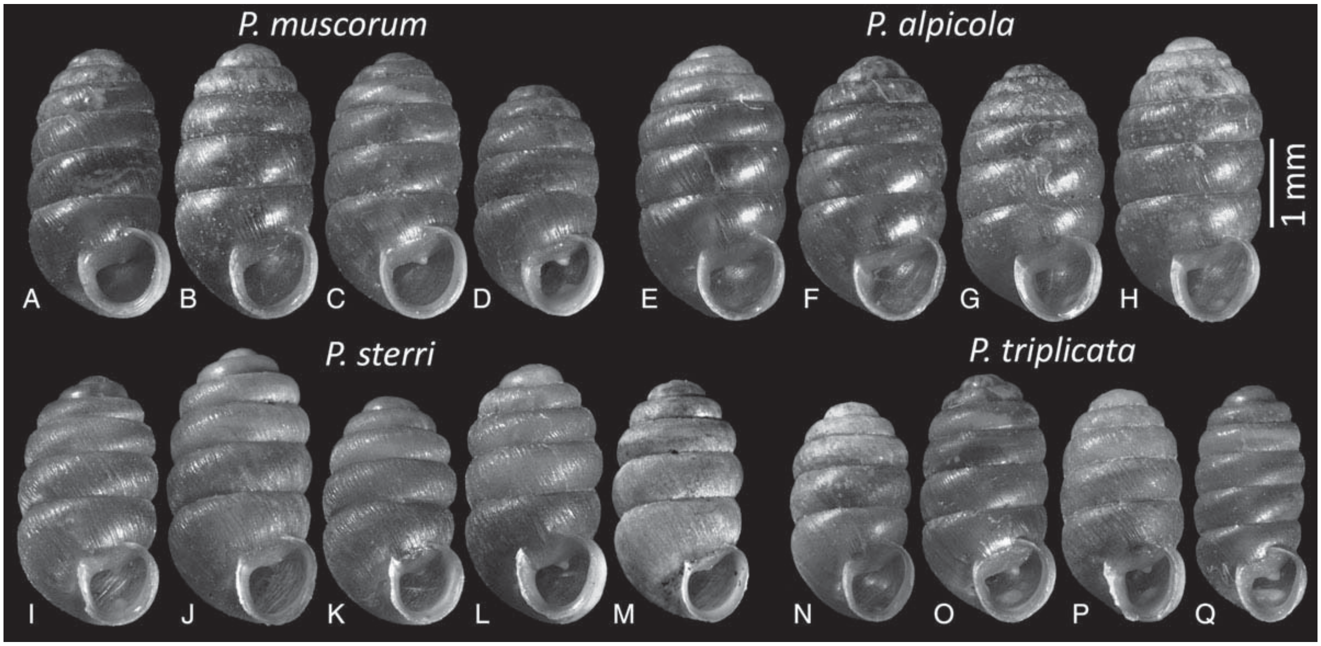

Figure 3. *Pupilla* species of primarily European distribution. Names are those supported by DNA sequence analysis. **A–D.** *P. muscorum*. **A.** Cedar Rapids, Iowa, USA (22). **B.** Brno, Moravia, Czech Republic (mtG-Pup) **C.** Syracuse, New York, USA (AP26). **D.** Pont, Calvados, France (AP25). **E–H.** *P. alpicola*. **E.** Rakša, Slovakia (H6). **F.** Belyashi, Altai, Russia (AP12). **G.** Závod, Slovakia (H5). **H.** Pozděchov, Moravia, Czech Republic (H1). **I–M.** *P. sterrii*. **I.** Verkhne Bikberda, Bashkortostan, Russia (AP15). **J.** Periferi Dibre, Albania (AP16). **K.** Klentnice, Moravia, Czech Republic (AP22). **L.** Pavlov, Moravia, Czech Republic (H8). **M.** Valaská Dubová, Slovakia (AP21). **N–Q.** *P. triplicata*. **N.** Hracholusky, Bohemia, Czech Republic (H17). **O.** Ozero Kureevo, Altai, Russia (AP32). **P.** Pavlov, Moravia, Czech Republic (H9). **Q.** Cahors, Dordogne, France (AP31).

