


Mitogenome of an endemic Andean *Daphnia titicacensis* Birge, 1909 in the context of evolutionary history of the *D. pulex* complex (Cladocera: Daphniidae)


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Abstract

The genus *Daphnia* O.F. Mueller, 1776 (Crustacea: Cladocera: Daphniidae) holds a prominent place among the iconic model organisms in modern biological science. At the same time, it is a well-known example of a genus with a complicated and often confused taxonomy, for which there are multiple objective and subjective reasons. Such confusion is specially obvious in the *Daphnia* (*D.*) *pulex* group, specially *D. pulex* complex within the latter, despite its species being numerous, globally distributed, and ecologically significant. We had an opportunity to annotate a mitogenome of *D. titicacensis* Birge, 1909 from Lake Titicaca and discuss the evolutionary history of this group based on new genomic data. We found that: (1) The *D. pulex* complex is subdivided into *D. pulex* s.str., "European *D. cf. pulicaria*", *D. titicacensis* and *D. pulicaria* species flock. These four portions of the complex are clearly subdivided by the barcoding gaps, first three ones represent "good" biological species. *D. titicacensis* is a distinct, and already named, South American species found in high mountains of Peru and Bolivia. (2) There are no barcoding gaps within the *D. pulicaria* species flock. Theoretically, whole this assembly could be regarded as belonging to a single species *D. pulicaria* Forbes, 1893 in terms of the COI-based delimitation, all others are junior synonyms of the latter. In our understanding, to date it is better to speak about a "species flock". (3) Our phylogenetic trees and the haplotype network do not support the version of a Southern American origin of *D. pulex* complex, as well as of the *D. pulicaria* species flock (Mergeay *et al.*, 2008). Version about the Nearctic origin of the flock (Crease *et al.* 2012) agrees with our data.

Unfortunately, taxonomy of the *D. pulex* complex still remains to be dubious. We need to continue efforts to resolve numerous problems concerning the former combining different approaches, from genomics to morphology and ecology.

Key words Anomopoda, COI, high mountains, molecular phylogeny, taxonomy.

Introduction

The genus *Daphnia* O.F. Mueller, 1776 (Crustacea: Cladocera: Daphniidae) holds a prominent place among the iconic model organisms in modern biological science. Thousands of papers on this genus have been published in the fields of population ecology, genetics, environmental monitoring, and toxicology (Lampert 2011; Smirnov 2017). This genus attracts many researchers, and the number of annual publications continues to grow (Seda and Petrusek 2011).

At the same time, *Daphnia* is a well-known example of a genus with a complicated and often confused taxonomy, for which there are multiple objective and subjective reasons (Kotov 2015). Unfortunately, recent efforts to resolve these issues have been limited, largely due to a global crisis in taxonomy that originated in Western countries (Agnarsson and Kuntner 2007). Even molecular biologists have largely shifted their focus away from taxonomy-oriented studies, with a few notable exceptions (Petrusek *et al.* 2008; Petrusek *et al.* 2009; Ma *et al.* 2015; Ma *et al.* 2019). As a result, many widespread Holarctic species and species complexes still require comprehensive, step-by-step revision using integrative approaches, including morphology, molecular phylogeny, phylogeography, and population ecology.

This step-by-step approach is currently being applied in revisions of the subgenus *Daphnia* (*Ctenodaphnia*) Dybowski et Grochowski, 1895 (e.g., Petrusek *et al.* 2009; Pereboev *et al.* 2025), as well as several species groups within the subgenus *Daphnia* (*Daphnia*), including the *D. (D.) longispina* group (Taylor, Finston and Hebert 1998; Hebert, Witt and Adamowicz 2003; Adamowicz *et al.* 2009; Petrusek *et al.* 2008; Kotov *et al.* 2021). In contrast, the *D. (D.) pulex* group has received less attention, despite its species being numerous, globally distributed, and ecologically significant (Crease *et al.* 2012). Past research on this group has revealed fascinating phenomena, such as asexual lineages, male-less haplotypes, polyploid populations, and polyploid hybrids with high invasive potential (Hebert 1987; Mergeay *et al.* 2008; Mergeay, Verschuren and Meester 2006; Crease *et al.* 2012; Galimov, Walser and Haag 2011).

