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ddRAD Sequencing Sheds Light on Low Interspecific and High Intraspecific mtDNA Divergences in Two Groups of Caddisflies

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Abstract

Large-scale global efforts on DNA barcoding have repeatedly revealed unexpected patterns of variability in mtDNA, including deep intraspecific divergences and haplotype sharing between species. Understanding the evolutionary causes behind these patterns calls for insights from the nuclear genome. While building a near-complete DNA barcode library of Finnish caddisflies, a case of barcode-sharing and some cases of deep intraspecific divergences were observed. In this study, the *Apatania zonella* (Zetterstedt, 1840) group and three *Limnephilus* Leach, 1815 species were studied using double digest RAD sequencing (ddRAD-seq), morphology, and DNA barcoding. The results support the present species boundaries in the *A. zonella* group species. A morphologically distinct but mitogenetically nondistinct taxon related to parthenogenetic *Apatania hispida* (Forsslund, 1930) got only weak support for its validity as a distinct species. The morphology and genomic-scale data do not indicate cryptic diversity in any of the three *Limnephilus* species despite the observed deep intraspecific divergences in DNA barcodes. This demonstrates that polymorphism in mtDNA may not reflect cryptic diversity, but mitonuclear discordance due to other evolutionary causes.

Key words: Apatanidae, ddRAD sequencing, DNA barcoding, Limnephilidae, Trichoptera

The systematic gathering of DNA barcodes, i.e., the mitochondrial cytochrome oxidase (mtCOI) sequences (Hebert et al. 2003) during the last decade demonstrated that DNA barcodes can successfully be used to identify species of caddisflies (Trichoptera) (Zhou et al. 2016). In connection with the international efforts to build a global-wide reference library for caddisflies (Zhou et al. 2016), we built the mtCOI library for 214 out of the 218 Finnish species (dx.doi.org/10.5883/DS-TRIFI200). This national-wide library has been used to clarify some known taxonomic issues (Salokannel et al. 2010, 2012) and, especially, to identify unknown larvae and verify poorly characterized early larval instars to build a comprehensive larval key (Rinne and Wiberg-Larsen 2017). To assess the levels of intraspecific variation and to identify possible undetected cryptic species, we attempted to sequence multiple specimens per species, ideally from different locations.

Various unusual patterns observed in mtDNA, including deep intraspecific splits and barcode sharing between species, may result from different evolutionary phenomena such as introgression, retained ancestral polymorphism and incomplete lineage sorting, but may also reflect taxonomic inaccuracies, such as undetected cryptic diversity or oversplitting of species, or even sequencing or alignment errors or nuclear mitochondrial pseudogenes (Funk and Omland 2003, Mutanen et al. 2016). By providing multiple loci across the entire genome, genome-wide approaches have great potential to elucidate the underlying causes behind such mitochondrial anomalies. Reduced-representation sequencing (RRS) has become very popular in the last few years among scientists studying the genetic variation of nonmodel organisms (Andrews et al. 2016), with special emphasis on digestion-based techniques, commonly referred to as RAD (restriction-site associated DNA). In this study, we took of one of the most popular RRS methods, double digest RAD (ddRAD) sequencing, which enables rapid recovery of thousands of orthologous loci from samples with or without a reference genome (Peterson et al. 2012). While entire

genomes have recently been made available for some caddisfly species (Luo et al. 2018, Heckenhauer et al. 2019, Olsen et al. 2021), to our knowledge, no published studies have focused on species-level taxonomy of caddisflies based on high-throughput sequencing techniques.

We focused on two groups of caddisflies, both showing patterns in DNA barcodes incongruent with the current taxonomy. The genus *Apatania* Kolenati, 1848 (Apatanidae) consists of 13 valid species in Fennoscandia (Salokannel and Mattila 2018). Their larvae inhabit mostly cool running waters and feed primarily on diatoms, although some consume algae (Rinne and Wiberg-Larsen 2017). Parthenogenesis is common within the genus, including the *Apatania zonella* (Zetterstedt, 1840) group, in which only females are known for some species, whereas males are rare or unknown in some others (Salokannel and Mattila 2018). Although DNA barcodes and morphological characters mostly agree with the current delimitation of species in the Finnish material, unsolved taxonomic issues within the *A. zonella* group remain (Salokannel et al. 2010, Lecaudey 2013, Pálsson et al. 2016). The valid Finnish species within the group are *A. zonella*, *A. dalecarlica* (Forsslund, 1942), *A. auricula* (Forsslund, 1930), *A. forsslundi* Tobias, 1981, and *A. hispida*. While identification of these species is usually straightforward, specimens with intermediate or otherwise unclear morphology are found in alpine habitats in the North Finland. A regularly collected, but unclear taxon '*A.* nr. *hispida*' has a DNA barcode identical with *A. hispida* but differs by some characters of genital segments (Salokannel et al. 2010). Two more species of the *A. zonella* group, *A. scandinavica* Svensson & Tjeder, 1975, and *A. majuscula* McLachlan, 1872 are reported from Fennoscandia but were not included in the analysis because no material was available.

Limnephilus Leach, 1815 (Limnephilidae) is the most species-rich genus of caddisflies in the northern Europe; it consists of 39 species in Finland. Their larvae are mainly herbivorous, feeding on algae, decayed fine (FPOM), and coarse (CPOM) plant material (Rinne and Wiberg-Larsen 2017). *Limnephilus* larvae inhabit various waters, including small and temporary waters with the help of their well-developed multifilament gills (Rinne and Wiberg-Larsen 2017). Despite the relatively high number of species, the taxonomy of Finnish *Limnephilus* has been stable during the recent decades. However, the DNA barcodes of *Limnephilus flavicornis* (Fabricius, 1787), *L. sericeus* (Say, 1824) and *L. centralis* Curtis, 1834 each appeared to split into two distinct clusters (Salokannel and Mattila 2018).

