DNA analysis confirms the species *Parachaetocladius lenferringtoni* Bouchard *et* Namayandeh, 2024 (Chironomidae: Diptera)

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Abstract

In this study, we confirm the status of *Parachaetocladius lenferringtoni* Bouchard *et* Namayandeh, 2024, using molecular evidence. Using distance-based ABGD, ASAP, and K2P techniques, we determined that the minimum interspecific distance between sequences of *P. lenferringtoni* and *Parachaetocladius abnobaeus* (Wülker, 1959) was 14.8%, sufficient to maintain the species level. Based on the results obtained, we suggest that the threshold of 3% is sufficient to set the species limit for the *Parachaetocladius* species. Therefore, our molecular analyses of *P. lenferringtoni* specimens further support its status as a new Nearctic species distinguished from *Parachaetocladius abnobaeus* (Wülker, 1959).

Introduction

The genus *Parachaetocladius* is characterized by univoltine, cold-stenothermic species inhabiting spring and spring-fed lotic freshwaters (Ferrington 1987, Wülker, 1959, Bouchard et al., 2024). In the Nearctic, the genus is so far represented by five described species (Sæther and Sublette 1983, Namayandeh et al. 2020, Bouchard et al. 2024) and two morphospecies, described as pupae (Langton 2023). Bouchard et al. (2024) previously demonstrated morphological differences between the Nearctic *Parachaetocladius abnobaeus* (Wülker, 1959) (= *Parachaetocladius hudsoni* Sæther & Sublette, 1983) and those from the Palearctic, suggesting the restoration of *P. hudsoni* as a species. Bouchard et al. (2024) further described and erected a new species, *Parachaetocladius lenferringtoni* Bouchard & Namayandeh, based on adult males from Kansas and Manitoba previously identified as *P. abnobaeus* (= *P. hudsoni*), as well as additional adults and immatures from Minnesota, Nebraska, and Wisconsin. The characteristics of the adult male's head and hypopygium and the pupal tergal shagreen, spines, and anal lobe supported the delineation of a new species. At the time of their study, Bouchard et al. (2024) were unable to generate barcode sequences from DNA extracted from the type material. In this study, we successfully extracted sufficient DNA from the tissues of voucher specimens to amplify and provide molecular evidence for the presence of this new species.

Combining morphological and molecular methods has been beneficial to tease out new Chironomidae species, particularly those of cryptic species. For instance, by utilizing mitochondrial and nuclear DNA, Anderson et al. (2013) separated *Micropsectra subletteorum* Anderson, Stur & Ekrem, 2013 from other morphologically similar species in the *M. notescens* group and also separated *Micropsectra neoappendica* Anderson, Stur & Ekrem, 2013 from the morphologically similar *M. appendica* Stur and Ekrem, 2006. Additionally, by utilizing the mitochondrial cytochrome oxidase (COI) gene, Gilka et al. (2018) were able to erect *Tanytarsus latens* Giłka, Paasivirta, Gadawski & Grabowski, 2018 as a new species, which morphologically resembled *Tanytarsus occultus* Brundin, 1949 and *Tanytarsus desertor* Giłka & Paasivirta, 2007. Since separating some *Parachaetocladius* species is difficult, it may be necessary to utilize molecular methods combined with examination of the morphology of the adult and immature stages.

Materials and methods

Molecular analysis

Specimens were collected using the methods outlined in Bouchard et al. (2024). We extracted the genomic DNA of two *P. lenferringtoni* sequences (PCHAT-01, PCHAT-02) from the entire tissues of the adults using the Qiagen DNA Blood and Tissue Kit (Qiagen, Inc., Germantown, MD). A 658 base pair fragment of the cytochrome oxidase subunit 1 (COI) was amplified using the universal primers LCO1490 and HCO2198 (Folmer et al. 1994). DNA amplification was carried out in

24.5 µl reactions of 0.5 µl of each primer, 10 µl of PCR water, 12.5 µl of SSO Advanced SYBR Supermix, and 1.5 µl of template DNA. The amplification cycles were performed using an initial denaturation step of 95°C for four minutes, followed by 40 cycles of 95°C for 30s, 50°C for 30s, 72°C for 1.5 minutes, and a final extension at 72°C for ten minutes. This was followed by DNA denaturation steps that went from 65°C to 95°C with 0.5°C intervals. The above reactions were conducted in the laboratory of Dr. Jeffrey Ram, Department of Physiology, Wayne State University, Michigan, USA. The amplicons were shipped to GENEWIZ, a subsidiary of Azenta Life Sciences, for bi-directional Sanger Sequencing.