Problems with the taxonomy of this group are still obvious. The system for applying the species epithets "*D. pulex*" and "*D. pulicaria*" to European and American populations is poorly defined. Moreover, such identifications were not accompanied by any morphological evidence (but remember that both aforementioned species were described based on morphological characters, and everybody must follow just the author's understanding of these taxa rather than speculations of subsequent researchers (International Commission on Zoological Nomenclature (ICZN) 2000). Recently, several non-taxonomic investigations based on NGS data were published for the *D. pulex* species complex (e.g., Ye *et al.* 2021; Ye, Pfrender and Lynch 2023; Elguweidi and Crease 2024). Mostly, they were devoted to "Panarctic *D. cf. pulex*" and *D. pulicaria* with rare exceptions. Still prevalent use of "*D. pulex*" and "*D. pulicaria*" as catchall taxa for the entire group is confusing.

South America is a continent with rich and specific daphniid fauna, and with a long period of its study (Ekman 1900; Sars 1901; Daday 1902; Birge 1909). Several investigators made a great contribution to the morphology-based taxonomy of South American *Daphnia* (Paggi 1996; Paggi 1999), but little attention was paid to the *D. pulex* group. By the turn of the millennium, several valuable molecular studies were performed (Adamowicz, Hebert and Marinone 2004; Mergeay *et al.* 2008; Crease *et al.* 2012), including studies of the *D. pulex* complex (Aguilera *et al.* 2007; Mergeay *et al.* 2008). However, since that time such efforts were almost stopped, and no coordination with morphology-based taxonomy was made.

Mergeay *et al.* (2008) have concluded that "the *Daphnia pulicaria* group, a complex of predominantly North American species that has diversified rapidly since the Pleistocene, has its origin in South America". In reality, such conclusion was made from the tree based on three mitochondrial locus (12S+COI+ND5) sequences with many branches having quite moderate ML support, or no support. In contrast, Crease *et al.* (2012) have concluded that "based on the Holarctic distribution of the *D. pulex* species complex, it seems unlikely that it came from South America. A more parsimonious hypothesis is that it colonized the Nearctic from Asia and spread to South America soon thereafter".

Several mitogenomes of *D. pulex* complex have been sequenced since that time. We had an opportunity to annotate a mitogenome of *D. titicacensis* Birge, 1909 from Lake Titicaca and discuss the evolutionary history of this group based on new genomic data.

Materials and methods

Our sampling was conducted in a minimal quantity in a tourist locations of Lake Titicaca, without a protection status and did not involve any protected species. Three samples were collected from Puno Bay (-15.808361, -69.973300) and near Amantani Island (-15.652981, -69.719056) on 21.07.2022 through double vertical hauls from the surface to a depth of 2–3 meters using a 50 µm plankton net and were preserved in 96% ethanol. See further detail in Pereboev *et al.* (2025). Samples were pre-sorted in the laboratory under a dissecting microscope, cladoceran specimens were provisionally identified to species group level.

An individual parthenogenetic female of *Daphnia titicacensis* Birge, 1909 from the shore of Amantani Island was subjected to short-read whole-genome sequencing. Total DNA was isolated using the QiAmp Micro Kit (Qiagen) according to the manufacturer's protocol into 50 µL of eluent solution. Library preparation (including PCR whole genome amplification) and whole genome sequencing using the DNBSEQ Platform (ca. 89 million paired reads of 100 bp) were performed by BGI Genomics Co., Ltd. (China).

Raw reads were filtered by fastp 0.23.4 (Chen 2023) then assembled with GetOrganelle v1.7.7.1 (Jin *et al.* 2020) and annotated with MitoZ v3.6 (Meng *et al.* 2019). Manual correction of the annotated mitogenomes was performed as described in Pereboev *et al.* (2025). Gene map visualization was created using the Proksee web-service (Grant *et al.* 2023; Pereboev *et al.* 2025).