In this study, we examined both the *Apatania zonella* group and the three *Limnephilus* species supplemented with reference specimens of a fourth species, *L. marmoratus* Curtis, 1834 using ddRAD. We tested if genomic-scale data support the existing notion of the relationships of taxa within the *A. zonella* group and clarifies the status of *A.* nr. *hispida* as well as the identity of a selected morphologically unidentified specimen. We also tested if genome-wide data of nuclear loci recover the same pattern as the DNA barcode clustering of the three *Limnephilus* species, and particularly if the intraspecific deep divergences observed in mtCOI gene in three *Limnephilus* species are reflected in the nuclear genomes.

Materials and Methods

Along with the national DNA barcoding activities, altogether 1,133 specimens of 214 species were sequenced for the standard DNA barcoding fragment of the mitochondrial COI gene. Full collection and taxonomic data as well as photographs for most specimens are publicly available through the BOLD dataset DS-TRIFI200 accessible at dx.doi.org/10.5883/DS-TRIFI200. In short, for most cases all steps prior to DNA extraction, i.e., sample collection, preparation, tissue sampling, photography, and data entry to the BOLD database, were conducted in Finland by the authors and following guidelines of the BOLD Handbook (accessed at http://v4.boldsystems.org/libhtml_v3/static/BOLD4_Documentation_Draft1.pdf). DNA extraction, PCR, sequencing, sequence alignment and sequence upload to the BOLD database were conducted at the Canadian Centre for DNA Barcoding following the protocols of Hebert et al. (2003) and DeWaard et al. (2008). Partly, DNA sequencing was conducted at the University of Turku as explained in Salokannel et al. (2010). These data had previously revealed cases of deep intraspecific divergences in three species of *Limnephilus*, which were chosen as target species for this study. Similarly, DNA barcoding did not support the status of morphologically identifiable *Apatania* nr. *hispida* as a distinct species, leaving its taxonomic status open.

In total, 41 specimens were included in the genomic analyses (dx.doi.org/10.5883/DS-RADTRIC): *Apatania auricula* (2 specimens), *A. dalecarlica* (4), *A. forsslundi* (1), *A. hispida* (4), *A.* nr. *hispida* (2), *A. zonella* (4), *A.* sp. (1), *Limnephilus centralis* (7), *L. flavicornis* (6), *L. marmoratus* (2), and *L. sericeus* (8). The material was collected from Finland and Estonia in 2007–2016, supported by two reference specimens of *Limnephilus centralis* from Germany. Detailed information regarding each specimen is available in Table 1 and BOLD dataset DS-TRIFI200. The collection sites are shown on the map in Figure 1.

The initial identifications of the samples were based on specimen morphology. The adult specimens were identified using their genital characters, as defined in the European atlas (Malicky 2004) and keyed by Salokannel et al. (2010). In the morphological examination of *Limnephilus sericeus* specimens, the characters of North American *L. sericeus* species group (Ruiter 1995) were also studied: primarily the relative length of the intermediate and superior appendages in the males and the connection of the appendages of 10th segment in females (Ruiter 1995). Identification of the larvae followed the characters given in Trichoptera Larvae of Finland (Rinne and Wiberg-Larsen 2017).

Molecular Analysis

Molecular Analyses and Bioinformatics

DNA extractions were performed using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instruction. The quantity of genomic DNA extracts was checked using the Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes). The ddRAD library construction followed protocols described in Lee et al. (2018) with an exception: the size distribution was measured with Bioanalyzer (Agilent Technologies). Raw demultiplexed reads are archived in the NCBI SRA: PRJNA631141.

Raw paired-end reads were de-multiplexed with no mismatches tolerated using their unique barcode and adapter sequences using *ipyrad* v.0.7.23 (Eaton and Overcast 2020). All *ipyrad* defaults were used, with the following exceptions: the minimum depth at which majority rule base calls are made was set to 3, the cluster threshold was set to 0.85, and the minimum number of samples with data for a given locus to be retained in the final data set was set to 3 and 4. We also compiled a dataset of biallelic, unlinked SNPs by extracting a single SNP from each locus. The unlinked SNPs generated from the *ipyrad* datasets were analyzed using STRUCTURE analysis.