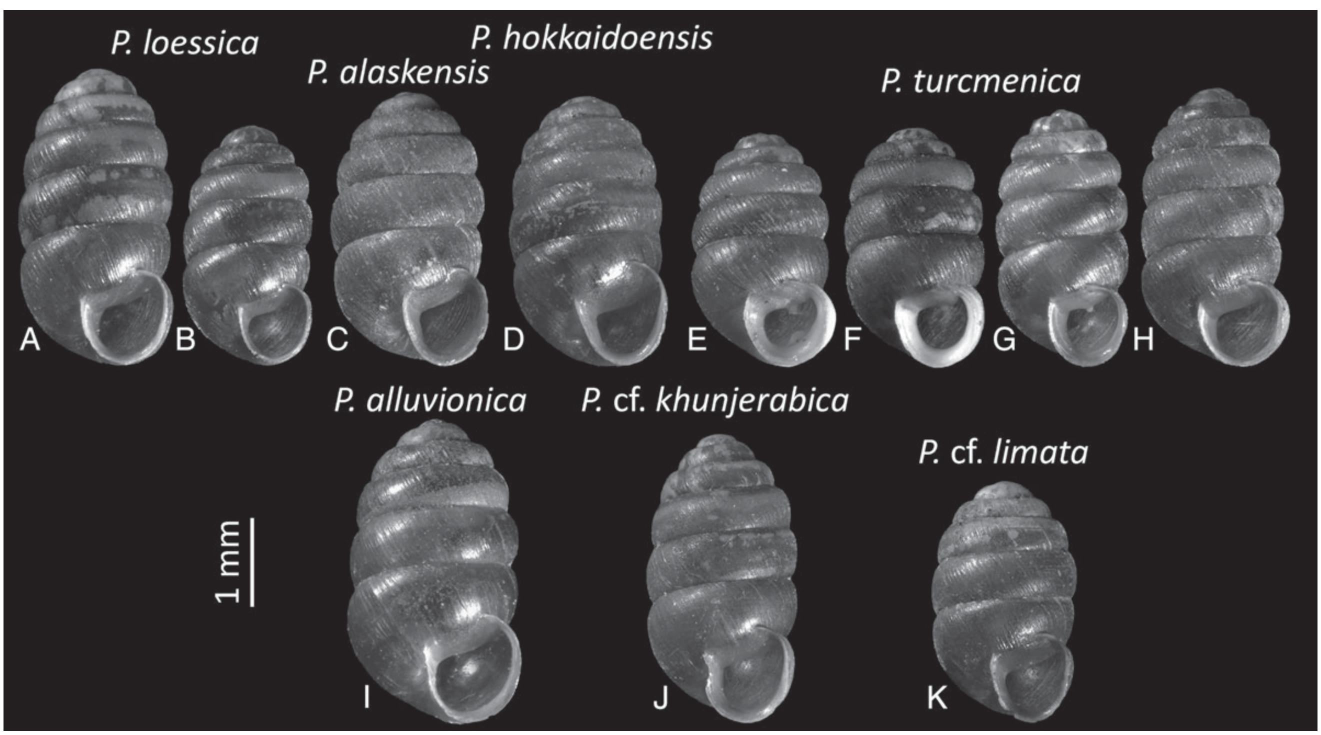

Figure 4. *Pupilla* species primarily of Asian/Beringian distribution. Names are those supported by DNA sequence analysis. **A, B.** *P. loessica*. **A.** Belyashi, Altai, Russia (AP7). **B.** Belyashi, Altai, Russia (AP9). **C.** *P. alaskensis*, Knik Island, Anchorage, Alaska, USA (AP29). **D.** *P. hokkaidoensis*, Toyokoro, Nakagawa, Hokkaido, Japan (VH29). **E–H.** *P. turcmenica*. **E.** Ust'-Muny, Altai, Russia (AP1). **F.** Kurai, Altai, Russia (AP17). **G.** Kurai, Altai, Russia (AP18). **H.** Kosh-Agach, Altai, Russia (AP3). **I.** *P. alluvionica*, Belyashi, Altai, Russia (AP13). **J.** *P.* cf. *khunjerabica*, Chagan-Uzun, Altai, Russia (AP11). **K.** *P.* cf. *limata*, Kapitonovka, Yakutia, Russia (AP39).

