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Research

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Taxonomy

High throughput sequence data for association and description of female *Calicnemia haksik* (Odonata: Platycnemididae)

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Nagoya Protocol: The authors attest that all legal and regulatory requirements, including export and import collection permits, have been followed for the collection of specimens from source populations at any international, national, regional, or other geographic level for all relevant field specimens collected as part of this study.

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High throughput sequencing is an effective method for associating sexually dimorphic species. Increasing the available taxonomic understanding of females is important for biodiversity and conservation efforts. Here, we confirm the association of females caught in copulation with *Calicnemia haksik* Wilson and Reels, 2003 males in Vietnam using high throughput sequencing (92 loci) and provide the description of the female.

Keywords: anchored hybrid enrichment, damselfly, Vietnam.

Introduction

Associating different life stages and sexes of organisms is required to fully understand the full breadth of taxonomy, biodiversity, ecology, and evolution in biological systems. Molecular associations (eg adult-larva, male-female) based on the barcoding region (COI) have often proven reliable among insects (Miller et al. 2005, Zhou et al. 2007, Renaud et al. 2012, Shashank et al. 2015, Azevedo et al. 2016, Kalkman and Orr 2016, Wilson et al. 2017, Toan and Phu 2019). Barcoding approaches have reduced some of the time-consuming and logistical challenges (eg rearing insects in the field) that come from more traditional morphological association methods. Large-scale identification and association efforts of different life stages using next-generation sequencing (NGS) barcoding workflows have demonstrated the ability to build barcoding libraries and simultaneously associate large amounts of data (Shokralla et al. 2014, Wang et al. 2018, Yeo et al. 2018). However, challenges to barcoding efforts are well documented (eg quality of reference library or sequence, high amplification requirements, interspecific overlap, co-amplification of pseudogenes and contamination, phylogenetic resolution, etc.) as they rely on a single molecular marker (Moritz and Cicero 2004, DeSalle et al. 2005, Song et al. 2008, Shokralla et al. 2014, Yeo et al. 2018, Cheng et al. 2023). Studies have argued that additional loci can help to overcome the limitations of barcoding and provide resolution, especially in complex taxonomic systems for various types of associations (Dupuis et al. 2012, Bourke et al. 2013, Dowton et al. 2014, Liu et al. 2017). Additional data generation does come with additional challenges around cost, efficiency, success of multiple PCRs, ultimately limiting the available specimens to those of high enough quality to reliably generate these markers with traditional methods. Overcoming some of these challenges is possible by using modern high-throughput sequencing tools that allow for

multiplexing many loci simultaneously for species identification and/or association.

Traditionally, methods of association (eg rearing nymphs in the field and observation of male/female copulation) for taxonomic description are complicated when the level of undescribed odonate diversity is high. This is especially true for females where the taxonomic literature can be sparse. The limitations are compounded by the relatively high rate of sexual dimorphism found across Odonata (Corbet 1999, Crowley et al. 2002). *Calicnemia* (Odonata: Platycnemididae) is currently composed of 23 species restricted to Southeast Asia, China, and India (Yu and Bu 2008, Paulson et al. 2024). Males are often characterized as having a black and yellow or red striped thorax with a black or red abdomen and can be divided into two groups by the genital ligula (Lieftinck 1984, Phan et al. 2017). As is common for many groups of insects, comparatively little taxonomic work focusing on females has been done (Phan et al. 2017). The male taxonomic bias has led to a lack of identification resources for females with only two, *Calicnemia akahara* Phan, Kompier & Karube and *Calicnemia uenoi* Asahina, being described to date (Lieftinck 1984, Phan et al. 2017, Yeo et al. 2018). Females, even when collected, are often not worked on taxonomically due to the difficulty of identifying them and the overall lack of literature describing female morphology. Unfortunately, this leads to a continued poor understanding of intraspecific variation and can increase the rarity problem faced in biodiversity surveys and both ecological and evolutionary studies (Yeo et al. 2018). Increasing female taxonomic information via associations and descriptions in a biodiversity hotspot such as Vietnam is important for biodiversity monitoring, conservation, and survey efforts (Yeo et al. 2018). Here, we use a high throughput, multilocus (92 total loci) method to associate females with males using both nuclear and mitochondrial markers to confirm that the association of females caught in copulation with *Calicnemia haksik* Wilson and Reels males and provide the scientific description of the female.