Table 1. Collection data summary for the specimens included in the analyses

Species	Sample ID	Life stage	Collection sites	Collection date	Latitude	Longitude	Elevation	Collector
Apatania auricula	ARin-2016R012	Adult	Punelia, Loppi, FINLAND	17-X-2010	60.6676	24.1952	180	Esko Viitanen
Apatania auricula	ARin-2016R013	Larva	Tyyrinvirta, Rautalampi, FINLAND	30-V-2013	62.6742	26.8371	97	Aki Rinne
Apatania dalecarlica	ARin-2016R003	Larva	Tolppaniityn lähde, Paltamo, FINLAND	10-V-2015	64.4652	27.6157	205	Aki Rinne
Apatania dalecarlica	ARin-2016R004	Larva	Tolppaniityn lähde, Paltamo, FINLAND	10-V-2015	64.4652	27.6157	205	Aki Rinne
Apatania dalecarlica	ARin-2016R005	Larva	Tolppaniityn lähde, Paltamo, FINLAND	10-V-2015	64.4652	27.6157	205	Aki Rinne
Apatania dalecarlica	ARin-2016R006	Larva	Tolppaniityn lähde, Paltamo, FINLAND	10-V-2015	64.4652	27.6157	205	Aki Rinne
Apatania forsslundi	JSlk-2016R010	Adult	Vatikuru, Muonio, FINLAND	02-VII-2012	68.0645	24.0517	560	Juha Salokannel
Apatania hispida	ARin-2014F108	Adult	Savukoski, FINLAND	05-VIII-2014	68.1594	28.5714	420	Jukka Salmela
Apatania hispida	ARin-2014F109	Adult	Savukoski, FINLAND	05-VII-2014	68.1594	28.5714	420	Jukka Salmela
Apatania hispida	ARin-2016R001	Larva	Muonio, FINLAND	24-IX-2010	68.0575	24.0568	480	Juha Salokannel
Apatania hispida	ARin-2016R002	Larva	Muonio, FINLAND	24-IX-2010	68.0575	24.0568	480	Juha Salokannel
Apatania nr. *hispida*	JSlk-2013F052	Adult	Kaunispää, Inari, FINLAND	30-VI-2013	68.4430	27.4110	324	Juha Salokannel
Apatania nr. *hispida*	JSlk-2013F053	Adult	Kaunispää, Inari, FINLAND	30-VI-2013	68.4430	27.4110	324	Juha Salokannel
Apatania zonella	ARin-2016R015	Larva	Iso-Malla, takalampi, Enontekiö, FINLAND	15-IX-2015	69.0898	20.6358	870	Juha Salokannel
Apatania zonella	JSlk-2016R008	Adult	Siilasjärvi, Enontekiö, FINLAND	03-VII-2012	69.0703	20.7551	484	Juha Salokannel
Apatania zonella	ME071	Adult	Pulmankijärvi, Utsjoki, FINLAND	03-VII-2007	69.9820	27.9790	13	Juha Salokannel
Apatania zonella	TRIFI-2015F19	Larva	Iso-Malla, takalampi, Enontekiö, FINLAND	15-IX-2015	69.0898	20.6358	870	Juha Salokannel
Apatania sp.	JSlk-2016R003A	Adult	Toskaljärvi, Enontekiö, FINLAND	02-VII-2014	69.1986	21.4481	710	Jukka Salmela
Limnephilus centralis	ARin-2016R007	Larva	Porkkalanniemi, Kirkkonummi, FINLAND	18-X-2015	60.0591	24.5672	2	Aki Rinne
Limnephilus centralis	ARin-2016R008	Larva	Tolppaniitty, Paltamo, FINLAND	09-V-2015	64.4652	27.6157	205	Aki Rinne
Limnephilus centralis	ARin-2016R009	Larva	Raappananmäki, Paltamo, FINLAND	09-V-2015	64.5049	27.5890	210	Aki Rinne
Limnephilus centralis	JSlk-2014F174	Adult	Pitkäniemi, Nokia, FINLAND	15-VII-2012	61.4820	23.5930	77	Juha Salokannel
Limnephilus centralis	JSlk-2016R006	Adult	Petäjäoja, Orivesi, FINLAND	31-VII-2015	61.7200	24.2900	130	Juha Salokannel
Limnephilus centralis	JSlk-2017R001	Adult	Wahnbach, GERMANY	14-VIII-2016	49.5744	6.8296	NA	Peter Neu
Limnephilus centralis	JSlk-2017R002	Adult	Hangweg, GERMANY	04-VII-2015	49.5291	7.0756	NA	Peter Neu
Limnephilus flavicornis	JSlk-2015M001	Adult	Iso-Kana, Tampere, FINLAND	08-VIII-2015	61.6169	23.8494	95	Ilari Rasimus
Limnephilus flavicornis	JSlk-2015M002	Adult	Iso-Kana, Tampere, FINLAND	08-VIII-2015	61.6169	23.8494	95	Ilari Rasimus
Limnephilus flavicornis	JSlk-2015M003	Adult	Iso-Kana, Tampere, FINLAND	19-VIII-2015	61.6169	23.8494	95	Ilari Rasimus
Limnephilus flavicornis	TRIFI-2015F20	Adult	Salovesi, Karkkila, FINLAND	30-VIII-2015	60.6096	23.9547	117	Esko Viitanen
Limnephilus flavicornis	TRIFI-2015F21	Adult	Salovesi, Karkkila, FINLAND	30-VIII-2015	60.6096	23.9547	117	Esko Viitanen
Limnephilus flavicornis	TRIFI-2015F23	Adult	River Volupe, Volupe, ESTONIA	10-VIII-2010	58.5731	22.7554	1	Esko Viitanen
Limnephilus marmoratus	ARin-2014F129	Larva	Läsäkoski, Kangasniemi, FINLAND	24-IV-2013	61.9080	26.8950	97	Aki Rinne
Limnephilus marmoratus	ARin-2016R010	Larva	Kura, Houtsala, FINLAND	02-V-2013	60.3336	21.5709	0	Aki Rinne

Table 1. Continued

Species	Sample ID	Life stage	Collection sites	Collection date	Latitude	Longitude	Elevation	Collector
Limnephilus sericeus	ARin-2012F370	Larva	Siilasranta, Enontekiö, FINLAND	03-VII-2012	69.0708	20.7578	484	Juha Salokannel
Limnephilus sericeus	ARin-2012F374	Larva	Siilasranta, Enontekiö, FINLAND	03-VII-2012	69.0708	20.7578	484	Juha Salokannel
Limnephilus sericeus	ARin-2014F140	Adult	Matalajärvi, Espoo, FINLAND	04-VIII-2014	60.2556	24.6944	22.9	Aki Rinne
Limnephilus sericeus	ARin-2014F141	Adult	Törmäoja, Savukoski, FINLAND	18-IX-2014	67.8334	29.4424	170	Jukka Salmela
Limnephilus sericeus	ARin-2014F152	Adult	Sorvanulkki, Rovaniemi, FINLAND	05-VIII-2014	66.2980	25.1310	150	Jukka Salmela
Limnephilus sericeus	ARin-2016F047	Adult	Ainijärvi, Savukoski, FINLAND	28-IX-2015	67.7701	29.4331	265	Jukka Salmela
Limnephilus sericeus	JSlk-2015F191A	Adult	Vuotso, Sodankylä, FINLAND	16-VII-2015	68.1486	27.0041	246	Esko Viitanen
Limnephilus sericeus	JSlk-2016R004	Adult	Isokangas, Kangasala, FINLAND	11-VIII-2015	61.3789	24.2081	140	Juha Salokannel

Data Analyses

Sequences were examined for fixed substitutions between species based on K2P distances in sequence alignments using MEGA v.7.0 (Kumar et al. 2016). The proportion of missing data was calculated using Mesquite (Maddison and Maddison 2017).