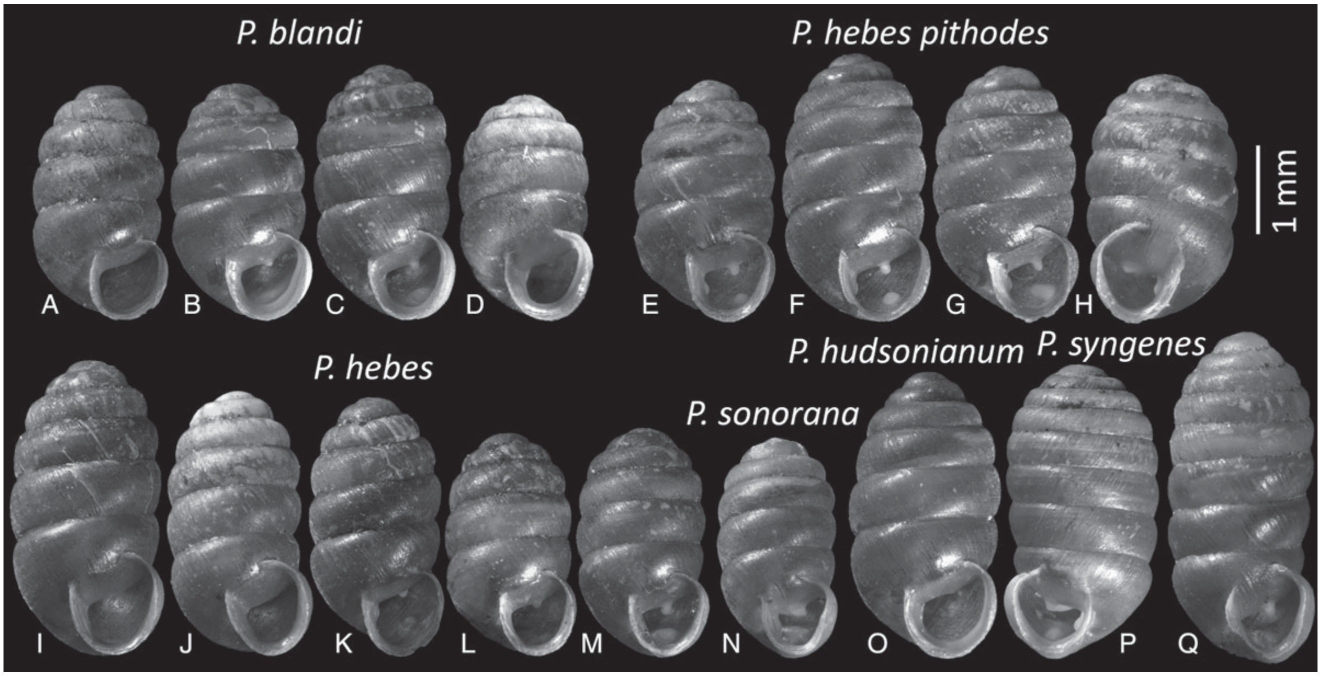

Figure 5. *Pupilla* species of North American distribution. Names are those supported by DNA sequence analysis. **A–D.** *P. blandi*. **A.** Ute Creek Canyon, Colorado, USA (AP37). **B.** Irvine, Alberta, Canada (AP34). **C.** Moose Jaw, Saskatchewan, Canada (AP35). **D.** Bannon Ranch, New Mexico, USA (image for ET7 was lost, so a similar shell from same population is figured). **E–H.** *P. hebes pithodes*. **E.** Tusas Ridge, New Mexico, USA (AP38). **F.** Bullion Canyon, Utah, USA (AP27). **G.** Bullion Canyon, Utah, USA (AP28). **H.** Bear Wallow, Arizona, USA (P6). **I–M.** *P. hebes hebes*. **I.** Loope East, California, USA (P14). **J.** Bullion Canyon, Utah, USA (P17). **K.** Ruby Mountains, Nevada, USA (P16). **L.** Kaibab Plateau, Arizona, USA (P1). **M.** East Tintic Range, Utah, USA (P2). **N.** *P. sonorana*, Sacramento Mountains, New Mexico, USA (P12). **O.** *P. hudsonianum*, Lake Bemidji, Minnesota, USA (AP33). **P, Q.** *P. syngenes*. P. Mogollon, New Mexico, USA (AP30). **Q.** Kaibab Plateau, Arizona, USA (P11).

