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ARTICLE

Taxonomy and biogeography of *Hemphillia* (Gastropoda: Pulmonata: Arionidae) in North American rainforests, with description of a new species (Skade's jumping-slug, *Hemphillia skadei* sp. nov.)

M.K. Lucid, A. Rankin, A. Espíndola, L. Chichester, S. Ehlers, L. Robinson, and J. Sullivan

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Abstract: Species diversity of the genus *Hemphillia* Bland and W.G. Binney, 1872 (jumping-slugs) was studied across its range in western North America's inland temperate rainforests. The taxonomic relationships among jumping-slug populations were clarified by integrating morphological, molecular, and biogeographic approaches. A new species, Skade's jumping-slug (***Hemphillia skadei*** sp. nov.), was discovered in this process and is described herein. We base this taxonomic decision on molecular comparison of representatives from other *Hemphillia* species and four morphological characters that distinguish *H. skadei* from its sister species, the pale jumping-slug (*Hemphillia camelus* Pilsbry and Vanatta, 1897). The distribution of *H. skadei* and *H. camelus* is described along with the notable lack of detection of the marbled jumping-slug (*Hemphillia danielsi* Vanatta, 1914) within the primary survey area.

Key words: biodiversity, forest, Gastropoda, *Hemphillia camelus*, *Hemphillia danielsi*, *Hemphillia dromedarius*, *Hemphillia glandulosa*, *Hemphillia malonei*, *Hemphillia skadei*, *Magnipelta mycophaga*, morphology, phylogeny, temperate, taxonomy, systematics.

Résumé : La diversité spécifique du genre *Hemphillia* Bland and W.G. Binney, 1872 (limaces-sauteuses) a été étudiée à la grandeur de son aire de répartition dans les forêts humides tempérées intérieures de l'ouest de l'Amérique du Nord. Les relations taxonomiques au sein des populations de limaces-sauteuses ont été précisées en intégrant des approches morphologiques, moléculaires et biogéographiques. Ce faisant, une nouvelle espèce, ***Hemphillia skadei*** sp. nov., a été découverte et est décrite. Nous basons cette décision taxonomique sur la comparaison moléculaire à des représentants d'autres espèces d'*Hemphillia* et quatre caractères morphologiques qui distinguent *H. skadei* de son espèce-sœur, la limace-sauteuse pâle (*Hemphillia camelus* Pilsbry et Vanatta, 1897). Les répartitions de *H. skadei* et *H. camelus* sont décrites, ainsi que l'absence notable de détection de limaces-sauteuses marbrées (*Hemphillia danielsi* Vanatta, 1914) dans la principale région d'étude. [Traduit par la Rédaction]

Mots-clés : biodiversité, forêt, gastéropodes, *Hemphillia camelus*, *Hemphillia danielsi*, *Hemphillia dromedarius*, *Hemphillia glandulosa*, *Hemphillia malonei*, *Hemphillia skadei*, *Magnipelta mycophaga*, morphologie, phylogénie, tempérée, systématique, taxonomique.

Introduction

The demographic and distribution data necessary for landscape-level species conservation are only usable in the context of accurate taxonomic frameworks (Wilson 2000). Within this context, spatially expansive inventory programs are a necessary component of species status assessments, particularly for data-deficient species (e.g., Bland et al. 2017), to determine if they should be targeted for conservation action. North American terrestrial gastropods are a case in point. Mollusk species comprise the second largest phylum (Mollusca) of Animalia, and this highlights their ecological impact on the biosphere. Mollusks serve a wide variety of ecological roles including decomposing organic matter, hosting parasites, vectoring disease, soil building, and providing a prey base for many species (Jordan and Black 2012). Although mollusks are listed as the third largest group of International Union of Conservation of Nature (IUCN) threatened animal species globally and are the most numerous group of IUCN threatened species in North America (http://www.iucnredlist.org/, accessed 27 February 2017), biological knowledge is limited and much remains to be discovered. Without thorough knowledge of the ecology, evolution, and systematics of these animals, it will be difficult to describe and conserve the diversity that mollusks represent.

Up to 25% of North American terrestrial gastropods await description (Nekola 2014) and new species and genera from the Pacific Northwest are described regularly (e.g., Leonard et al. 2003; 2011). Some areas of the Pacific Northwest have exceptionally high terrestrial mollusk diversity and endemism (Frest and Johannes 1995; Burke 2013), some of which may still be undescribed. Jumping-slugs, gastropods in the genus *Hemphillia* Bland and W.G. Binney, 1872 (Bland and Binney 1874), are endemic to North America's Pacific Northwest. The genus currently consists of five recognized species

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(Burke 2013) distributed in a mesic forest disjunct pattern (Brunsfeld et al. 2001).

The dromedary jumping-slug (*Hemphillia dromedarius* Branson, 1972), warty jumping-slug (*Hemphillia glandulosa* Bland and W.G. Binney, 1872), and malone jumping-slug (*Hemphillia malonei* Pilsbry, 1917) have coastal distributions, whereas the pale jumping-slug (*Hemphillia camelus* Pilsbry and Vanatta, 1897) (Pilsbry and Vanatta 1898) and marbled jumping-slug (*Hemphillia danielsi* Vanatta, 1914) are distributed in inland forests. The Burrington jumping-slug (*Hemphillia burringtoni* Pilsbry, 1948; also known as the keeled jumping-slug) (Branson, 1972), a sixth purported species, is not supported by molecular analysis (Wilke and Ziegltrum 2004). The panther jumping-slug (*Hemphillia pantherina* Branson, 1975), a seventh purported species, was described from a single coastal specimen (Branson 1975) but is now widely viewed as not warranting specific recognition (Burke 2015; T.E. Burke, personal communication).

The two inland *Hemphillia* species (*H. camelus* and *H. danielsi*) were included as part of a multitaxa inventory program in the inland Pacific Northwest (Lucid et al. 2016). Specifically, *H. camelus* was known to occur within the study area (Burke 2013), and *H. danielsi* was known to occur adjacent to (Hendricks 2003), as well as predicted to occur within (Burke 2013), the study area. Our objectives were to (*i*) identify the *Hemphillia* species diversity from northern Idaho and the surrounding regions, (*ii*) clarify the taxonomic relationships of those entities by integrating morphological, molecular, and biogeographic approaches, (*iii*) describe anatomical, molecular, distributional, and natural history characteristics of a new taxon discovered in this process.

Materials and methods

Study area

Centered on northern Idaho's Panhandle region, the study area encompassed portions of northeastern Washington and northwestern Montana, USA (Figs. 1A and 1B). It included parts of the Selkirk, Purcell, West Cabinet, Coeur d'Alene, and Saint Joe mountain ranges. The topography is mountainous, ranging in elevation from 702 to 2326 m, with a climate characterized by mild summers and wet and moderately cold winters. The heavily forested area is dominated by a diverse mix of conifer species and is characterized as supporting inland temperate rainforest (DellaSala et al. 2011).