Phylogenetic trees were constructed for both mtCOI and ddRAD data using the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at; Trifinopoulos et al. 2016). We used the IQ-TREE integrated ModelFinder (Kalyaanamoorthy et al. 2017) with 286 DNA substitution models, which selected 'GTR+G+I' as the best-fit model according to the Bayesian information criterion for mtCOI data, 'TVM+F+I' for *Apatania* ddRAD data, and 'TN+F+R5' for *Limnephilus*. To reconstruct the phylogenetic tree, ML analysis with ultrafast bootstrap approximation model (1,000 replicates) was applied (Minh et al. 2013). In addition, a coalescent SVDquartets analysis (Chifman and Kubatko 2014) was conducted in PAUP 4.0a169 (https://paup.phylosolutions.com/; last accessed 2 April 2021) on the SNP data. We used default settings to yield a species tree and ran 1,000 bootstrap replicates. The trees were generated using FigTree v.1.4.2 (Rambaut 2015) and modified using Adobe Illustrator CS6.

We inferred population clustering with admixture from unlinked SNPs data to visualize genomic variation between individuals in STRUCTURE v.2.3.1 (Pritchard et al. 2000). Ten replicates were run at each value of K between 1 and 7 for *Apatania* group and K = 1 to 4 for *Limnephilus* group. Each run had a burn-in of 10K generations followed by 20K generations of sampling. We used StrAuto to automate Structure processing of samples (Chhatre and Emerson 2017). Replicates were permuted in CLUMPP (Jakobsson and Rosenberg 2007) according to the ad hoc ΔK statistics (Evanno et al. 2005), which is the second-order rate of change of the likelihood function. Structure results were visualized using DISTRUCT (Rosenberg 2004).

We used four-taxon D-statistics (Durand et al. 2011) to distinguish introgression from incomplete lineage sorting. All D-statistics were calculated in pyRAD v.3.0.64 (Eaton 2014). In order to run interactive data analysis, the Python Jupyter notebooks (https://jupyter.org) were used. The python script for D-statistics is available from the Dryad Digital Repository at https://doi.org/10.5061/dryad.b883mf8 (Dincă et al. 2019a).

Results

Apatania zonella Group

Species recognition using the morphological characters was in line with the key provided by Salokannel et al. (2010), except for the specimen JSlk-2016R003A. *Apatania zonella* group specimens included in the analysis represent the five valid Finnish species and *A.* nr. *hispida*. The specimen JSlk-2016R003A has a ventrally blunt-rounded supragenital plate similar to *A. zonella*, but the upper part of the last segment is protruded, making it laterally similar to *A.* nr. *hispida*. Such combination of characters is not clearly associated with any valid taxon, so the sample was named as *Apatania* sp.

Sequence variation of mtCOI within species was close to zero (0–0.15%) except in *A. zonella* with 1.16% variation. *A. hispida* and *A.* nr. *hispida* samples have identical DNA barcode. *Apatania* sp. (JSlk-2016R003A) has the closest match (0.15%) to the specimens of *A. forsslundi*. Otherwise, the inter-species variation between the valid species ranges from 1.05 to 2.31% (Table 2).

We generated a genome-wide SNP dataset from 18 individuals of the *A. zonella* group using ddRAD sequencing. We obtained 3.2 million reads per individual on average, of which 3 million reads per individual (93.3%) were retrieved after quality filtering step

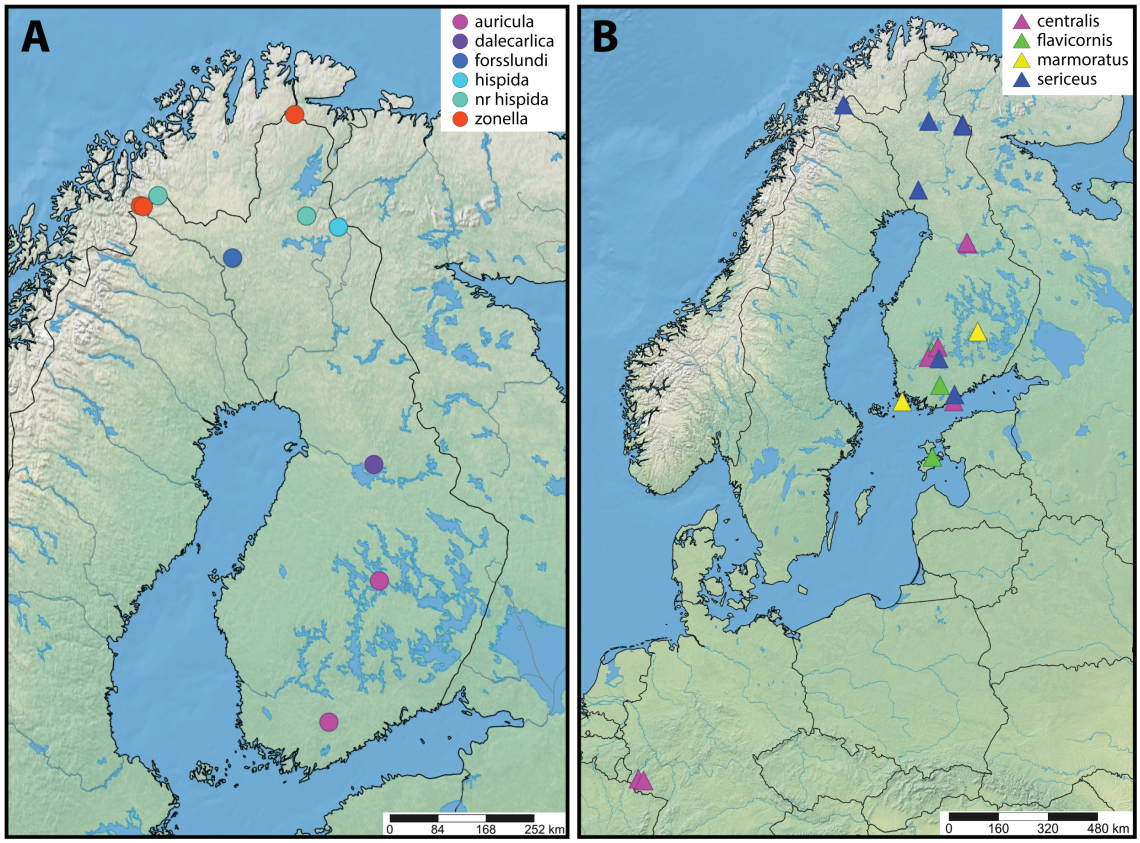

Fig. 1. Geographical sampling maps for (A) *Apatania* and (B) *Limnephilus*.