lamellae generally absent (Fig. 7G, H), but a weak parietal (Fig. 7E) and vestigial plate-like columellar lamella (Fig. 7A, F) occasionally present.

Geographical distribution: Currently documented from DNA sequence data from the foothills of the Rockies in western Alberta, Canada east through the northern (Churchill, Manitoba, Canada) and southern (Lake Bemidji, Minnesota, USA) taiga limits in central North America to the north shore of the Gulf of St Lawrence in Quebec. Shell lots at the Academy of Natural Sciences at Drexel University (ANSP 106909, 141759, 141770, 141776, 141783, 150006, 150026), Carnegie Museum (CM 86989, 87010, 62.20823), Museum of Comparative Zoology (MCZ 048304, 201542), National Museum of Canada (NMC 2892, 69132), Royal Ontario Museum (ROM 21464) and University of Michigan Museum of Zoology (UMMZ 55951, 109819, 109829, 168485, 180110, 180112) indicate that *P. hudsonianum* occurs across the southern shore of Hudson Bay in Ontario and along the Gulf of St Lawrence shore from the Gaspé and Anticosti Island in Quebec to the west shore of Newfoundland All reports of *P.* '*muscorum*' from Pleistocene sediments in central North America (Hubricht, 1985) represent *P. hudsonianum* (Fig. 6J).

Habitat: This species occurs has been found in mesic taiga, calcareous fens, dry sandy lakeshores and tundra-like turfs on shoreline limestone pavements.

Remarks: *Pupilla hudsonianum* is most readily distinguished from *P. muscorum*, with which it has been previously confused, by its deeper suture, less massive and more yellow apertural callus, and sharp, fine striae which give the shell a matte luster (Table 5).

Pupilla hokkaidoensis **Nekola, Coles & S. Chiba, n. sp.**

(Fig. 8A–K)

Types: holotype (Fig. 8A–D, K): ANSP 458642, Toyokoro, Nakagawa District, Hokkaido Prefecture, Japan (42°36′18″N, 143°33′23″E). Paratypes: 10 shells, ANSP 458643, collected with holotype; 11 shells, NMW.Z.2014.013.00061, collected with holotype; 5 shells, ANSP 458644, Kushiro Marsh, Kushiro District, Hokkaido Prefecture, Japan (43°2′2″N, 144°23′24″E); 12 shells, NMW.Z.2014.013.00062, same loc. as preceding; 5 shells, ANSP 458645, Betsukai, Notsuke District, Hokkaido Prefecture, Japan (43°20′50″N, 145°19′6″E); 5 shells, ANSP 458646, Hama-koshimizu, Shari District, Hokkaido Prefecture, Japan (43°56′1″N, 144°26′38″E); ~25 shells, NMW.Z.2014. 013.00064, same loc. as preceding.

Zoobank registration: urn: lsid:zoobank.org:act:891CB0E8-E4AA-43BB-9BDB-8F579461E48A.

Other material examined: 6 shells NMW.Z.2005.011.03876, 03878; 4 lots from Nekola collection (176 individuals).

Etymology: The specific name *hokkaidoensis* refers to the island of Hokkaido, where all known populations reside.

Diagnosis: Shell small, ovoid-cylindrical, similar to *P. hebes* but differentiated by a more ovate shell with shallower sutures and shell surface sculpture of coarse, anastomosing, radial striae.

GenBank: KM518566, KM518488, KM518411, KM518333.