A total of 56 sequences were used in the phylogenetic analyses including: 33 *Pseudorthocladius* (*Pseudorthocladius*) *curtistylus* (Goetghebuer, 1921) and *Pseudorthocladius* (*Pseudorthocladius*) *pilosipennis* Brundin, 1956, 16 *Georthocladius*, 6 *P. abnobaeus* and *Parachaetocladius akanoctavus* Sasa *et* Kamimura, 1987*,* and 1 *Orthocladius carlatus* (Roback, 1957) as an outgroup. We submitted new sequences to the BOLD database (http://dx.doi.org/10.5883/DS-PCHAT). The list of all sequences studied, and BOLD species IDs are provided in Supplementary Material Table S1.

The phylogenetic trees were obtained based on two methods: Neighbour-Joining (NJ) and Maximum Likelihood (ML). DNA sequences were aligned using Clustal X version 2.1 software to construct the BI tree (Larkin et al. 2007). We determined the best model for nucleotide substitution using ModelTest-NG v0.1.7 by the Exelixis Lab (Darriba et al. 2020) and chose the best model using the Akaike Information Criterion (AIC). We constructed the ML trees using raxmlGUI 2.0 software (Edler et al. 2021) and with 10,000 Bootstrap repeats. The Neighbour-Joining tree with 10,000 bootstrap replications and K2P distances were produced in MEGA X (Kumar et al. 2018). Trees constructed in the NJ and ML models were visualized in FigTree v. 1.4.2 (Rambaut 2014).

To determine the limits of "molecular species", three species delimitation methods were used based on distance: Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012), Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2021), and K2P distances. ABGD was run with P min = 0.001, P max = 0.1, and a gap width of 1.5, all for 10 steps to calculate the barcode gap in the distribution of pairwise differences. ASAP was run with Kimura (K80) ts/tv = 2.0 setting. For the K2P distance, we set the threshold to 3%. Because the number of *Parachaetocladius* sequences available in BOLD and NCBI was limited, we used sequences from its closest genera, *Pseudorthocladius* and *Georthocladius,* to determine the intraspecific and interspecific K2P distances between the three species in *Parachaetocladius*.

Results and Discussion

The length of the sequences was found to be 685 base pairs. The nucleotide composition was as follows: Cytosine (C) constituted 18%, Guanine (G) constituted 17%, Adenine (A) constituted 26%, and Thymine (T) constituted 39%. No additions or deletions were observed in the sequence. Furthermore, the GC content was calculated and determined to be 35%.

The phylogenetic trees generated in our study indicate the presence of six species. The sequences belonging to the three species of *Parachaetocladius, P. lenferringtoni*, *P. abnobaeus*, and *P. akanoctavus*, cluster within their own groups. As we expected from the previous morphological assessment, the molecular results confirm the presence of a new species, *P. lenferringtoni* (Fig. 1).

Based on the intraspecific K2P distances obtained for the analyzed sequences in this study, the lowest distances belonged to *P. lenferringtoni* (0.0%) and *P. akanoctavus* (0.0%), and the highest to *P. pilosipennis* (1.0%). The intraspecific K2P distance for the remaining species is as follows: *P. curtistylus*, 0.6%; *P. abnobaeus,* 0.84%; and *Georthocladius* sp., 0.95%. The average interspecific distance between all analyzed sequences was 11.0%. The minimum interspecific distance between sequences of *Parachaetocladius* was 14.8%, which belonged to *P. lenferringtoni* and *P. abnobaeus*, the closest species in this genus. This value is sufficient to maintain the species level (Ekrem et al. 2007, 2010, Montagna et al. 2016).

Using ABGD, there is a gap between the highest intraspecific K2P distance (0.03 or 3%) and the lowest interspecific K2P distance (0.13 or 13%). This gap (i.e., for sequences used) suggests that if the distance between two sequences is less than 3%, the sequences belong to the same species. If it is more than 13%, the sequences belong to two different species (Fig. 2). The ASPA result indicates the same results as those of ABGD (Supplementary file 1-Figure S1). Based on the results obtained from these tests, all five species analyzed in this study are placed in the correct classification and represent separate species. While it is premature to establish a criterion for *Parachaetocladius* species in the absence of a more complete DNA library for this genus, this study's 10% gap, determined