Field methods

We stratified our study area into 920 sampling cells that were 5 km × 5 km each and conducted 992 surveys in 879 of those cells. Our survey sites were either randomly selected but biased to roads and trails (n = 842) or subselected from random forest inventory and analysis plots (n = 150) (Bechtold and Patterson 2005) based on site characteristics (stand age, elevation, and distance to road; for details see Lucid et al. 2016). Gastropod surveys were conducted from May to November, 2010–2014. Each site was surveyed once during this time period with each survey consisting of two repeat visits. Each survey consisted of (*i*) three 30 cm × 30 cm cardboard cover board traps (Boag 1982) from which specimens were collected after a 14 day deployment period, (*ii*) collection, freezing, and drying of 1 L of leaf litter (Coney et al. 1981) from which gastropods were sorted, and (*iii*) two 15 min timed visual searches conducted 14 days apart. Air temperature was recorded every 90 min at survey sites for 1 year as described in Lucid et al. (2016). Field personnel collected all terrestrial gastropods encountered, drowned each specimen in water, and then preserved specimens in 70% ethanol.

Morphological identification

To identify the species present in the area, we identified all ethanol preserved specimens following Burke (2013). Of 177 preserved specimens identified as *Hemphillia* spp., we measured total body length and mantle length of 34. Our dissection sample size was limited because we restricted specimens selected for dissection to those with body length ≥10 mm to maximize the potential for specimens to possess mature genitalia. Thirty-four specimens genetically identified (see below) as *H. camelus* (n = 22) or ***Hemphillia skadei*** sp. nov. (Skade's jumping-slug; n = 12) were dissected under a binocular dissecting microscope (Table 1). Microsurgical scissors and fine dental probes were employed to access the internal organs, with special emphasis on the reproductive system. The technique was modified from Gregg (1958).

Molecular identification

For initial analyses, DNA was extracted at Wildlife Genetics International (WGI) using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) per the manufacturer's protocols. WGI developed a species test for this group of organisms using a portion of the mitochondrial *16s* rRNA gene that was compared with reference data. WGI downloaded *16s* rDNA sequences from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) for *H. dromedarius* (AY382638), *H. camelus* (AY382639), and the pygmy slug (*Kootenaia burkei* Leonard, Chichester, Baugh and Wilke, 2003) (AY382640, AY382641, AY382642, and AY382643). Using these sequences, primers were designed to target two conserved regions across a range of slug species that flanked the highly variable portion of the *16S* rRNA gene (16sbr-H 5′-CCGGTCTGAACTCAGATCACGT-3′ and 16sar-L 5′-CGCCTGTTTATCAAAAACAT-3′). Polymerase chain reaction (PCR), electrophoresis, and Sanger sequencing were used to produce sequence profiles of a highly variable portion of the *16S* rDNA region. These sequences were afterwards compared with reference data from GenBank, using the program BLAST (Altschul et al. 1997).

Extended molecular analyses

Based on the results of the molecular species ID and comparative anatomy analyses (see below), we sampled additional individuals to subject to phylogenetic and DNA barcoding analyses. All laboratory work described henceforth was performed at the University of Idaho. Data were obtained from 30 *H. camelus* specimens (plus one from GenBank AY382632_AY382639) and 28 *H. skadei* specimens originating from multiple localities in northern Idaho and the surrounding regions. In addition, nine specimens from *H. danielsi*, eight from *H. glandulosa*, five from *H. dromedarius*, and seven from *H. malonei* were obtained (Table 2, Fig. 1B). The magnum mantleslug (*Magnipelta mycophaga* Pilsbry, 1953) was included as an outgroup. Total DNA was extracted from the foot of each specimen (10–15 mg) using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) per the manufacturer's protocols. Partial sequences of the mitochondrial *COI* gene, mitochondrial *16S* rRNA gene, and nuclear *ITS1* marker were amplified by the PCR with the primers LCO1490/HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ and 5′-GGTCAACAAATCATAAAGATATTGG-3′; Folmer et al. 1994), 16Sar-L/16Sbr-H (described above), and ITS1F/ITS1R (5′-GCTGCGTTCTTCATCGATGC-3′ and 5′-TAACAAGGTTTCCGTAGGTGAA-3′; Armbruster et al. 2000; Mumladze et al. 2013), respectively. All PCRs were carried out in 50 μL reactions containing 3 μL DNA, 37.75 μL water, 5 μL buffer, 1 μL of 25 mmol/L $MgCl_2$, 1 μL of 10 mmol/L dNTPs, 1 μL of 10 mmol/L forward and reverse primers, and 0.25 μL of 5 U/μL of New England Biolabs *Taq* polymerase. The PCR reactions consisted of an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of a denaturation step at 95 °C for 35 s, an annealing step (52 °C for *COI*, 47.5 °C for *16s* rDNA, and 52 °C for *ITS1*) for 60 s, and an elongation step at 72 °C for 45 s, and finalized with a final extension step at 72 °C for 5 min. Amplicons were electrophoresed in a 1% agarose gel to verify the amplifications and were cleaned up using the Qiaquick PCR Cleanup Kit (Qiagen). PCR products were sequenced in both the forward and reverse directions, and sequences were visually examined and edited with Chromas version 2.6.2 (Technelysium Pte Ltd.; http://www.technelysium.com.au/chromas.html). Nuclear *ITS1* electropherograms showed

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Fig. 1. *Hemphillia* sampling. (A) Survey area and visited sampling cells. Dashed cells: visited cells; dotted outlines: Skade's jumping-slug (*Hemphillia skadei*) found; gray cells: pale jumping-slug (*Hemphillia camelus*) found. (B) Samples used in our phylogenetic study. Shapes and colors indicate species.