Description: Shell 3.0–3.1 mm tall × 1.7–1.8 mm wide; opaque to translucent, yellow-brown; *c.* 6 whorls; apical whorls conical

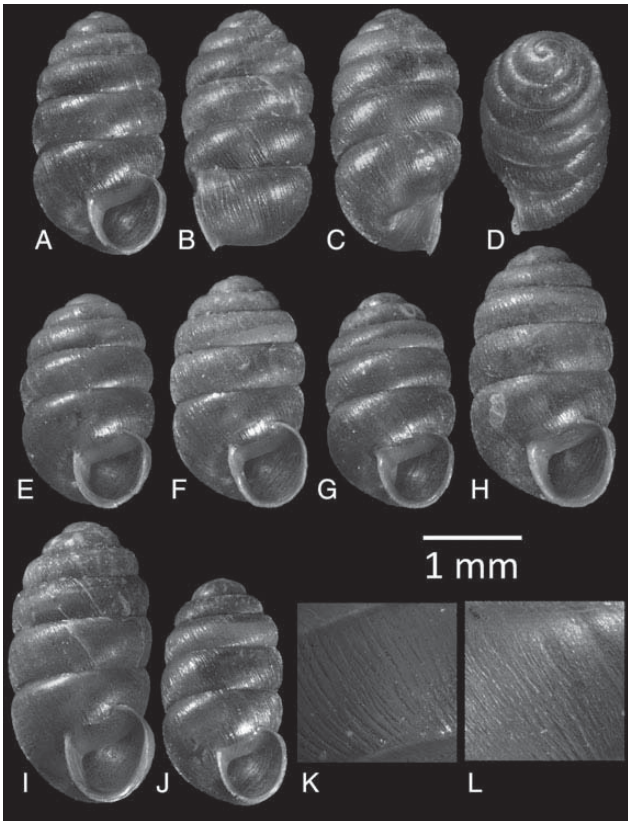

Figure 6. A–H. *Pupilla alaskensis*, n. sp. **A–D, K.** Holotype, ANSP 458632; Happy Valley, North Slope Borough, Alaska, USA. **E.** Paratype, ANSP 458633; Happy Valley, North Slope Borough, Alaska, USA. **F.** Paratype, ANSP 458635; Livengood East, Yukon-Koyukuk Census Area, Alaska, USA. **G.** Paratype, ANSP 458634; Sukakpak Mountain, Yukon-Koyukuk Census Area, Alaska, USA. **H.** Paratype, ANSP 458636; Knik I., Matanuska-Susitna Borough, Alaska, USA. **I, L.** *P. hebes*, JCN 17254; Loope East, Alpine Co., California, USA. **J.** *P. loessica*, Belyashi, Altai Republic, Russia; 49°16′8″N, 87°59′2″E.