To reconstruct mitogenomic phylogeny, we composed a dataset of eight mitogenomes (seven from the *Daphnia pulex* species group, and *D. cf. obtusa* as an outgroup, Table 1) with 13196 nucleotide sites (8470 constant, 2449 parsimony informative). Individual genes were aligned using MAFFT v7.525 (Katoh and Standley 2013), with the L-INS-i algorithm, and poorly aligned regions were removed using trimAl v1.4.rev15 (Capella-Gutiérrez, Silla-Martínez and Gabaldón 2009) with the -automated1 option. We used IQ-TREE 3.0.1 (Wong *et al.* 2025) with automatic model selection (Kalyaanamoorthy *et al.* 2017) to build the phylogenetic tree based on the concatenation of both ribosomal genes and full nucleotide sequences of all protein-coding genes. We conducted the analyses with 1000 ultrafast bootstrap replicates (Hoang *et al.* 2018) and 1000 SH-aLrt replicates (Guindon *et al.* 2010). To reconstruct the COI + 12S + ND5 tree, corresponding genes were extracted from mitogenomic dataset and combined with reference data (Table 2), based on Ma *et al.* (2019), the phylogenetic analysis was performed as above. This dataset included 26 sequences with 3982 nucleotide sites (2748 constant, 771 parsimony informative). The COI alignment for minimal clade containing *D. titicacensis* and *D. pulicaria* was extracted and all gaps were trimmed out with trimAl. This alignment was used to construct the TCS haplotype network (Clement, Posada and Crandall 2000) by PopART (<https://popart.maths.otago.ac.nz/>).

The original genome is deposited in the NCBI GenBank (accession number PX647830).

Table 1. Mitogenomic data used in the study.

Species: strain	GenBank acc. no.	Reference
<i>D. cf. obtusa</i>	LS991524.1	Cornetti et al., 2019
<i>D. pulex</i> : CH-H	LS991506.1	Cornetti et al., 2019
<i>D. cf. pulex</i> : PA42	LS991507.1	Cornetti et al., 2019
<i>D. cf. pulex</i> : TCO	LS991508.1	Cornetti et al., 2019
<i>D. cf. pulicaria</i> : CZ-RIM1-1	LS991509.1	Cornetti et al., 2019
<i>D. pulicaria</i> : CLO001	n.a.	Ye et al., 2022; https://osf.io/km8w4/
<i>D. mitsukuri</i>	MW699926.1	n.a.
<i>D. titicacensis</i>	PX647830.1	this study

Table 2. Reference sequence data for the COI + 12S + ND5 tree. Sequences mistakenly named in the GenBank marked by an asterisk (*). Invalid names are designated by placing the specific epithet in quotation marks.

Species: strain	COI	12S	ND5
<i>D. pulex</i> : PXBE	EU152320	EU152311	EU152309
Panarctic <i>D. cf. pulex</i> : Africa	AY745244	AY745243	n.a.
Panarctic <i>D. cf. pulex</i> : NZPL486	HM622591	JX150980	n.a.
Panarctic <i>D. cf. pulex</i> : CHI-03	JX532787	n.a.	JX532797
<i>D. cf. middendorffiana</i>	FJ427496	FJ427429	n.a.
<i>D. «melanica»</i>	FJ427495	FJ427428	n.a.
South American <i>D. cf. pulicaria</i> A: ARG2	EU152329	EU152319	n.a.
South American <i>D. cf. pulicaria</i> A: BOLA1	EU152328	EU152318	n.a.
<i>D. pulicaria</i> : CAN	FJ427505	FJ427448	n.a.
<i>D. pulicaria</i> : MI-14	JX532742	n.a.	JX532831
<i>D. «arenata»</i>	FJ427493	FJ427424	n.a.
South American <i>D. cf. pulicaria</i> B: BOLB1	EU152324	EU152316*	EU152304
South American <i>D. cf. pulicaria</i> B: BOLB2	EU152323	EU152317*	EU152305
South American <i>D. cf. pulicaria</i> B: BOLB3	EU152325	EU152315	EU152306
South American <i>D. cf. pulicaria</i> C: BOLC2	EU152327	EU152314*	n.a.
<i>D. cf. tenebrosa</i>	FJ427506	FJ427450	n.a.
European <i>D. cf. pulicaria</i> : PUCZ1	EU152322	EU152312	EU152307
Japanese <i>D. cf. pulex</i> : sp. 1	GU595191	GU595176	n.a.