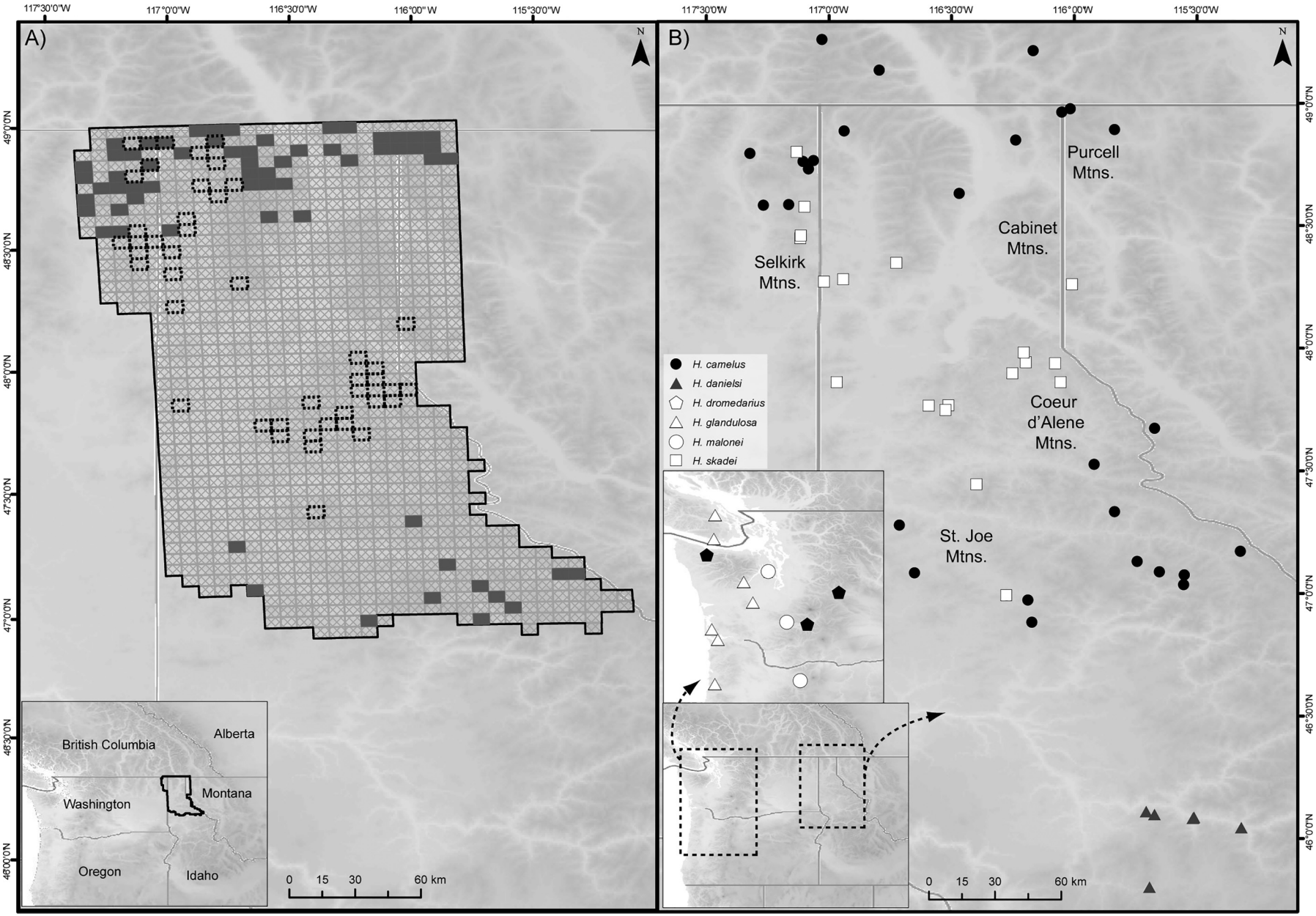

Table 1. Dissected specimens of the pale jumping-slug (*Hemphillia camelus*) and Skade's jumping-slug (*Hemphillia skadei*) specimens.

Specimen	Genetic ID	Length (mm)		Maturity	Collection date[a]
		Body	Mantle		
C113GTSD	*H. camelus*	23	13.5	Mature	7/16/2010
C50GTSV2D	*H. camelus*	10	8	Immature	8/29/2010
C97GTS	*H. camelus*	20	15	Immature	8/17/2010
C113GTSC	*H. camelus*	27	19	Mature	7/16/2010
C2GBV2	*H. camelus*	30	16	Mature	8/28/2010
C20GTS	*H. camelus*	31	21	Mature	8/14/2010
C137GTSA	*H. camelus*	21	18	Mature	8/19/2010
GDNA2	*H. camelus*	28	19	Mature	8/3/2012
GDNA3	*H. camelus*	28	18	Mature	8/3/2012
GDNA4	*H. camelus*	28	17	Mature	8/3/2012
GDNA5	*H. camelus*	21	13	Mature	8/3/2012
GDNA6	*H. camelus*	24	16.5	Mature	8/3/2012
GDNA7	*H. camelus*	24	13	Immature	8/3/2012
GDNA8	*H. camelus*	32	21	Mature	8/3/2012
GDNA9	*H. camelus*	37	18	Mature	10/1/2012
GDNA10	*H. camelus*	27	16	Mature	10/1/2012
GDNA11	*H. camelus*	31	19	Mature	10/1/2012
GDNA12	*H. camelus*	39	20	Mature	10/1/2012
GDNA13	*H. camelus*	30	21	Mature	10/1/2012
GDNA14	*H. camelus*	40	20	Mature	10/1/2012
GDNA15	*H. camelus*	32	21	Mature	10/1/2012
GDNA16	*H. camelus*	27	20	Mature	10/13/2012
C108GTS	*H. skadei*	12	8	Immature	8/12/2010
C43GTSV2A	*H. skadei*	17	12	Immature	8/27/2010
C50GTSV2A	*H. skadei*	20	11	Immature	8/29/2010
C95GTSA	*H. skadei*	15	13	Immature	7/16/2010
C92GTS	*H. skadei*	25	14	Mature	8/13/2010
C26GTSV2	*H. skadei*	21	11	Immature	8/27/2010
GDNA1	*H. skadei*	24	12.5	Immature	6/21/2012
FIA1180GTSV3A	*H. skadei*	24	16	Mature	9/20/2014
FIA76GTSV3AB	*H. skadei*	21	14	Mature	9/24/2014
FIA76GTSV3B(1)	*H. skadei*	11.5	9	Immature	9/24/2014
FIA125GTSV3B(1)	*H. skadei*	14	8	Immature	9/25/2014
FIA125GTSV3B(2)	*H. skadei*	17.5	5	Immature	9/25/2014

[a]Collection date format is month/day/year.

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low levels of heterogeneity, and a consensus sequence was generated. The mitochondrial *COI* gene and mitochondrial *16S* rRNA gene were concatenated into a single data set, and the resulting sequence data were assembled into two separate sets: the concatenated *COI-16S* and the *ITS1*. All new sequences are deposited on GenBank (accession Nos. MG640377-MG640475, MG640486-MG640561, MG641787-MG641883; additional details, including sample names and collection localities, are available in Supplementary Table S1[1]).

Phylogenetic analyses

Multiple alignments were constructed between *H. camelus*, *H. skadei*, *H. danielsi*, *H. dromedarius*, *H. glandulosa*, *H. malonei*, and *M. mycophaga* using MAFFT online (https://www.ebi.ac.uk/Tools/msa/mafft/). In both data sets, many regions were too divergent to be aligned across lineages (i.e., parts of *16S* and *ITS1*). Therefore, we used the Gblocks algorithm (Castresana 2002) to eliminate ambiguous regions and extract the conserved regions from each alignment, resulting in 992 bp for the concatenated *COI-16S* and 390 bp for *ITS1*.

The mitochondrial *COI-16S* data matrix was subjected to maximum likelihood (ML) and Bayesian phylogeny estimation. We used the decision theoretic approach (DT; Minin et al. 2003) implemented in PAUP* version 4.0a152 (Swofford 2003) to select a model of nucleotide sequence evolution using the Bayesian information criterion (BIC). ML analyses were performed in Garli (Zwickl 2006) using the TrN+I+Γ model and default parameters. The ML tree was first determined by conducting 10 replicate runs with random starting trees, and nodal support was then assessed using 100 bootstrap replicates using two tree searches per bootstrap. Bootstrap values were viewed in PAUP* by constructing a majority rule consensus tree. Bayesian analyses with the Metropolis-coupled Markov chain Monte Carlo method were performed in MrBayes version 3.2.5 (Huelsenbeck and Ronquist 2001) using the TrN+I+Γ model and default parameters. The analysis was run twice with four chains per run for 2 million generations, and trees were saved every 200 generations. Convergence was assessed by the standard deviation of split frequencies. We assumed runs had converged when this value reached 0.01. We also assessed parameter estimates with Tracer version 1.4 (Rambaut and Drummond 2007) to assure that these values had stabilized. We discarded the first 25% of samples from each run and built a majority-rule consensus tree from the remaining trees. All tree output files were viewed and summarized using Figtree version 1.3 (Rambaut and Drummond 2014).

For the nuclear *ITS1* data set, an ultrametric tree was estimated in BEAST version 2 (Bouckaert et al. 2014), under a strict molecular clock with the mean substitution rate set to 1, so that time would be reported in units of substitutions per site. The Yule speciation

[1]Supplementary Table S1 is available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjz-2017-0260.