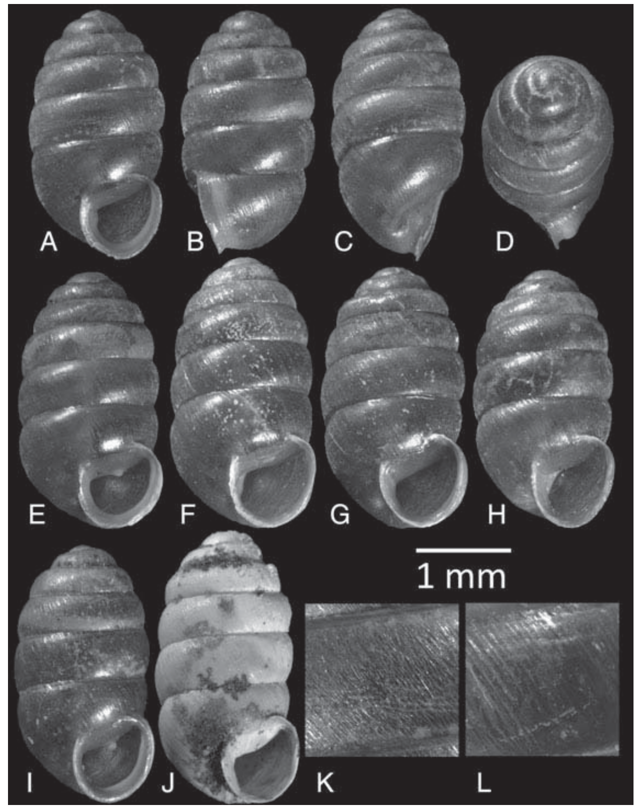

Figure 7. A–H, J, K. *Pupilla hudsonianum*. **A–D, K.** Holotype, ANSP 458637; Lake Bemidji State Park, Beltrami County, Minnesota, USA. **E.** Paratype, ANSP 458638; Lake Bemidji State Park, Beltrami County, Minnesota, USA. **F.** Paratype, ANSP 458641; La Grande Pointe, Duplessis District, Quebec, Canada. **G.** Paratype, ANSP 458639; East of Benchlands, Bighorn #8 Municipal District, Alberta, Canada. **H.** Paratype, ANSP 458640; Goose Creek Road, Churchill, Manitoba, Canada. **J.** Pleistocene loess fossil, Wenig Road, Cedar Rapids, Linn Co., Iowa, USA; 42°0′8″N, 91°40′40″ W; JCN 3650. **I, L.** *P. muscorum*; Syracuse University South Campus, Syracuse, Onondaga Co., New York, USA; 43°0′27″N, 76°6′38″W; JCN 13955.

in outline, remainder ovoid-cylindrical giving shell slight barrel shape; suture shallow; shell surface silky in general appearance, post-neanic whorls bearing irregular, anastomosing radial striae most strongly developed on mid whorls, superimposed on a minutely and irregularly papillate surface (Fig. 8K); aperture *c.* $\frac{1}{4}$ of shell height, ranging in shape from approximately circular (Fig. 8A, E, H) to taller than wide (Fig. 8F, G), in profile ascending onto body whorl (Fig. 8B); umbilicus closed by preceding whorls (Fig. 8C); peristome interrupted by body whorl; apertural lip flared (Fig. 8B–D), shell slightly contracted behind; crest absent or weakly developed (Fig. 8D), callus absent; apertural lamellae absent.

Geographical distribution: Currently known only from the eastern coast of Hokkaido, Japan.

Habitat: This species was found in beach grasslands, wetland margins and old fields.

Remarks: *Pupilla hokkaidoensis* differs from *P. loessica* in its more ovate shell, more yellow shell colour, shallower suture and coarser, more widely spaced and irregular striae. It differs from *P. alaskensis* in its more yellow shell colour, shallower suture and more irregular striae. It differs from *P.* cf. *limata* from Yakutia, Siberia, in its larger size, lighter shell colour and presence of anastomosting striae.

DISCUSSION

These analyses show that in three widely separated geographic regions the understanding of species-level taxonomy within the genus *Pupilla* has been hampered by the traditional reliance on a suite of highly plastic shell apertural features that are of little taxonomic value. As a result, too many species have been described in Europe and central Asia, and too few species in North America and eastern Asia, with confusion existing about actual species ranges and ecological tolerances. However, DNA sequence analysis also confirms that most previously described taxa have biological merit, with alternative conchological traits such as shell sculpture and architecture being able accurately to distinguish these entities.

Because traditional taxonomic concepts within *Pupilla* have been based on unstable shell features, larger patterns regarding biodiversity, biogeography and ecology must also be reconsidered. While we cannot deal here with these issues for the entire genus, the current analysis does allow for reconsideration within each of our three study regions.

Reassessment of Pupilla *biodiversity*

In Europe oversplitting has been predominant, with both *P. bigranata* and *P. pratensis* having been differentiated from *P.*