Materials and Methods

Taxon Sampling

We included 14 out of 23 (60%) species within *Calicnemia*. Fresh male and potential female representatives of *C. haksik* (two mated pairs) were collected and preserved in 95% EtOH in June 2022 from Dong-Song Ky Thuong and Bach Ma National Parks in Vietnam. The outgroup consisted of representatives from a closely related genus, *Coeliccia* (Gassmann 2005, Dijkstra et al. 2014). The remaining specimens included were identified and provided by Brigham Young University (BYU), the Naturalis Biodiversity Center (RMNH), and the Florida State Collection of Arthropods (FSCA). Images were taken with Helicon Remote 4.4.4 using a Canon EOS 6D camera fitted with 100 mm and 65 mm lens and stacked with Helicon Focus 8.2.2.

DNA Extraction and Sequencing

Thoracic flight muscle was extracted using a Qiagen DNeasy Blood and Tissue kit (Valencia, CA), following manufacturer protocols. Specimens were deposited in the BYU genomic collection (Provo, UT) as vouchers or returned to the original institution. The extraction was quantified using a high-sensitivity qubit fluorometer before being sent to LGC Genomics for anchored hybrid enrichment (AHE) sequencing. Probes were previously designed by Bybee et al. (2021) to capture 92 loci (~20 KB) representing both nuclear (90) and mitochondrial (2) conserved regions across Odonata.

Tree Reconstruction

The bioinformatics pipeline was adapted from Breinholt et al. (2018) to analyze the raw reads received from LGC Genomics using the BYU supercomputer. The raw reads were assembled using SPAdes (Bankevich et al. 2012), and contamination was eliminated (BLAST). Top orthologs for each taxon and loci were selected based on bit score, length, and coverage. Alignments for each locus were generated using MAFFT v.7.45 (Katoh and Standley 2013) and cleaned with Aliscore 22.ii.2012 (Misof and Misof 2009, Kück et al. 2010) and AliCUT v2.31 (Kück 2009). PartitionFinder (Lanfear et al. 2017) and ModelFinder (Kalyaanamoorthy et al. 2017) were run to produce the optimal partition and model scheme according to BIC scores. A maximum likelihood reconstruction was produced using IQTree v2.2.0 (Minh et al. 2020) with 1000 ultra-fast bootstraps (Hoang et al. 2018) as support. iTOL (Letunic and Bork 2021) and Adobe Illustrator 28.3 were used for tree visualization and annotation. Male-female association was primarily determined based on monophyly within the phylogenetic result.

Distance Analysis

The concatenated alignment was imported into Geneious v. 2023.2.1 where a genetic distance (similarity) matrix was generated. Male-female association was confirmed based on the percentage similarity between the sequences. There has been a 1% to 3% dissimilarity threshold used for barcoding studies in the past, and here we will use a 1% cutoff (Koroiva and Kvist 2018, Zhang and Bu 2022).

Results

The DNA alignment for phylogenetic reconstruction was based on a total of 21,683 bp of concatenated nucleotide sequence. All representatives of *Calicnemia haksik* were recovered together with maximal support, indicating strong support for the species association. The distance matrix revealed that the females were >99.7% similar to males of *C. haksik* (Fig. 1), confirming copulation observations made in the field.