Table 2. Mean genetic distances of ddRAD (below diagonal) and mitochondrial COI data (above diagonal) of within and between species of *Apatania*

	1	2	3	4	5	6	7
1. *A. auricula*	0.45 / 0.15	1.49	2.31	1.73	1.75	1.66	2.16
2. *A. dalecarlica*	2.38	0.15 / 0.10	2.01	1.28	1.29	1.05	1.87
3. *A. forsslundi*	0.82	0.80	NA	1.66	1.67	2.19	0.15
4. *A. hispida*	1.88	2.91	1.92	0.12 / 0	0	1.59	1.51
5. *A. nr. hispida*	1.57	2.51	0.64	0.94	0.66 / 0	1.60	1.52
6. *A. zonella*	1.51	2.53	1.55	1.93	1.29	0.62 / 1.16	2.19
7. *A.* sp.	1.54	2.70	1.14	1.79	1.39	1.06	NA

NA, not applicable, single specimen.

(Supp Table 1 [online only]). After clustering at 85% sequence similarity, 26,790 putative orthologous loci shared across more than four samples were retained, for a total length of 2,158,568 bp (Table 3). These data included 59,486 SNPs, of which 38,366 are parsimony informative. The proportion of missing data was 71.4% across all loci.

At the genomic level (ddRAD data), within and between species divergences partly overlapped in *A. zonella* group. Within species divergences ranged from 0.12 (*A. hispida*) to 0.66% (*A.* nr. *hispida*), whereas the mean interspecific distances varied from 0.64 (between *A.* nr. *hispida* and *A. forsslundi*) to 2.91% (between *A. hispida* and *A. dalecarlica*) (Table 2).

In the ML tree based on mtCOI sequences, most species in the *A. zonella* group did not clearly separate as distinct clades, except for *A. auricula* (Fig. 2A). *Apatania* nr. *hispida* completely intermixed with *A. hispida*, while *A. zonella* diverged into two lineages and was found closely related to *A. dalecarlica*. In the ddRAD ML tree, the lineages that correspond to *A. hispida*, *A. auricula*, and *A. dalecarlica* were strongly supported as monophyletic (BS 100%) (Fig. 3A). *Apatania* nr. *hispida* appeared paraphyletic with respect to its putative sister taxon *A. hispida*. The unidentified sample, *Apatania* sp., was closely related to *A. zonella*. The species tree estimation analyses recovered a rather similar topology to the ML tree, but *A. auricula* was found sister to *A. zonella* and *Apatania* sp. (Supp Fig. 1 [online only]).

The population clustering analyses revealed substantial heterogeneity in proportions of admixed ancestry between the species. The best supported model (K = 6) clustered populations into six major clades (Fig. 3A; Supp Fig. 2 [online only]). The STRUCTURE analysis revealed that much of common ancestry of *Apatania* nr. *hispida* is shared through apparent admixture with the closely related

Table 3. Comparison between mtCOI and ddRAD-seq data sets

	mtCOI		ddRAD-seq	
Matrix	*Apatania* (18 ind./7 spp.)	*Limnephilus* (23 ind./4 spp.)	*Apatania* (18 ind./7 spp.)	*Limnephilus* (23 ind./4 spp.)
Number of loci	1	1	12,337	20,003
Alignment length	670 bp	670 bp	2,158,568 bp	3,407,678 bp
SNPs	27 (4.0%)	152 (22.7%)	59,486 (2.8%)	81,701 (2.4%)
Informative sites	25 (3.7%)	149 (22.2%)	38,366 (1.8%)	27,557 (0.8%)
Missing data	0%	0%	71.4%	72.7%

See details for all data sets in Supp Table S1 (online only).