Results

We have assembled the complete circular mitochondrial genome of *D. titicacensis* (Figure 1), using ca. 20 million reads, with average base-coverage = 1165.9. The mitogenome includes all the 37 expected genes (2 rRNAs, 22 tRNAs, 13 PCGs) in the same order as reported for the *Daphnia* (*Daphnia*) subgenus (Castellucci, Luchetti and Mantovani 2022). Length of the mitogenome is 15 964 bp, GC content is 29.37%. The plus strand is a majority strand with 23 genes (9 PCGs, 14 tRNAs), and a light strand with T+G=46.8% (AT-skew = 0.02, GC-skew = -0.17).

In the mitogenomic phylogeny, all clades are recovered with maximal support. *D. mitsukuri* is confirmed as a sister group to the *D. pulex* species complex as defined in Ma *et al.* (2019). Inside the latter, *D. pulex* s.str. is placed in basal position, and European *D. cf. pulicaria* branches next. *D. titicacensis* is a sister group of closely related *D. pulicaria* and *D. cf. pulex*.

The COI + 12S + ND5 tree (Fig. 3) is fully concerted with the mitogenomic one. Distinct lineages of the *D. pulex* species complex not represented in the mitogenomic phylogeny (except *D. cf. tenebrosa* combined in one well-supported clade with European *D. cf. pulicaria*) are grouped with *D. pulicaria* s.str. in a sister clade to Panarctic *D. cf. pulex*, albeit with only moderate support. Nonetheless, the maximal clade includes Panarctic *D. cf. pulex* and *D. pulicaria* s.s. but not *D. titicacensis* has the maximal support and here we define it as the *D. pulicaria* species flock.

We have found that *D. titicacensis* possesses exactly the same Folmer COI haplotype as BOLC1 strain of South American "*D. cf. pulicaria* C" from Mergeay *et al.* (2008). Moreover, we have discovered that some sequences seem to be mistakenly named in the GenBank. Namely, the sequence data for 12S gene were confused between BOLB1-2 and BOLC1-2 lineages, as it was inferred on the basis of

discordant gene trees, and similarity of BOLB3 12S gene to allegedly BOLC1-2 ones, as well as 12S gene of *D. titicacensis* to allegedly BOLB1-2 ones (in fact the "BOLB1" one was identical). In the tree, *D. titicacensis* is grouped with another strain of South American "*D. cf. pulicaria* C" with a maximal support. We believe that South American "*D. cf. pulicaria* C" is in fact *D. titicacensis* (Fig. 3).