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Table 2. Specimens of the pale jumping-slug (*Hemphillia camelus*), Skade's jumping-slug (*Hemphillia skadei*), marbled jumping-slug (*Hemphillia danielsi*), dromedary jumping-slug (*Hemphillia dromedarius*), warty jumping-slug (*Hemphillia glandulosa*), malone jumping-slug (*Hemphillia malonei*), and magnum mantleslug (*Magnipelta mycophaga*) used in phylogenetic and DNA barcoding analyses, with asterisks denoting sequences from GenBank.

Sample	ID	State or province[a]	Latitude (°N)	Longitude (°E)	Collection date[b]
C798GTSV2	*H. camelus*	ID	47.2795	–116.71259	7/14/2011
C833GTSV1A	*H. camelus*	ID	47.08485	–116.65096	8/2/2013
C1110GTSV1A	*H. camelus*	ID	46.97431	–116.18865	7/21/2013
FIA1539BGNCA	*H. camelus*	ID	47.036	–115.555	7/27/2013
C798GN	*H. camelus*	ID	47.2795	–116.71259	7/14/2011
FIA1343GTSV1A	*H. camelus*	ID	47.222	–115.878	7/16/2013
C4GTSV2A	*H. camelus*	WA	48.79582	–117.32204	8/31/2010
FIA83GTSV2B	*H. camelus*	ID	48.888	–116.937	6/15/2013
C32GNAC	*H. camelus*	WA	48.76273	–117.10503	6/15/2013
C1291GW	*H. camelus*	ID	48.96342	–116.05133	7/28/2011
C31GW	*H. camelus*	WA	48.73202	–117.08286	8/14/2010
C1152GN	*H. camelus*	ID	48.85086	–116.23873	7/4/2011
C1110GTSV2A	*H. camelus*	ID	46.97431	–116.18865	8/4/2013
C1428GW	*H. camelus*	MT	48.89293	–115.83614	7/3/2011
C1110GNCB	*H. camelus*	ID	46.97431	–116.18865	8/4/2013
FIA1539AGTSV3BA	*H. camelus*	ID	47.052	–115.580	9/13/2014
C1336PGA	*H. camelus*	MT	48.9783	–116.01607	7/28/2011
C1336GWA	*H. camelus*	MT	48.9783	–116.01607	7/28/2011
C584BGTSV1	*H. camelus*	WA	48.58734	–117.1638	7/27/2011
C1743GTSV2A	*H. camelus*	ID	47.17297	–115.32325	7/26/2013
C167GTSB	*H. camelus*	ID	48.63165	–116.46842	7/3/2010
FIA1436GNB	*H. camelus*	ID	46.989	–115.696	7/28/2013
C1489GTSV1C	*H. camelus*	ID	47.08875	–115.65327	7/12/2013
C541GTSV2B	*H. camelus*	WA	48.58376	–117.26717	6/13/2013
C1304GTSV1A	*H. camelus*	ID	47.5275	–115.91911	6/22/2013
UI05	*H. camelus*	ID	46.883395	–116.172734	9/11/2015
AY382632_AY382639*	*H. camelus*	WA			
51111666	*H. camelus*	MT	47.6738	–115.673	
RBCM-013-00058-002	*H. camelus*	BC	49.260754	–117.02835	9/12/2010
RBCM-998-00267-001	*H. camelus*	BC	49.135821	–116.794549	9/21/1998
RBCM-998-00287-001	*H. camelus*	BC	49.215358	–116.167895	9/20/1998
C25BGTSV2C	*H. skadei*	WA	48.44899	–117.11639	8/11/2011
FIA1131GTSV1C	*H. skadei*	ID	47.943	–116.198	6/28/2013
C1223GNCB	*H. skadei*	ID	47.93804	–116.07764	7/12/2013
FIA883GTSV1	*H. skadei*	ID	47.765	–116.594	6/9/2013
FIA1080PGA	*H. skadei*	ID	47.813	–116.270	6/8/2013
C1222GTSV2	*H. skadei*	ID	47.86182	–116.0567	7/13/2013
FIA116GTSV3BC(1)	*H. skadei*	ID	48.349	–116.725	9/27/2014
C919GTSV1B	*H. skadei*	ID	47.7672	–116.51242	5/25/2013
FIA1127GTSV3A	*H. skadei*	ID	47.771	–116.192	9/22/2014
C919GNCA	*H. skadei*	ID	47.7672	–116.51242	6/7/2013
C697GTSV1B	*H. skadei*	ID	48.28077	–116.94193	5/30/2013
C918GNBB	*H. skadei*	ID	47.748	–116.52481	6/7/2013
C1132GNAC	*H. skadei*	ID	47.98217	–116.20507	7/13/2013
FIA992GTSV2B(1)	*H. skadei*	ID	47.856	–116.405	7/14/2013
C25BGTSV2A	*H. skadei*	WA	48.44899	–117.11639	8/11/2011
C918GNBA	*H. skadei*	ID	47.748	–116.52481	6/7/2013
C1223GNBC	*H. skadei*	ID	47.93804	–116.07764	7/12/2013
C688GTSV2A	*H. skadei*	ID	47.86127	–116.96848	6/25/2013
C918GTSV2A	*H. skadei*	ID	47.748	–116.52481	6/7/2013
C25GTSC	*H. skadei*	WA	48.45904	–117.11486	8/17/2010
C28GTSV2A	*H. skadei*	WA	48.57685	–117.09885	8/26/2010
C28GTSV2B	*H. skadei*	WA	48.57685	–117.09885	8/26/2010
C25GTSE	*H. skadei*	WA	48.45904	–117.11486	8/17/2010
C33GW	*H. skadei*	WA	48.8012	–117.13055	8/14/2010
C982GTSV2	*H. skadei*	ID	47.44566	–116.39922	6/14/2013
C697GNB	*H. skadei*	ID	48.28077	–116.94193	6/13/2013
C658GTSV2B	*H. skadei*	ID	48.27209	–117.01922	6/13/2013
FIA116GTSV3A	*H. skadei*	ID	48.349	–116.725	9/27/2014
BLdan01	*H. danielsi*	ID			9/12/2010
BLdan07	*H. danielsi*	ID	46.77501	–11548071	9/14/2010
UI01	*H. danielsi*	ID	46.085226	–115.51515	2/–/2015
UI03	*H. danielsi*	ID	46.083801	–115.516957	5/23/2016

Table 2 (*concluded*).