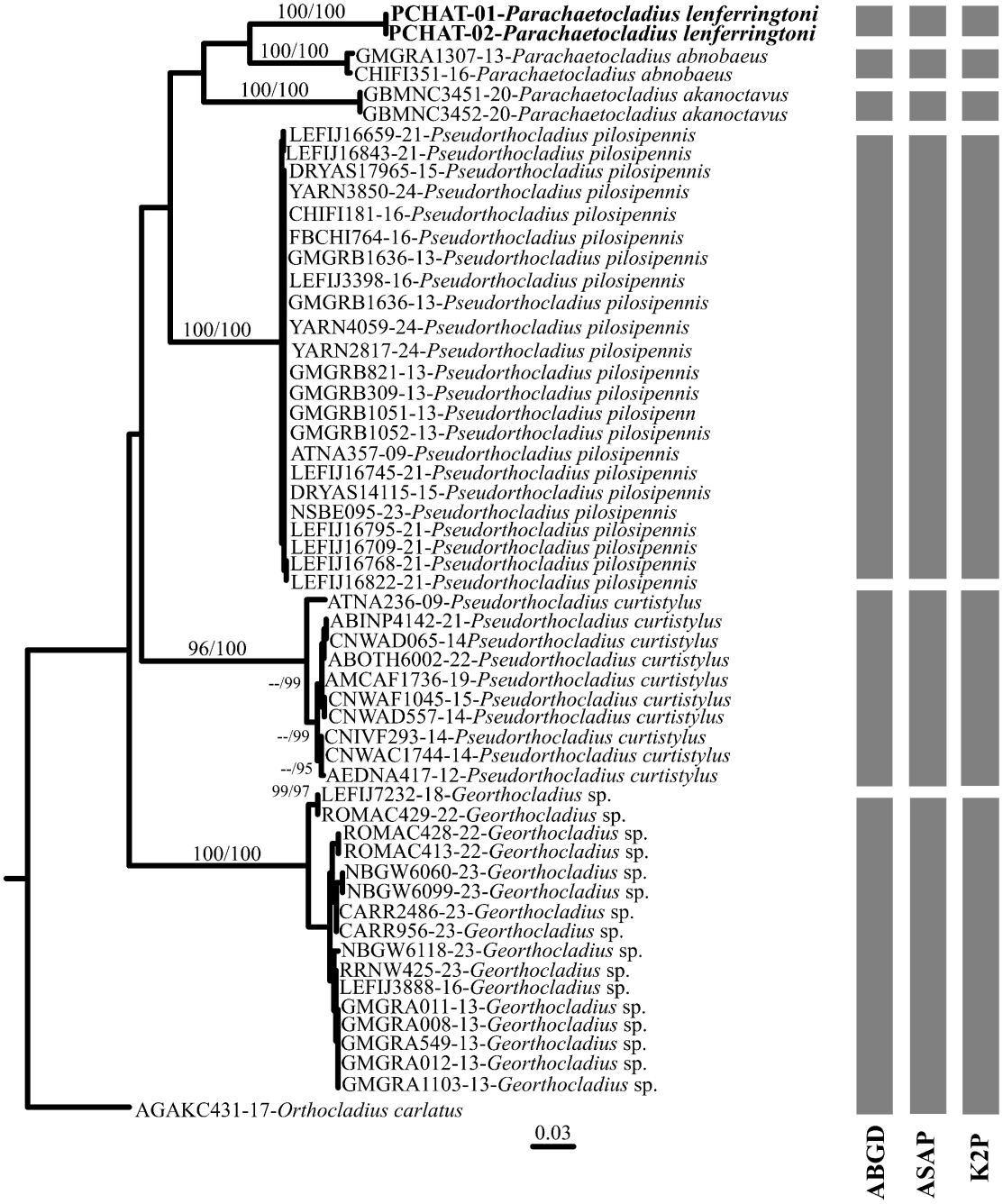

Figure 1. Neighbor-Joining (NJ), Maximum Likelihood (ML) trees of the genera *Georthocladius* Strenzke, 1941, *Parachaetocladius* Wülker 1959, *Pseudorthocladius* Goetghebuer, 1932, and one outgroup *Orthocladius carlatus* (Roback, 1957) inferred from the COI nucleotide sequence data (658 bp). Numbers on branches represent the bootstrap value for Neighbor-Joining (NJ) and Maximum Likelihood (ML) (10000 replicates; values < 95 were omitted). The gray bars on the right are clusters estimated using three molecular species delimitation methods: ABGD, ASAP, and K2P (species delimitation based on the K2P distance, new species threshold value of 3% for sequences used).