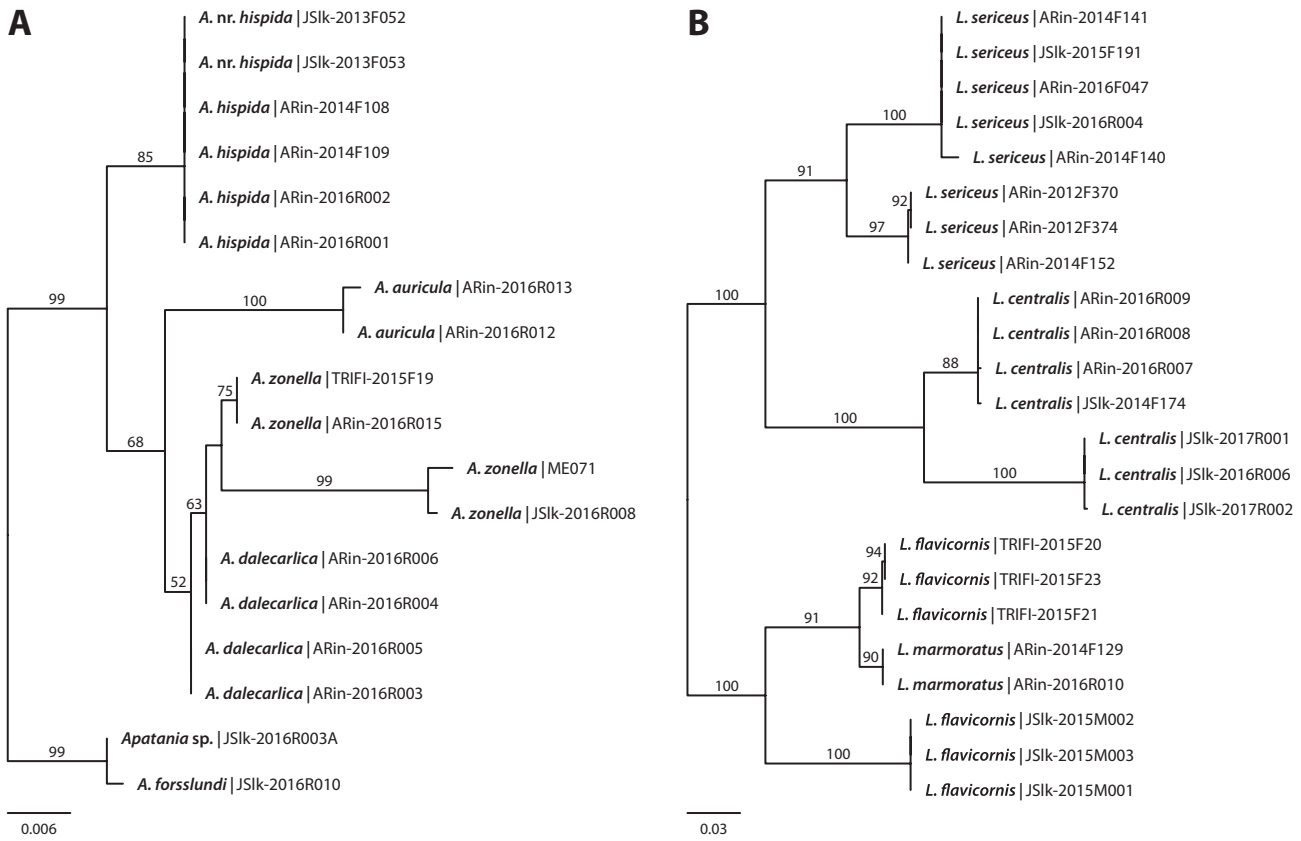

Fig. 2. Maximum likelihood trees based on mitochondrial COI sequences of *Apatania* (A) and *Limnephilus* (B). The bootstrap values are indicated near the branches (only shown for nodes supported with more than 50% of BS).

A. hispida and *A. auricula* (Fig. 3A). However, D-statistics estimated insignificant levels of gene flow between *A. hispida* and *A. auricula*, but significant only between *A. hispida* and *A. nr. hispida* (Supp Table 3 [online only]). *Apatania* sp. also shared significant common ancestry with *A. zonella* and *A. auricula*, and did not form a distinct cluster even when the number of assumed genetic clusters was increased to 7.

Limnephilus spp.

The morphological characters were well in line with the literature and we found no clear indications of cryptic species. Only minor differences in the shapes and setae arrangement of genital segments and their appendages were detected, whereas species-level differences between North European Limnephilids are regularly clearly more significant (Malicky 2004). Also, when examining *L. sericeus* adults, we found no characters of closely related North American *L. sericeus* group of species (Ruiter 1995).

The samples of each three *Limnephilus* species were split into two DNA barcode clusters: (i) *Limnephilus sericeus* with mean Kimura-2 parameter (K2P) divergence of 6.8% between the clusters, and mean intra-cluster K2P divergence of 0.3%; (ii) *Limnephilus centralis* with mean K2P divergence of 8.8% between the clusters and mean intra-cluster K2P divergence of 0.1%; and (iii) *Limnephilus flavicornis* with mean K2P divergence of 10.1% between the clusters, and mean intra-cluster K2P divergence of 0.1%. One of *L. flavicornis* clusters is closer (mean K2P divergence 2.2%) to the DNA barcodes of a related species *L. marmoratus* than the second cluster of *L. flavicornis*.