Sample	ID	State or province[a]	Latitude (°N)	Longitude (°E)	Collection date[b]
UI04	*H. danielsi*	ID	46.085226	–115.51515	2/–/2015
CM-64157	*H. danielsi*	ID	45.803047	–115.6942	9/14/2002
Ma12DG01380	*H. danielsi*	ID	46.099668	–115.67396	12/15/2015
Ma16AG01403_1	*H. danielsi*	ID	46.111797	–115.707343	1/20/2016
UN5CG01604_3	*H. danielsi*	ID	46.046097	–115.320409	12/15/2015
CM-63984_1	*H. dromedarius*	WA	47.223244	–121.144828	7/13/2001
CM-63984_2	*H. dromedarius*	WA	47.223244	–121.144828	7/13/2001
CM-68014	*H. dromedarius*	WA	47.68067	–122.68133	11/28/2003
CM-64708	*H. dromedarius*	WA	46.534204	–121.831656	4/12/2003
AY382631_AY382638*	*H. dromedarius*	WA			
RBCM-014-00268-001	*H. glandulosa*	BC			8/25/2001
RBCM-016-00167-001	*H. glandulosa*	BC			6/7/2015
CM-63982_1	*H. glandulosa*	WA	46.41185	–123.906166	10/22/2002
CM-63982_2	*H. glandulosa*	WA	46.41185	–123.906166	10/22/2002
CM-80083	*H. glandulosa*	OR	45.224833	–123.838667	4/12/2007
CM-64903	*H. glandulosa*	WA	47.44667	–123.21056	2/20/2003
CM-63972	*H. glandulosa*	WA	47.002005	–123.01096	10/22/2002
AY382630_AY382637*	*H. glandulosa*	OR			
CM-63526_2	*H. malonei*	WA	46.578892	–122.273635	9/4/2001
CM-63526_3	*H. malonei*	WA	46.578892	–122.273635	9/4/2001
CM-70373_2	*H. malonei*	WA	47.68067	–122.68133	10/29/2004
CM-70373_3	*H. malonei*	WA	47.68067	–122.68133	10/29/2004
CM-70373_4	*H. malonei*	WA	47.68067	–122.68133	10/29/2004
AY357609_AY357656*	*H. malonei*	OR			
FIA121GTSV3B	*M mycophaga101*	ID	48.574	–116.732	9/26/2014
FIA1234PGA	*M. mycophaga102*	ID	48.438	–116.120	7/8/2013
C896GTSV2	*M. mycophaga103*	ID	48.36953	–116.66483	7/15/2013
C1144GTSV1A	*M. mycophaga104*	ID	48.52729	–116.20406	6/25/2013
C154GNA	*M. mycophaga105*	ID	48.96629	–116.62474	8/2/2013

Note: CM prefix denotes specimens from the Carnegie Museum and RBCM denotes specimens from the Royal British Columbia Museum. Coordinates for the samples with the FIA prefix have been purposely altered in compliance with a U.S. Forest Service legal agreement but are within 500 m of the actual location.

[a]State or province abbreviation (listed alphabetically within country): ID, Idaho; MT, Montana; OR, Oregon; WA, Washington; BC, British Columbia.

[b]Collection date format is month/day/year.

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model was used as a tree prior with the JC+Γ model (selected using DT; Minin et al. 2003). The chain was run for 10 million steps, sampling every 1000 steps. The BEAST output was analyzed using Tracer version 1.4 and the first 25% of samples were discarded as burn-in. TreeAnnotator was used to produce a maximum clade credibility tree from the post-burn-in trees. The ultrametric gene tree was used as a guide tree in the generalized mixed Yule-coalescent (GMYC) model (Pons et al. 2006; Fontaneto et al. 2007; Fujisawa and Barraclough 2013) methodology to delimit independently evolving lineages. We applied the single-threshold model GMYC method using the splits package (Ezard et al. 2009) in R (R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org).

Intraspecific and interspecific genetic distance

We examined the overlap between intraspecific and interspecific genetic distance between *H. camelus* and its candidate sister species using the concatenated *COI-16s* data set and the *ITS1* data set separately. A genetic distance matrix was produced in PAUP* (using an appropriate nucleotide substitution model selected using the DT approach) for pairwise sequence divergences calculated separately for both intraspecific and interspecific comparisons. We report the mean intra- and inter-specific distances, as well as the smallest and largest inter- and intra-specific distances.

Results

Six species of coastal (*H. dromedarius*, *H. glandulosa*, and *H. malonei*) or inland (*H. camelus*, *H. danielsi*, and *H. skadei*) distributed species can be distinguished by the morphological or molecular characters that we examined. *Hemphillia skadei* holotype OSAC_AC_2017_06_09-01-001 was collected on 13 August 2010 in the Selkirk Mountains, Boundary County, Idaho, USA (48.75886°N, –116.84624°E; 763 m elevation). Paratypes may also be accessed via the above accession number.

Table 3. Differences (upper) and similarities (lower) in genitalia of mature dissected specimens.

Pale jumping-slug, *Hemphillia camelus*	Skade's jumping-slug, *Hemphillia skadei*
Penis peanut-shaped	Penis barrel- or acorn-shaped
Penis pigmented	Penis unpigmented
Free oviduct long	Free oviduct short
Spermatophore robust	Spermatophore slender
Long highly coiled epiphallus	
Base of spermathecal duct very swollen in mature animals	
Penis contains a large lobe-like stimulator	
Ovotestis, hermaphroditic duct, and spermoviduct alike	

Etymology of *H. skadei*

In Norse mythology, the goddess Skaði (often anglicized as Skadi or Skade) is associated with winter, mountains, skiing, and bow hunting (Sturluson 1916). The specific name *skadei* recognizes the relatively cool air temperatures with which this mountain-dwelling species is associated, while acknowledging the cultural and recreational value skiing and hunting provide to many of the people who share the range of *H. skadei*. We suggest the common name "Skade's jumping-slug".

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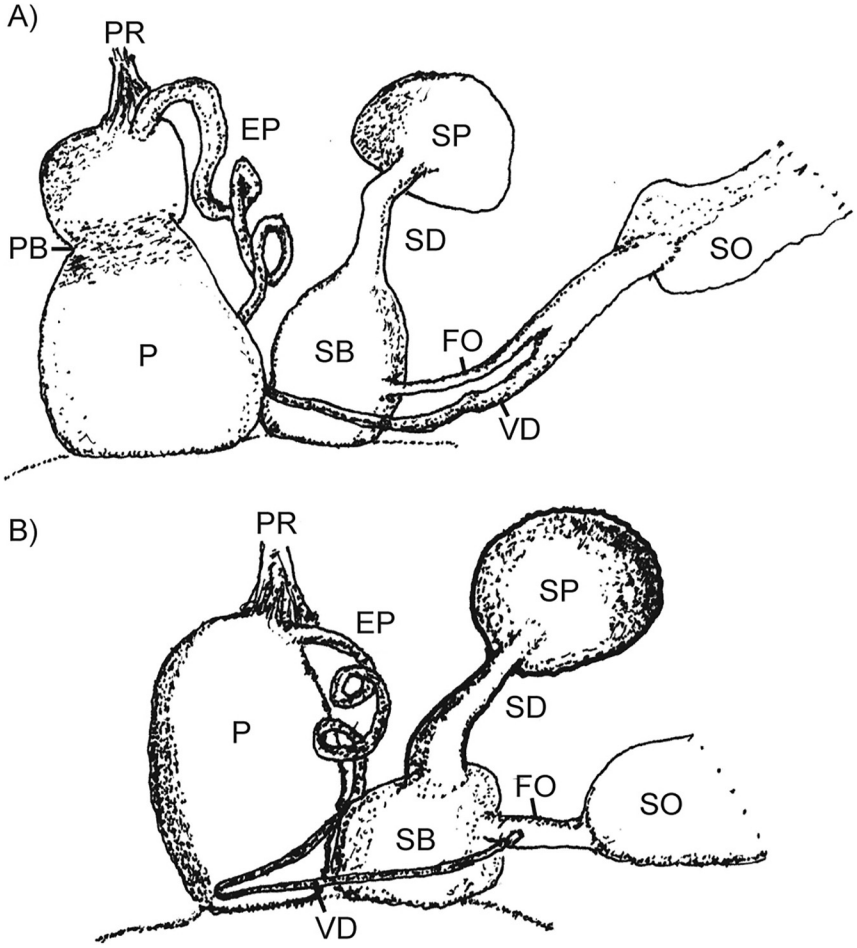
Fig. 2. Distal genitalia of (A) pale jumping-slug (*Hemphillia camelus*) and (B) Skade's jumping-slug (*Hemphillia skadei*). SB: swollen base of spermathecal duct at its junction with free oviduct; SP: spermatheca (bursa copulatrix); P: penis; PR: penis retractor muscle; EP: epiphallus; SD: spermathecal duct; VD: vas deferens; SO: spermoviduct (common duct); FO: free oviduct (vagina); HD: hermaphroditic duct; OV: ovotestis; PB: pigmented band. Modified from Lucid et al. (2016) and reproduced with permission of M.K. Lucid, Idaho Department of Fish and Game, Coeur d'Alene.