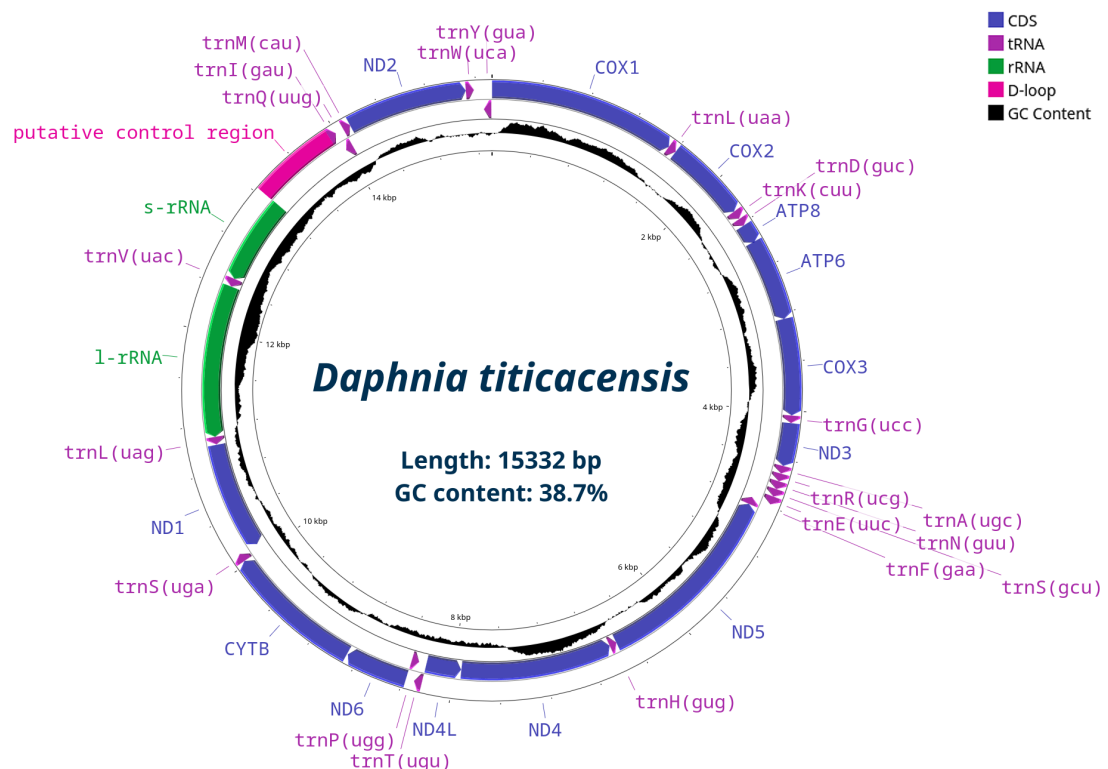


Figure 1. Mitochondrial genome of *Daphnia titicacensis*.

We can see a clear separation (>6%) between *D. titicacensis* and the cluster composed of all the sequences of the sister clade (the *D. pulicaria* species flock) in the TCS network based on the Folmer region of the COI gene (Fig. 4). In fact, it is about the barcoding gap usually found in cladocerans. In contrast, the *D. pulicaria* species flock seems to be composed of "a main axis" (or "core") with several offshoots, and there is no hint of any barcoding gap within the *D. pulicaria* species flock. Moreover, all South American haplogroups represent lateral offshoots from the "main axis".

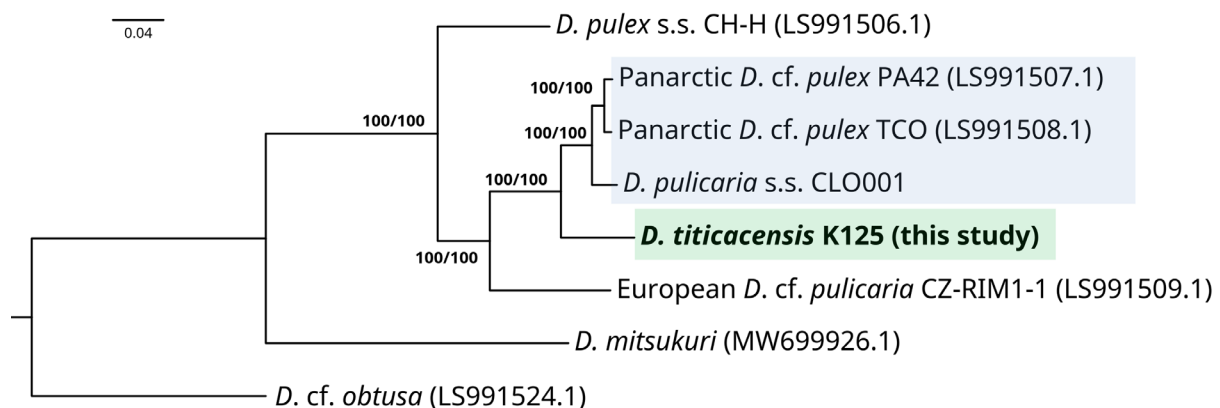


Figure 2. Maximum likelihood mitogenomic tree of the *Daphnia pulex* species group based on both ribosomal genes and full nucleotide sequences of all protein-coding genes. Ultrafast bootstrap (Ufboot) / SH-like approximate likelihood ratio test (SH-aLRT) supports are given at the nodes. Supports less than 50 are not shown. Supports for nodes with Ufboot ≥ 95 and SH-aLRT > 80 are highlighted in bold. The tree is rooted with *Daphnia cf. obtusa* as an outgroup. *D. titicacensis* is marked by green box *D. pulicaria* species flock is marked by blue box.