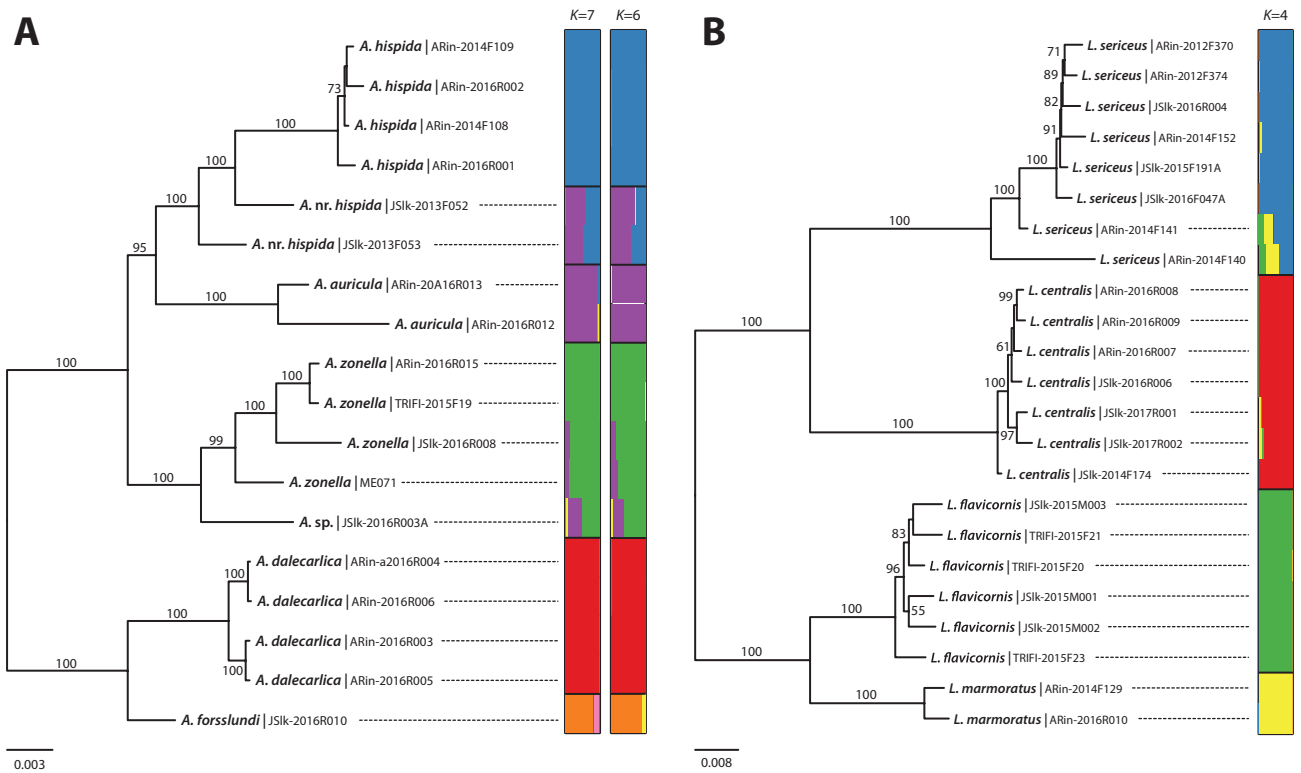

Fig. 3. Maximum likelihood trees and population structuring of *Apatania* (A) and *Limnephilus* (B) based on ddRAD SNP data. ML trees were inferred from RAxML analysis with 1,000 bootstrap replicates. The bootstrap values are indicated near the branches. Results of population STRUCTURE analyses with posterior probability plots of individual assignments to the inferred genetic clusters for $K = 6$ and 7 for *Apatania* and $K = 4$ for *Limnephilus*.

For ddRAD data of *Limnephilus* from 23 individuals, an average of 2.3 million raw reads was obtained (Supp Table 1 [online only]). We recovered a total of ca. 3.4 million base pairs with 20,003 loci and 81,701 SNPs, with average cluster depth of 207 (Table 3; Supp Table 1 [online only]). The proportion of missing data was 72.7% across all loci.

Pairwise distances of ddRAD data were consistent with the current species taxonomy. Mean within species divergence varied from 0.38% (*L. centralis*) to 0.95% (*L. sericeus*), whereas distances among species were more than three times higher, i.e., from 2.86 (between *L. flavicornis* and *L. marmoratus*) to 5.26% (between *L. flavicornis* and *L. sericeus*).

In the mtCOI ML tree, three *Limnephilus* species (*L. centralis*, *L. sericeus*, and *L. flavicornis*) showed a deep intraspecific split. Two samples of *L. marmoratus* were sister and closely related to one of the *L. flavicornis* clades (Fig. 2B). In the ddRAD ML tree, all four species of *Limnephilus* were fully supported as monophyletic clades (Fig. 3B). The phylogenetic tree analyses supported *L. flavicornis* as a sister species to *L. marmoratus*.

The STRUCTURE identified four clusters (Fig. 3B), which are perfectly compatible with the ddRAD ML tree. Some signs of admixture were observed between a few heterospecific samples. Interestingly, two samples (ARin-2014F140 and ARin-2014F141) of *L. sericeus* showed some admixture with both *L. flavicornis* and *L. marmoratus*. Tests of admixture using D-statistics confirmed the significant levels of gene flow between these two species (Supp Table 3 [online only]).

Discussion

Our study is among the first to provide a nuclear genomic insight into the deviant patterns of intra- or interspecific variability observed in the standard DNA barcode fragment of the mitochondrial genome. Patterns similar to what we observed in the focal caddisfly species are, however, not rare, as numerous DNA barcode data release papers report cases of deep intraspecific divergences and barcode sharing (e.g., Dincă et al. 2011; Hausmann et al. 2011; Huemer et al. 2014; Pentinsaari et al. 2014; Zahiri et al. 2014, 2017; Saitoh et al. 2015; Astrin et al. 2016; Yang et al. 2016; Morinière et al. 2017). Janzen et al. (2017) demonstrated, based on nuclear genomic data, that presumably a single species of butterfly showing three distinct barcodes actually represents three distinct cryptic species. Some other studies have also focused on exploring evolutionary causes of high levels of mitochondrial DNA variability within a species (Garg et al. 2016, Weiss et al. 2018, Dincă et al. 2019b, Hinojosa et al. 2019, Marchán et al. 2020) or its reversal, namely barcode sharing across species, but vast majority of detected cases of high intra or low interspecific variability remain unstudied by genomic or other means. While morphology or few-loci approaches may be useful to reveal cryptic diversity, they may not be powerful enough to reveal all cryptic species. They also may not provide compelling evidence for the conspecificity when identical barcodes result from the taxonomic oversplitting of species. Genomics methods, ddRAD sequencing included, possess high potential to provide an accurate overview of evolutionary (e.g., introgression) and operational (e.g., taxonomic inaccuracy) causes behind the unexpected patterns of mtDNA variability.

In this study, the three flagged cases of deep intraspecific barcode splits are likely to be explained by biological rather than operational causes. In the one studied case of barcode sharing between two morphologically supported putative species, the evidence remained more ambiguous as we cannot exclude the possibility that they represent two, genetically very closely related but biologically distinct species. Next, we discuss the taxonomy of both species groups in more detail.

Apatania zonella Group

The present DNA barcoding results are similar to the study by Salokannel et al. (2010), even if these studies had only one common sample (ME071): (i) despite relatively small divergences, each valid species of *A. zonella* group has its own cluster and appear monophyletic; (ii) *A.* nr. *hispida* specimens have identical DNA barcode with *A. hispida*; and (iii) specimens identified as *A. zonella* show more diversity than the other taxa of the group (Fig. 2A and Table 2). Also, the ddRAD-seq results are mainly in line with the mtCOI results, supporting the existing taxonomic notion of the presently valid species.