Morphology

Reproductive anatomy of H. skadei

The ovotestis consists of a small number of darkly pigmented lobules located in the rearmost portion of the visceral hump. The hermaphroditic duct is highly convoluted and long (about 9 mm). The yellow albumen gland is 4 mm or more in length. The bicolored spermoviduct is about 15 mm in length. The distal end of the free oviduct joins the swollen base of the spermathecal duct (bursa copulatrtix). The vas deferens originates just proximal to this junction. It is a very fine duct loosely adherent to the free oviduct (vagina). In one specimen, the duct formed a small loop and re-entered the wall of the oviduct only to emerge again as a separate duct a short distance away. The vas deferens continues to the base of the penis. It then follows the junction of the penis with the body wall before doing a 180° turn to join the epiphallus. The epiphallus is a stout, highly coiled and folded duct that joins the penis terminally or subterminally. The penis is an unpigmented barrel-shaped structure about 5 mm in length. The penis retractor inserts either on the penis or the epiphallus, or on both. The bisected penis reveals a large stimulator and a complex verge. There is a shallow atrial depression at the genital pore into which the penis and the vagina open.

Comparative anatomy

We found no external anatomical character that differentiated *H. skadei* from *H. camelus*. Both species are tan to gray in color. The genital opening is behind the right tentacle and the pneumostome is on the right side of the third quarter of the mantle. The mantle forms a visceral hump and clearly has an exposed portion of shell on the dorsal portion. Both slug species are of medium size, but the preserved, mature *H. skadei* specimens were smaller than the preserved, mature *H. camelus* specimens. Mature *H. skadei* specimens (n = 3) had a mean body length of 23 mm (minimum 21 mm, maximum 25 mm) and a mean mantle length of 15 mm (minimum 14 mm, maximum 16 mm) (Table 1). Mature *H. camelus* specimens (n = 19) had a mean body length of 29 mm (minimum 21 mm, maximum 40 mm) and a mean mantle length of 18 mm (minimum 13.5 mm, maximum 21 mm). Anatomical characters differentiating the two species are limited to mature genitalia. *Hemphillia skadei* has an unpigmented barrel- or acorn-shaped penis, short free oviduct, and slender spermatophore. *Hemphillia camelus* has a pigmented peanut-shaped penis, long free oviduct, and robust spermatophore (Table 3; Figs. 2A and 2B).

Molecular analysis

Molecular species identification

There were 54 *16S* rDNA sequences produced by WGI, which identified two distinct genetic clusters corresponding to the groups

Fig. 3. Phylogenetic reconstruction of the concatenated mitochondrial data matrix. Node supports show maximum likelihood bootstrap values and Bayesian posterior probabilities. Scale bar indicates number of substitutions per site.