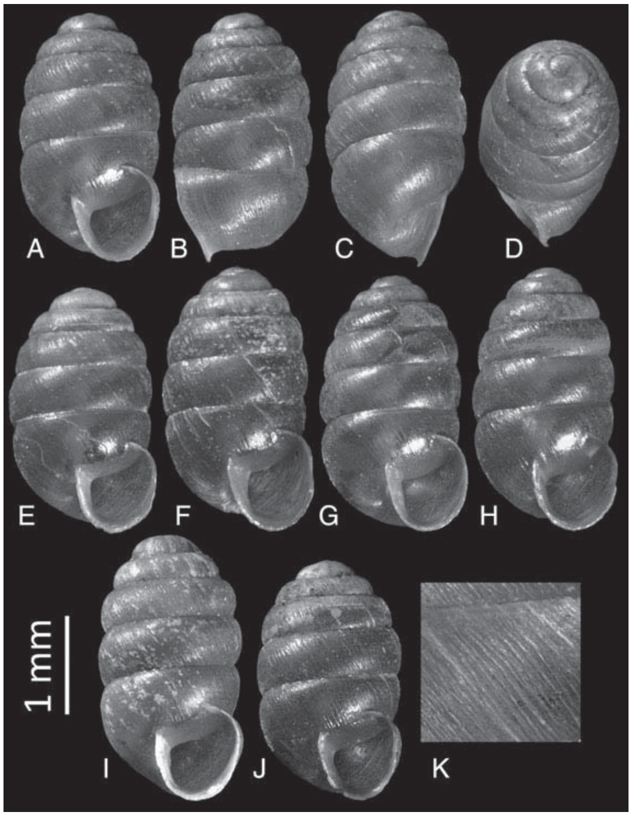

Figure 8. A–H. *Pupilla hokkaidoensis*. **A–D, K.** Holotype, ANSP 458642; Toyokoro, Nakagawa District, Hokkaido Prefecture, Japan. **E.** Paratype, ANSP 458643; Toyokoro, Nakagawa District, Hokkaido Prefecture, Japan. **F.** Paratype, ANSP 458644; Kushiro Marsh, Kushiro District, Hokkaido Prefecture, Japan. **G.** Paratype, ANSP 458646; Hama-koshimizu, Shari District, Hokkaido Prefecture, Japan. **H.** Paratype, ANSP 458645; Betsukai, Notsuke District, Hokkaido Prefecture, Japan. **I.** *P. hebes*, Charleston, Elko Co., Nevada, USA; 41°41′12″N, 115°30′22″W; JCN 18292. **J.** *P.* cf. *limata*, Kapitonovka, Yakutia Republic, Russia; 62°19′45″N, 129°55′42″E.

muscorum and *P. alpicola*, respectively, based upon unstable shell apertural characters. It is fortunate that degree of apertural calcification has never been used to split *P. triplicata*, as its development of lamellae can range from three very strong (French Pyrenees) to absent (basalt talus in northern Bohemia).

In central Asia, oversplitting has also been an issue. While some recently described taxa are strongly demarcated (e.g. *P. alluvionica* and *P.* cf. *khujerabica*), others (*P. altaica* and *P. pratensis*) appear to represent high-calcification endpoints in apertural development within previously described species (*P. turcmenica* and *P. alpicola*, respectively). Perhaps it is not surprising that such high-calcification shell forms tend to originate from drier, lower elevation sites, which would have higher calcium availability due to lower leaching and higher potential evapotranspiration rates (Lapenis *et al*., 2008).

In North America, overlumping and ignorance of taxonomically valid shell traits has led to considerable confusion. First, *P. muscorum* is not a native North American species, with all examined putative native populations representing either *P. hebes* (southwestern USA) or *P. hudsonianum* (central/eastern taiga and tundra) and with *P. muscorum xerobia* being a junior synonym of *P. blandi*. *Pupilla blandi* should be distinguished from other North American species not by apertural features, but rather by its weak to obsolete striation and shining shell lustre. *Pupilla hebes* should be distinguished by its strong thread-like striae and narrow, columnar shell. *Pupilla hebes pithodes* is most closely related to *P. hebes*, but differs in its wider and more barrel-shaped shell. Additionally, *P. alaskensis* has been variously regarded as *P. muscorum* or *P. hebes* in spite of its coarser striation, ovate shell shape and deeper suture than either of these species.