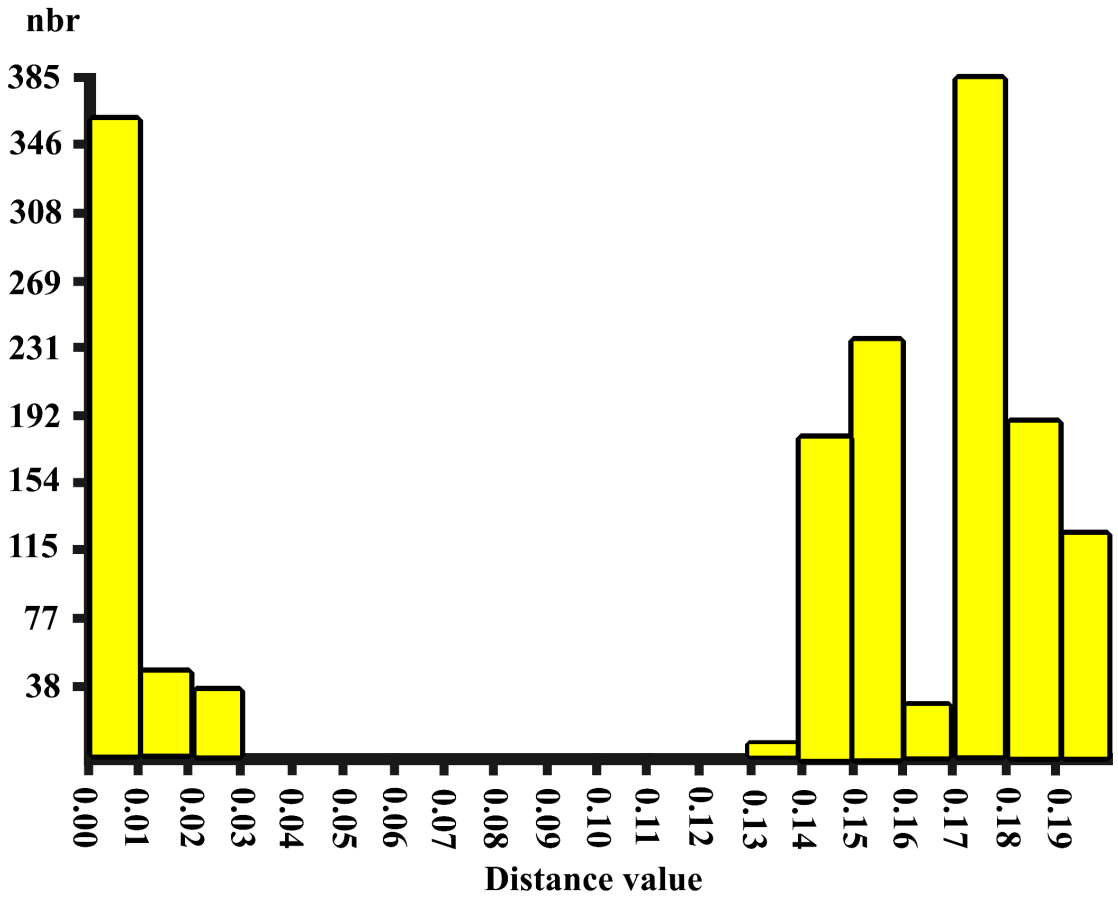

Figure 2. Histograms of genetic distance estimates from ABGD (Automatic Barcode Gap Discovery) for a partition analysis of 56 cytochrome oxidase subunit1 sequences of the *Georthocladius*, *Parachaetocladius,* and *Pseudorthocladius* spp. nbr = number of runs.

using distance-based techniques (such as ABGD and ASAP), is large enough (i.e., much larger than previous studies) to support delimitation of *P. lenferringtoni* from that of *P. abnobaeus* and *P. akanoctavus*. The tree-based and distance-based genetic approaches have been successfully used in recent studies to illustrate the delimitation of Chironomidae species. However, the species threshold (maximum intraspecific distance) of the distance-based methods between chironomid genera varies considerably. For example, the threshold for the *Tanytarsus* species was set at 4–5% by Lin et al. (2015), and within the same genus, the threshold was set at 1–6% for the *Tanytarsus mendax* group by Giłka et al. (2018). For other genera, the selected thresholds have also varied, including 5–8% for *Polypedilum* species (Song et al. 2018) and 4-6% for *Stenochironomus* species (Song et al. 2022). Based on the results obtained in this study, we suggest that the threshold of 3% is sufficient to set the species limit for the *Parachaetocladius* species. The results of the molecular analyses in this study further support our morphological findings in confirming the validity of *P. lenferringtoni.*

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