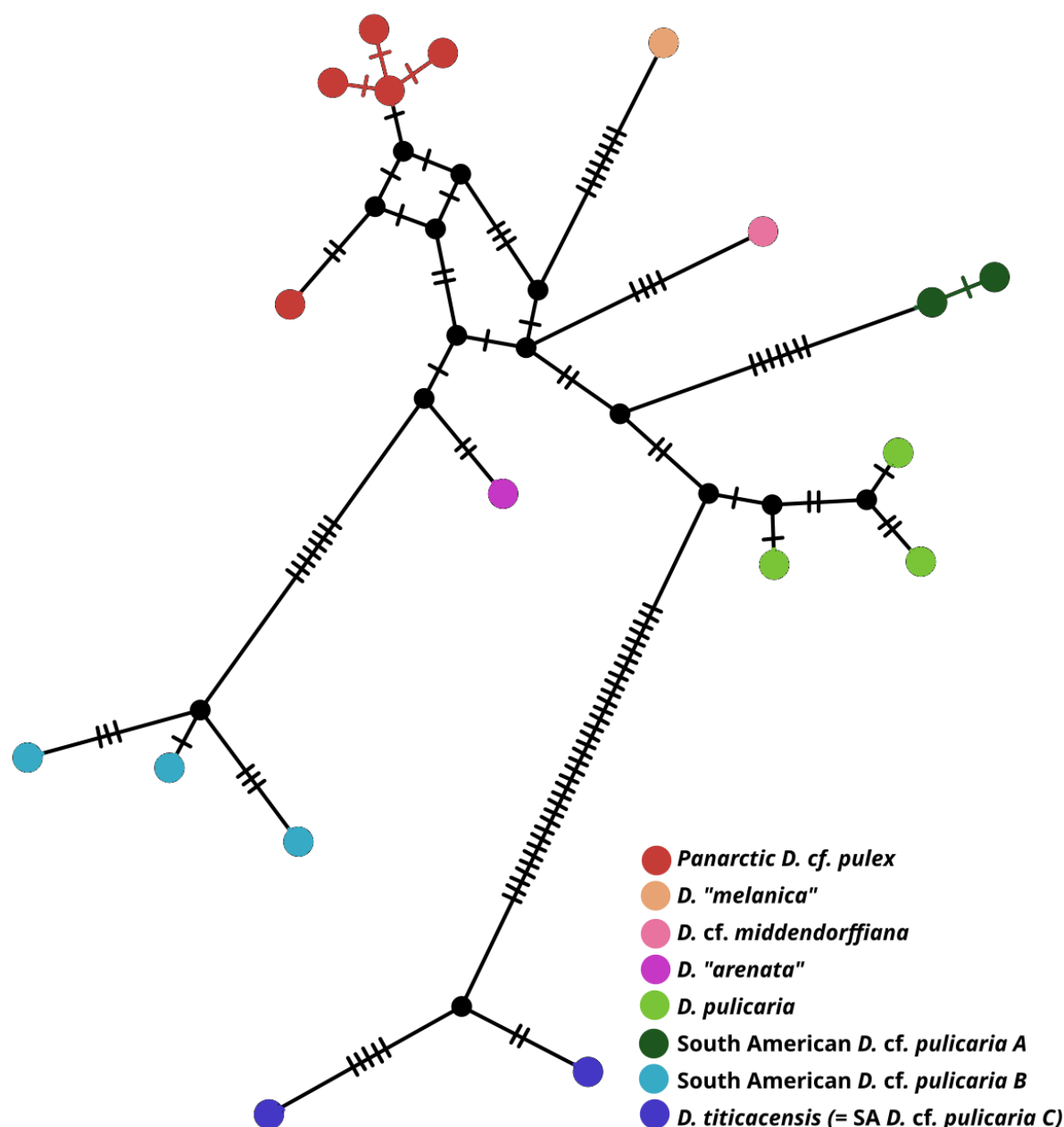


Figure 4. The TCS haplotype network of the minimal clade containing *D. titicacensis* and *D. pulicaria* species flock.