Reassessment of Pupilla *biogeography*

In Europe, *P. alpicola* cannot be considered a central European endemic with a disjunct set of populations in the Altai (Horsák *et al*., 2010). Rather, it extends continuously northwest into southern Scandinavia and Ireland (as the former *P. pratensis*) and east into central Asia. Although demarcation between the central Asian and European populations is evident in nDNA, the central Asian subpopulation extends at least as far west as Bohemia. The amount of mixing of these two populations during full glacial stages is thus unclear. *Pupilla triplicata* occurs as far east as the Altai in central Asia. *Pupilla sterrii* is the western sibling of *P. turcmenica*, with populations extending from central Europe east to the Urals. *Pupilla muscorum* is not a Holarctic species, but is a European endemic with confirmed Pleistocene fossil occurrences in loess deposits of central Europe.

In the Altai, species status for two putative central Asian endemics (*P. alluvionica* and *P.* cf. *khunjerabica*) was established. However, another (*P. altaica*) was found to be simply a shell form within *P. turcmenica*, which ranges from western China and Tibet to the Iran–Turkmenistan border (Pilsbry, 1921). *Pupilla loessica* was shown to be a member of a Beringian group that also includes *P. hokkaidoensis* and *P. alaskensis*.

In North America, the lack of true *P. muscorum* as a Pleistocene fossil suggests that it is an exotic species ranging from the western Great Lakes east to Virginia and north into the Canadian maritime provinces. The identical COI haplotype of the Brno and Cedar Rapids *P. muscorum* specimens suggest that both populations were sourced from the same pool. This is not surprising given that extensive immigration from the Czech Republic to eastern Iowa happened during the mid-1800s. *Pupilla hudsonianum*, which has been previously regarded as *P. muscorum*, extends west from the north shore of the St Lawrence River in Quebec to the southern border of Hudson Bay, northwestern Minnesota and the foothills of the Rockies in Alberta. It also represents the putative Pleistocene fossil '*P. muscorum*' reported by Hubricht (1985) across the central Midwestern USA and Plains. *Pupilla blandi* is limited to the Plains (NE New Mexico to NW Minnesota to southern Saskatchewan and Alberta) and rarely penetrates west into the Rockies as far as the continental divide. *Pupilla hebes* is characteristic of the Great Basin from Arizona and California to Utah and Idaho, with a well-demarcated subpopulation from the Colorado Plateau being demonstrated by mtDNA. While this subpopulation would equate to *P. hebes kaibabensis*, its shells and nDNA do not differ in any meaningful way from typical *P. hebes*, and we have not chosen to recognize it here. *Pupilla hebes pithodes* is found to the south and east of typical *P. hebes*, ranging from eastern Arizona and SE Utah to the eastern foothills of the Rockies in Colorado and New Mexico. *Pupilla alaskensis*, formerly confused with *P. hebes*, is actually a sibling of the western Beringian *P. loessica*.

Reassessment of Pupilla *ecology*

The existence of so much apertural variation within species across *Pupilla* begs for an explanation. In particular, how much of these differences are due to genetic variation and how much to ecophenotypic response? Little empirical data exist to address this question. However, we typically noted limited variation in apertural features within populations. Populations expressing a poorly developed apertural callus and lamellae tended to be found in sites with low calcium availability, such as *P. blandi*

(AP37) on acid metamorphic rock in the Colorado Rockies, and *P. triplicata* on basalt talus slopes in Bohemia (e.g. H10). In contrast, the most heavily calcified *P. turcmenica* in the Altai tend to be restricted to xeric, low-elevation steppe, often on calcium-rich metamorphic rock or limestone. Individual age and the season when maturity is reached may also play important factors. While these observations suggest that ecophenotypic or developmental response is responsible for much of the observed variation, different shell forms have nevertheless been observed in co-occurring individuals that share identical mtDNA and nDNA haplotypes (e.g. AP27, AP28), suggesting that multiple factors may be operating.

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We initially embarked on this project to discern whether *P. triplicata* with vestigal or absent lamellae from the basalt screes of northern Bohemia warranted erection as a new species. Had our initial hypothesis been validated, we intended to name this taxon after Dr Ložek. While this work ultimately documented an entirely different story, we would still like to thank Dr Ložek for his lifetime of work on Eurasian land snails, including the initial description of *P. loessica*.

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