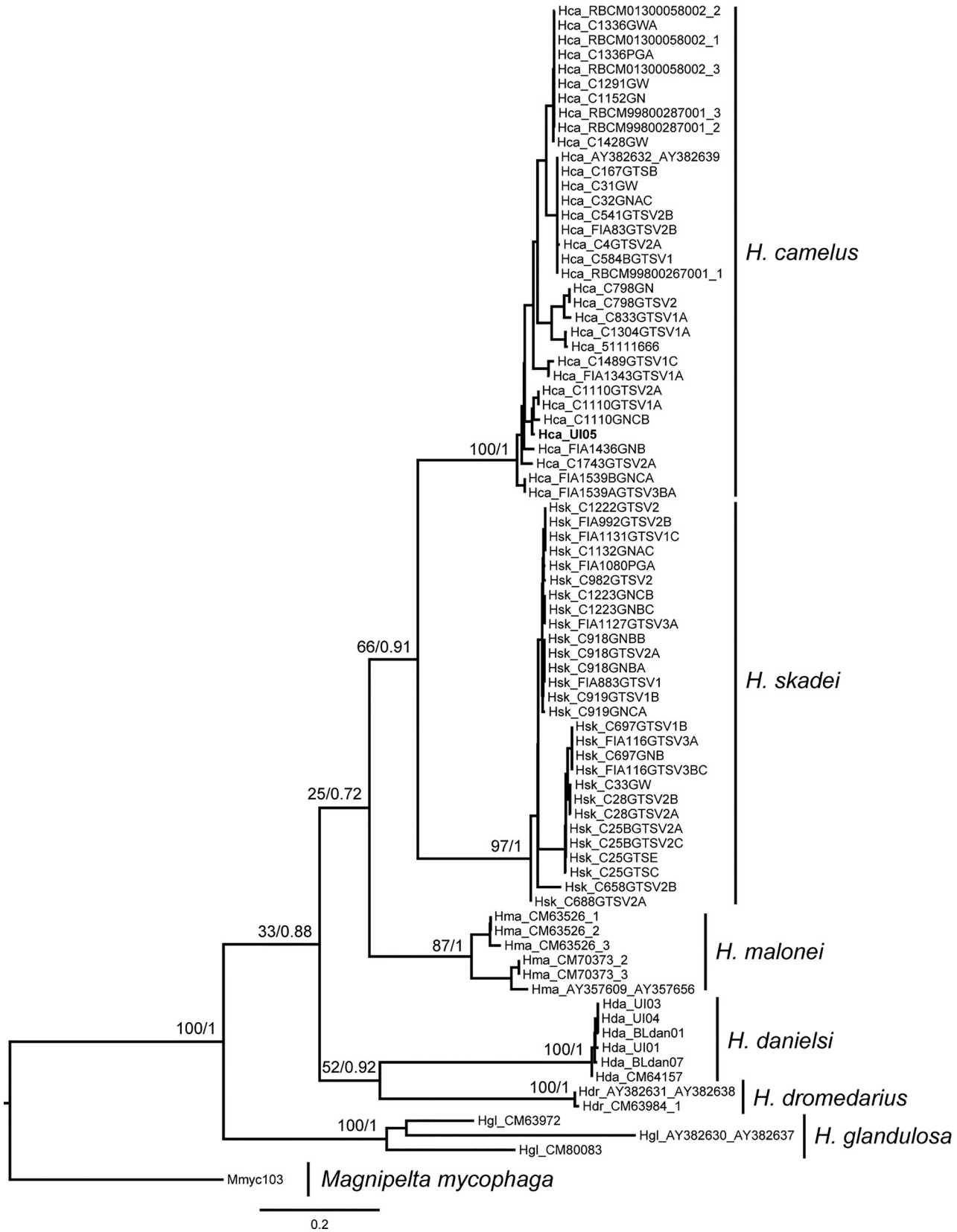


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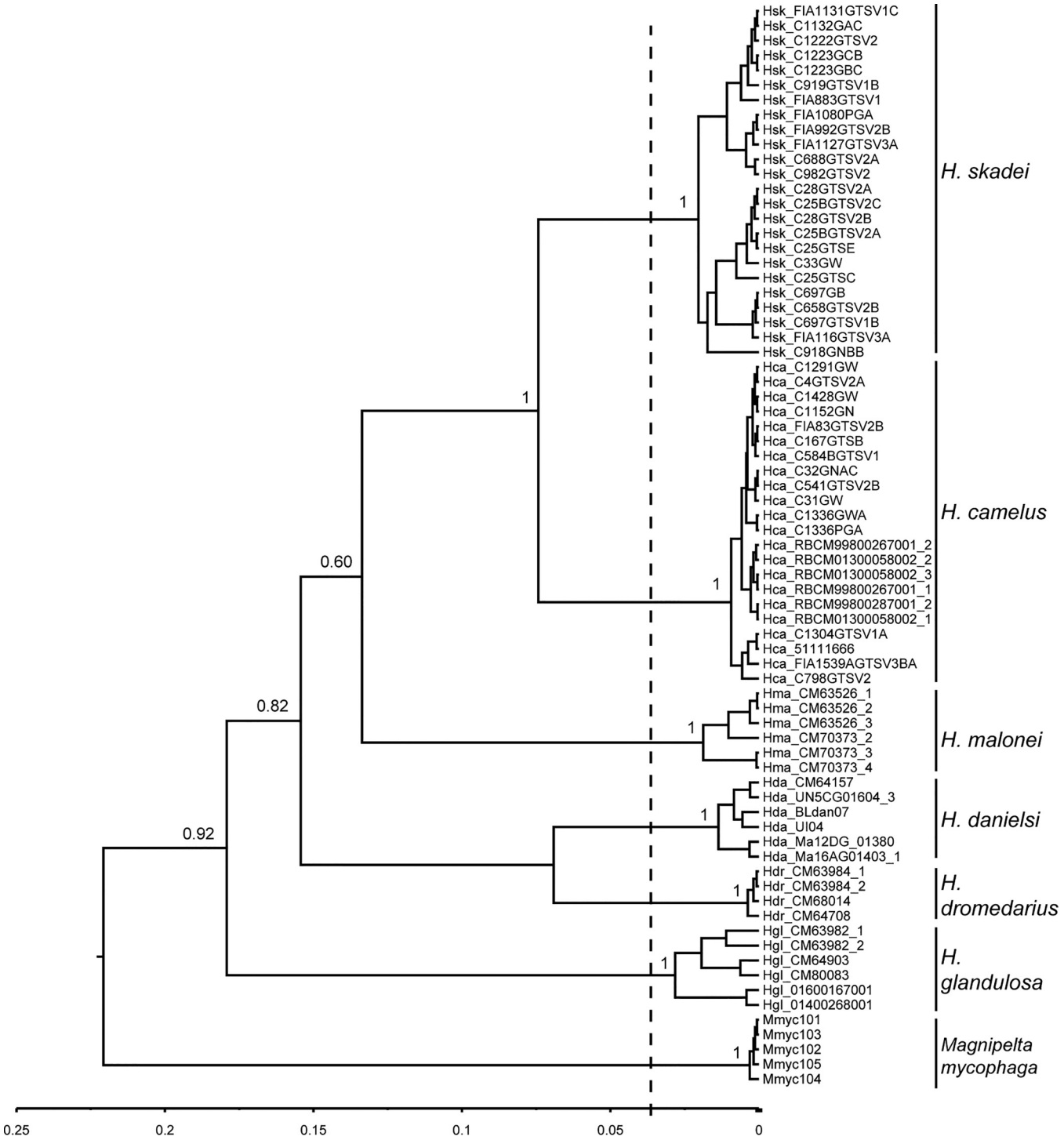
Fig. 4. Phylogenetic reconstruction of the *ITS1* data set using the Yule speciation prior and JC+G model. Node supports show Bayesian posterior probabilities. Vertical broken line indicates the inferred transition from interspecific speciation events to the intraspecific coalescent events.



identified in the morphological identification (33 *H. camelus* sequences and 21 *H. skadei* sequences) (Figs. 3 and 4). Of approximately 424 nucleotides, there were 45 variable sites. Thirty-five of these exhibit fixed differences between the two taxa and therefore distinguish the two groups perfectly.

Intra- and inter-specific genetic distance

For the concatenated *COI-16S* data matrix, the mean interspecific HKY+I+Γ distance was estimated to be 0.427 substitutions/site (0.312–0.619 substitutions/site) (Fig. 5). The mean intraspecific distances for *H. camelus* and *H. skadei* were estimated to be 0.057 substitutions/site (0–0.133 substitutions/site) and 0.046 substitutions/site (0–0.131 substitutions/site), respectively. For the nuclear *ITS1* marker, the mean interspecific JC+Γ distance was 0.179 substitutions/site (0.141–0.238 substitutions/site; Fig. 5), whereas the mean intraspecific distances for *H. camelus* and *H. skadei* were estimated to be 0.0037 substitutions/site (0–0.0114 substitutions/site) and 0.0208 substitutions/site (0–0.0554 substitutions/site), respectively. Therefore, there is no overlap between intra- and inter-specific

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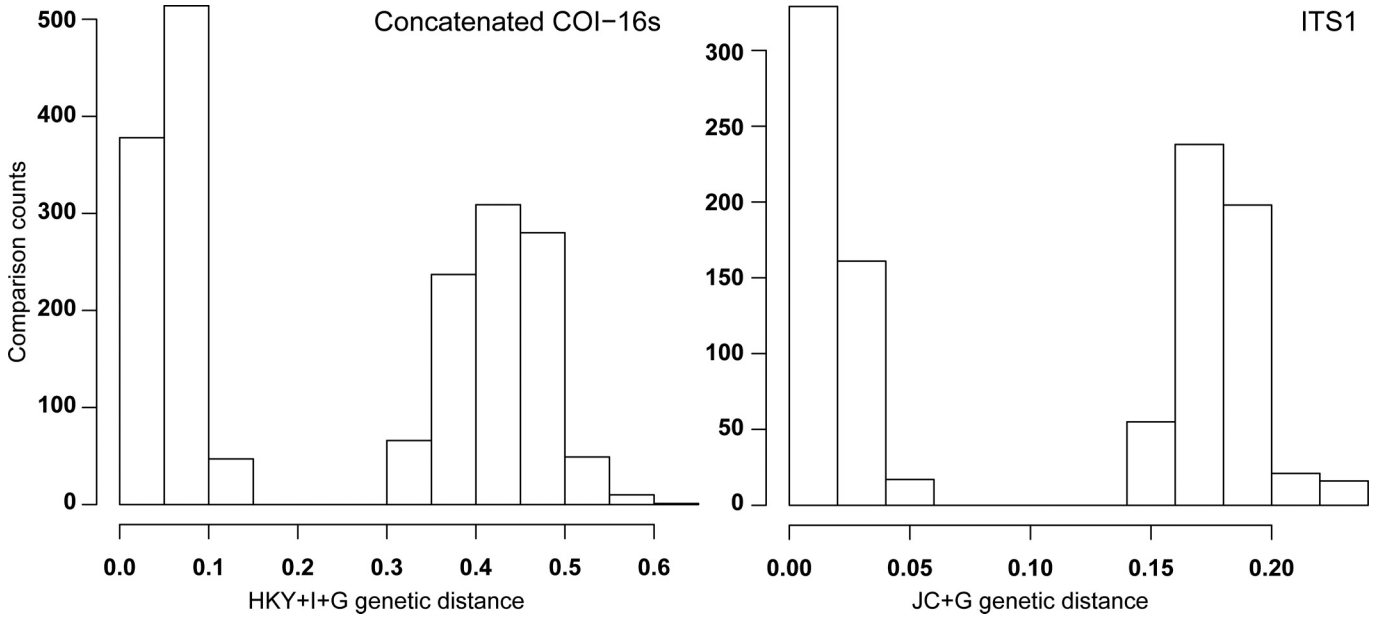
Fig. 5. Distribution of intraspecific and interspecific genetic distances using the concatenated mitochondrial (left) and the nuclear (right) data sets.