3) Our phylogenetic trees and the haplotype network represented in Fig. 4 do not support the version of a Southern American origin of the *D. pulex* complex, as well as of the *D. pulicaria* species flock. Indeed, South American clades appear as lateral deviations from the "main axis" of the network. The latter is represented by a series of Holarctic haplotypes interconnected through a few mutations. The version of Crease *et al.* (2012) about the Nearctic origin of the flock agrees with our data. Note that we can make the same conclusion from the Fig. 3A in Ma *et al.* (2019).

We in general accept the naming suggested by Ma *et al.* (2019) as a starting point for unravelling the taxonomic chaos within *D. pulex* complex. Some taxa deserve more attention, e.g. *D. "arenata"* which has not been validly described (Adamowicz *et al.* 2009), and there is apparent confusion of lineage presented by Adamowicz *et al.* (2009) and the TCO strain of *D. pulex*: the isolate belongs to an incipient lineage of *D. pulex*, endemic to an area west of the Cascade Mountains, called *D. arenata*" (Colbourne *et al.* 2011). Further investigations are awaited to understand the true nature of *D. "arenata"*, as well as

D. "melanica", and *D. cf. middendorffiana* as they all are placed as distinct lineages with unclear affinities inside the *D. cf. pulicaria-pulex* species flock.