The specimens of *A.* nr. *hispida* differentiate from *A. hispida* in the ddRAD-seq data, but not as significantly as the other analyzed species differentiate from each other. They also do not form reciprocally monophyletic entities. The combination of different morphology, identical mtCOI and slightly deviated ddRAD-seq suggests that *A.* nr. *hispida* and *A. hispida* are very closely related, but genetically distinct taxa. Because of parthenogenetic nature of these taxa (no males are known), their taxonomic status cannot be assessed under the biological species concept. A possibility remains that they represent relatively old, diverged asexual strains. Taxonomic delineation of asexual taxa is often difficult and inherently arbitrary. Considering that these two taxa appear as fully asexual, it is surprising that the D-statistics we conducted suggested introgression to have happened between them. Two possible scenarios might explain this situation. First, signs of introgression may originate time before they turned asexual. Our phylogeny suggests asexuality being a derived feature in this group. Second, *A. hispida* and *A.* nr. *hispida* might represent two independent shifts from sexual to asexual mode of reproduction, as has been demonstrated in a similar system in *Dahlica* moths (Elzinga et al. 2013). A question follows: what could this sexual 'stem' species then be? While not sampled here, an excellent candidate could be the sexual *A. majuscula*. Its DNA barcodes available in the BOLD systems (incl. our own data) reveals it being very closely related to the *A. hispida* complex, with barcodes being partially phylogenetically nested within the *A. hispida*—*A.* nr. *hispida* cluster. Its female genitalia also show close resemblance to those of *A. hispida* and *A.* nr. *hispida*. While we unfortunately did not include *A. majuscula* in our ddRAD sampling, we find the scenario of *A. hispida* and *A.* nr. *hispida* representing two asexual strains of *A. majuscula* appealing. Further studies are needed to address this question.

The unidentified specimen JSlk-2016R003A with exceptional morphology clusters with *A. forsslundi* in mtCOI, but ddRAD suggests admixture of between *A. zonella* and *A. auricula*. This is also biologically possible as both species, especially *A. zonella*, are known to produce males regularly in its Nordic populations. Altogether, the DNA results suggest hybridization between two or more species. Also, the hybridization may have occurred already in the past and the hybrid line might have survived, supported by parthenogenesis. Such hybrid lines could also explain other cases of morphologically intermediate or aberrant specimens that have caused issues for identifiers over the decades (Solem 1985, personal observations).

Limnephilus spp.

The three studied *Limnephilus* species seem to make an exception within the Finnish caddisfly fauna with distinct polymorphism in their DNA barcodes, with minimum Kimura-2 parameter divergences between the clusters varying from 6.8 to 10.1%. However, no clear morphological differences nor division of ddRAD sequences were detected in this study. A likely explanation for the genetic polymorphism in their mtDNA is historical admixture between closely related species, resulting in mitochondrial introgression and subsequent co-occurrence of two distinct lineages of COI within a species. Alternatively, this pattern could have resulted from retained historical genetic polymorphism. The former explanation would get indirect support if one of the clusters was observed being genetically closer to any other related species than to the other cluster. We explored this possibility in our own data, but also using all data accessible to us in the BOLD database.

One of the two mtCOI clusters of *L. flavicornis* is both genetically and phylogenetically closer to its morphological sister species *marmoratus* than the other intraspecific mtCOI cluster. This observation suggests introgression of mtCOI from *L. marmoratus* to *L. flavicornis* in the past, although the evidence for this scenario cannot be considered as compelling. The supposedly introgressed, yet then diverged COI haplotype is now widespread in *L. flavicornis* populations, as among our material (n = 18) both haplotypes seem common.

One of two mtCOI clusters of *L. sericeus* is almost identical (divergence <0.5%) with that of the public barcodes available (Zhou et al. 2016) for *L. abbreviatus* Banks, 1908 (Supp Table 2 [online only]), which is one of the Nearctic species of *L. sericeus* group (Ruiter 1995). This suggests a relatively recent introgression of mtCOI from *L. abbreviatus* to *L. sericeus*. However, it is surprising that the haplotype would have spread all the way from North America through Siberia to Finland. Such a spread could potentially have been promoted by endosymbiotic bacteria, *Wolbachia* in particular (Hurst and Jiggins 2005, Smith et al. 2012), but we do not have evidence that being happened as we did not screen *Wolbachia*.

Neither of the mtCOI clusters of *L. centralis* is closely associated with other taxa with public DNA barcodes available (Zhou et al. 2016) or other data accessible to us in BOLD. The closest match (about 4%) of the second cluster is Central European *L. italicus* McLachlan, 1884 (Supp Table 2 [online only]), which is originally described as a variety of *L. centralis* (McLachlan 1884). The second mtCOI cluster may suggest introgression from *L. italicus* to *L. centralis* long in the past or an introgression from an unknown species to *L. centralis*. So far, the second mtCOI cluster specimens were found only from the continent of Finland, while the first cluster sequences appear widely in Europe (Supp Table 2 [online only]), including South West Finland.

Conclusions

This study highlights the importance of DNA barcoding multiple specimens per species, preferably with good geographic coverage, to reveal the extent of intraspecific variability and possible genetic polymorphism in COI. We demonstrate that the genome-wide ddRAD sequencing provides a powerful method to uncover the cases with mitonuclear discordance and, although not detected here, undoubtedly also cryptic diversity. We did not obtain strong evidence to lift *Apatania* nr. *hispida* (=*A. kaisilai* nomen nudum) as a valid species, although this possibility remains. In case of three *Limnephilus* species with mitochondrial polymorphism, no evidence of cryptic diversity was obtained. Our observations suggest that the co-presence of two distinct mitochondrial lineages result from historical hybridization and introgression between species at least in two of the three studied cases.

Supplementary Data

Supplementary data are available at *Insect Systematics and Diversity* online.

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Author contributions

JS: Conceptualization; Data curation; Formal analysis; Investigation; Resources; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing. KML: Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Software; Validation; Visualization; Writing – original draft; Writing – review & editing. AR: Data curation; Investigation; Resources; Writing – review & editing. MM: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Project administration; Resources; Supervision; Validation; Writing – original draft; Writing – review & editing.

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