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variability; there is a distinct barcoding gap in the distribution of divergences, with higher interspecific variation than intraspecific variation, thereby distinguishing *H. camelus* and *H. skadei*.

Phylogenetic analyses

ML and Bayesian phylogenetic estimates were similar in topology (Fig. 3) and congruent with morphology; that is, *H. camelus* forms a strongly supported phylogroup that is sister to a strongly diverged, well-supported clade that contains all individuals of *H. skadei*. Additionally, *H. glandulosa*, which is distributed along the coast, is sister to all other *Hemphillia*. The inland-species *H. danielsi* is sister to coastally distributed *H. dromedarius*, whereas *H. malonei*, which occurs in coastal locations, is sister to the *H. camelus* – *H. skadei* complex.

The GMYC analysis rejected the null model of single coalescence (i.e., all sequences belong to the same species) model for the nuclear *ITS1* Yule tree (Fig. 4). The likelihood-ratio statistic for the comparison of the single coalescent (null) model versus the GMYC model was 10.24 ($p = 0.006$), indicating support for more than one species. The analysis estimated seven clusters (independent lineages) consistent with previous taxonomic assignment and with the addition of the new taxon, *H. skadei*, as a different entity.

Distribution, intraspecific genetic structure, and natural history

We detected *H. skadei* in 47 (5%) and *H. camelus* in 64 (7%) of 879 surveyed cells (Fig. 1A). *Hemphillia skadei* was the only species detected in the West Cabinet and Coeur d'Alene mountain ranges, whereas *H. camelus* was the only species detected in the Purcell Mountains. *Hemphillia skadei* was detected in one cell in the northern St. Joe Mountains, whereas all other detections in that range were *H. camelus*. In the Selkirks, *H. skadei* was detected in 26 cells and *H. camelus* was detected in 38 cells. Both species were detected in five Selkirk cells and in one instance both species were detected on the same Selkirk cover board trap.

In the mtDNA phylogeny (Fig. 3), *H. camelus* showed a monophyletic "northern" Selkirk–Purcells clade, which is nested within "southern" samples from Saint Joe and Coeur d'Alene. *Hemphillia skadei* shows an unresolved polytomy among a monophyletic "southern" Coeur d'Alene–Saint Joe clade, a "northern" Selkirk group, and one sequence (C658GTSV2B) from the Selkirks. An additional sequence (C688GTSV2A) from the Selkirks is sister to this group. Likewise, in the *ITS1* phylogeny, *H. camelus* shows reciprocally monophyletic "southern" Coeur d'Alene–Saint Joe and "northern" Selkirk–Purcells clades, whereas *H. skadei* shows reciprocally monophyletic "southern" Coeur d'Alene–Saint Joe and "northern" Selkirk clades (apart from C688GTSV2A from the Selkirks, which groups with the Coeur d'Alene clade).

Although either *H. skadei* or *H. camelus* were present in most parts of the study area, the exception appears to be the West Cabinet Mountains, where *H. skadei* was detected in just one cell (Figs. 1A and 1B). With no reason to suspect our techniques would lead to disparate detection rates, we conclude *H. skadei* occurs at relatively low densities and *H. camelus* may not occur in this mountain range.

Both species were typically collected in forested areas and most often found under logs or rocks. Occasionally both species could be found on the forest floor surface, especially during rain events. During particularly dry conditions, specimens could be found by breaking open decomposing logs and searching for moist sections. This suggests that both species retreat toward moist refuges as required by drying conditions. The latest collection date for immature specimens was 29 August for *H. camelus* and 25 September for *H. skadei* (Table 1). Late-season collection of immature specimens is suggestive that neither are annual species and likely overwinter as immature and mature animals. Mean annual air temperature at *H. camelus* (4.28 °C, $n = 51$) and *H. skadei* (5.16 °C, $n = 34$) collection sites was >1 °C lower than the study area's mean annual air temperature (6.17 °C). Future research should focus on whether these cooler than average air temperature sites are merely associative or ecological requirements and what plasticity the species may or may not possess for climate change adaptation. Both species occur more often at elevations greater than the study average (1112 m) with *H. camelus* found at elevations averaging 1383 m (605–1827 m, $n = 71$ sites) and *H. skadei* at 1199 m (723–1833 m, $n = 48$ sites).

Discussion

Hemphillia in northern Idaho and surrounding regions

Prior to our study, two described species (*H. camelus* and *H. danielsi*) were known to occur within or adjacent to our study area (Burke 2015; Hendricks 2012); however, survey effort in the

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area had been limited. Our results indicate that *H. danielsi* is not present in our survey area (Fig. 1A). Furthermore, our morphological and molecular analyses show what was formerly considered *H. camelus* represents in fact two distinct taxa: *H. skadei* and *H. camelus*, which are themselves distinct from *H. danielsi*.

Hemphillia skadei was detected in Washington, Idaho, Montana, and within 10 km of British Columbia, which suggests that it likely occurs in that Canadian province. *Hemphillia camelus* populations appear to exhibit a disjunct range, which are separated by a range inhabited by *H. skadei*. Although the distribution of the two species is predominately allopatric, there appears to be a northern contact zone in the Selkirk Mountains. Given these observations, it seems that biological or ecological barriers between these two groups are present and genetically isolate the lineages. Past populations may have been isolated within separate mountain refugia in which divergence was fostered by limited gene flow and genetic drift (Brunsfeld et al. 2001). Refugium populations may have since spread from their area of origin, but since making secondary contact, it appears that reproductive barriers may prevent gene flow.

Considering the possible contact zone and differences in penis structure, we suggest that future research investigates interbreeding capability of the two taxa. The divergence in genital shape can contribute to reproductive isolation, and future studies should investigate how this divergence affects reproductive success between *H. camelus* and *H. skadei*. For example, variation in mating behavior might indicate precopulatory reproductive barriers if individuals from separate lineages exhibit mechanical difficulty in genital insertion (Masly 2012). Additional contributing factors to consider may be the ability of individuals from separate lineages to recognize one another as potential mates (Rundle and Nosil 2005), differences in the timing of sexual maturity, and (or) the degree of parentage success given successful interspecific crosses.

Conservation implications

Prior to our study, populations of *H. camelus* in Idaho were listed as a NatureServe S2 ranked imperiled species (IDFG 2005) and considered to be in decline (Frest 1999). *Hemphillia camelus* was known from just 13 sites across the entire state of Idaho and only 4 Idaho sites within our study area (IDFG 2005). We documented three times the number of occupied Idaho sites in a single survey effort because we included this species in a broad multitaxa inventory program. We also demonstrated the importance of genetically screening a portion of samples to detect the presence of cryptic species. Without the genetic screening component of our survey, we would not have detected *H. skadei*, nor demonstrated that *H. danielsi* is not likely to occur within our study area. Modelling efforts proliferate as ways to determine range, distribution, and conservation status of species. However, our results indicate incorporation of a wide variety of taxonomic groups into broad-scale inventory and monitoring programs is not only feasible and practical, but necessary, to measure species distribution and conservation need.

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