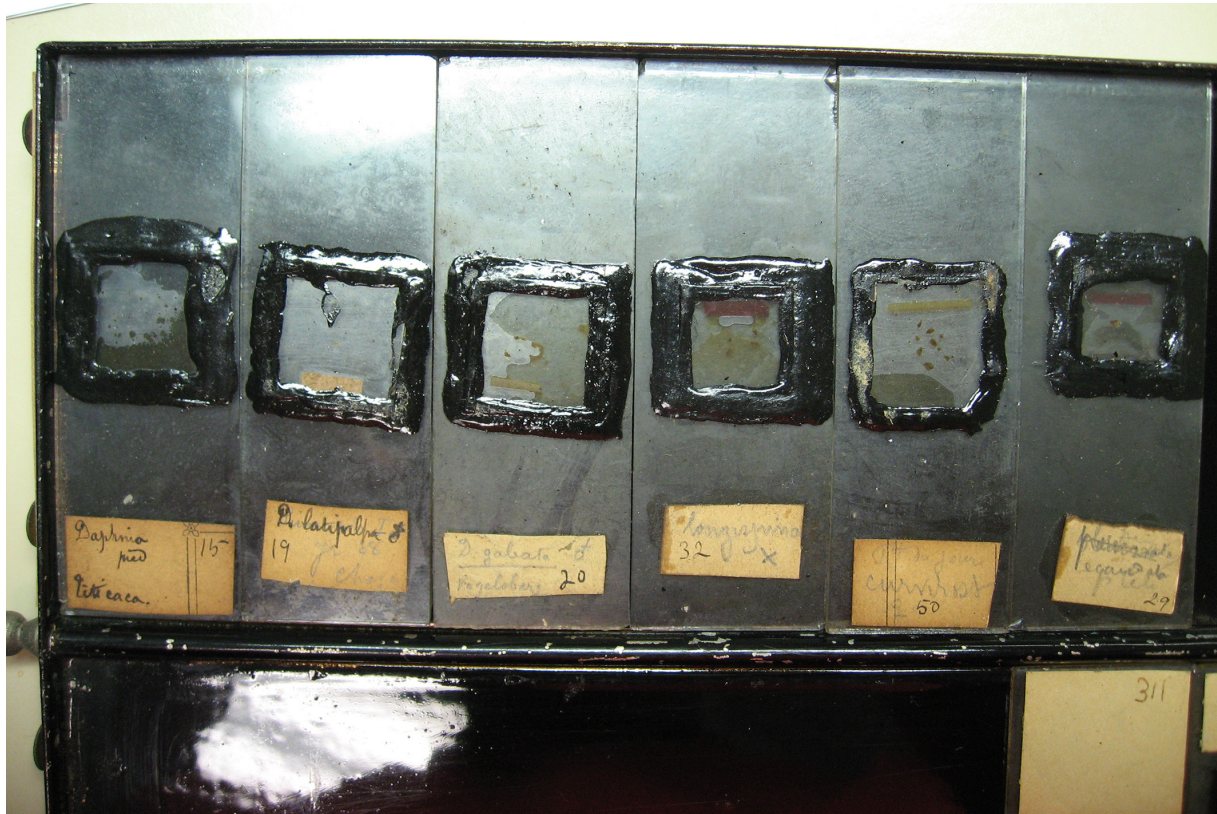


Figure 5. Recent state of slides in the metal cabinet of E.A. Birge in the Smithsonian Institution's National Museum of Natural History, U.S.A. See "*Daphnia pulex*, Titicaca" on the left side.

In this study, we examined only mitochondrial data on the *pulex* species complex. Certainly, this approach has its own limitations and, strictly speaking, is applicable only for the maternal lineages. However, mitochondrial markers analysed here suggest that Andean *D. titacacensis* represents a distinct, and already named, South American species. Although Lake Titicaca is a unique and ancient ecosystem with numerous endemic animal species, *D. titacacensis* is proved to have a broader Andean distribution. Therefore, some other 'endemic' taxa from Titicaca may also have ranges extending beyond this lacustrine basin, potentially representing endemics of the broader Andean Plateau.

Aguilera *et al.* (2007) and Mergeay *et al.* (2008) suggest the Bolivian samples of the BOLC clade are asexual tetraploid lineages of hybrid origin. We cannot rule out such a possibility for our sample. Nevertheless, the attempts to isolate different allelic variants of *LdhA*, *rab4* and ITS from our full-genome assembly failed (data not shown). It could be explained by the incompleteness of the assembly or by genuine homozygosity. Moreover, using of nuclear markers *LdhA* and *rab4* for phylogenetic reconstructions (e.g. Marková *et al.* 2013) is somewhat problematic and vulnerable for criticism. Firstly, lactate dehydrogenases A (*LdhA*) is a gene being under selection and different alleles are fixed in different environments, suggesting their adaptive value, moreover, there are signs of recombination inside the gene (Crease *et al.* 2011). On the other hand, the small GTPase *rab4* has insufficient phylogenetic signal and provides only weak to moderate support for nodes of interest, whereas the topology drastically differs (data not shown). We think, it would be misleading to rely on these gene trees as evidence of mito-nuclear discordance, and proper investigations based on traditional nuclear loci such as the ITS region, SNPs, and even phylogenomic data are needed urgently, especially for taxa outside the *D. pulicaria* and *D. cf. pulex*. It was not the task of our present study.

On the other hand, conclusions of Aguilera *et al.* (2007) were based on indirect evidence (number of microsatellite alleles and allele dosage analysis for ploidy; mendelian segregation of alleles in dormant eggs for asexuality). However, the conclusions would be more robust if more direct methods

(e.g., flow cytometry, karyotyping using cytogenetic methods) also were applied for the ploidy analysis. Mergeay *et al.* (2008) considered apparent mito-nuclear discordance as proof of hybrid origin. In our opinion, minimum-spanning network based on the restricted set of microsatellite data does not possess enough phylogenetic power to support such claim reliably, especially taking into account that there are no shared multilocus genotypes between major clades, and all groups of different subclades are clustered together.

In Cladocera, cryptic speciation, interspecific hybridization, polyploidy, and transitions to asexuality are not unique for the *D. pulicaria* species flock. For example, a very similar situation is described for the *D. carinata* species complex (Colbourne, Wilson and Hebert 2006). This complex is endemic to Australia, and belongs to another subgenus, *Daphnia* (*Ctenodaphnia*), and therefore most likely demonstrates the convergent evolution of such traits. In our opinion, it will not be surprising if hybridization, polyploidy, and transitions to asexuality are common phenomena in Cladocera undetected in many taxa only due to insufficient attention of researchers.

Unfortunately, taxonomy of the *D. pulex* complex still remains to be dubious. We need to continue efforts to resolve numerous problems concerning the former combining different approaches, from genomics to morphology and ecology.

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