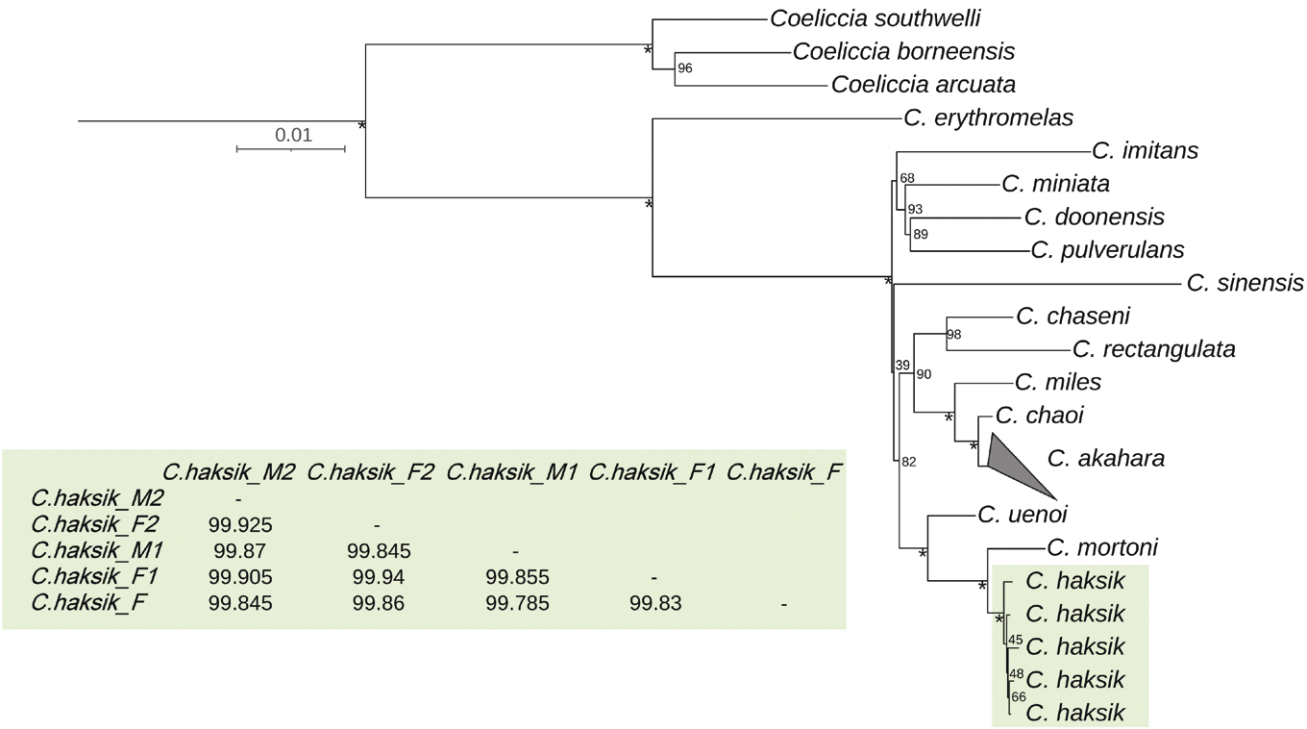
Female Description of *Calicnemia haksik* (Fig. 2)

Head

Occiput black with two pale spots originating at the two posterior ocelli and extending to the antennae (Fig. 2D and E). Frons black, vertex black, antennae black, postclypeus black with pale spots at the posterior edge, anteclypeus pale green with dark spots close to the posterior edge (Fig. 2D, E, and F). Genae pale green. Labrum pale green, labium pale blue-gray. Eyes posterior black, anterior pale green (Fig. 2A and E).

Thorax

Prothorax black with propleuron completely yellow, pronotum black, mesostigmal plate black (Fig. 2A, C, and G). Pterothorax with black carina, mesepisternum black with yellowish stripe start from anterior to the posterior, mesepimeron black, metepisternum ⅔ pale yellow dorsal from anterior to posterior and ⅓ black ventral starting from the under of spiracle from the base interpleural suture up to the edge, metepimeron mostly pale yellow with black extending to the dorsal of interpleural suture, mesinfraepisternum and metinfraepisternum pale yellow, mesocoxa and metacoxa yellow (Fig. 2A and C). Coxae yellow, trochanters mostly yellow with ⅔ linear spot on dorsal and ⅓ triangular spot on distal, femora pale yellow fading halfway to dark brown to black on dorsal to the distal end, hairs pale yellow ventral, tibia black at the proximal, brown and black at the distal end, two short spines close to the basal of tibia, tarsi brown with black edges,



	C.haksik_M2	C.haksik_F2	C.haksik_M1	C.haksik_F1	C.haksik_F
C.haksik_M2	-				
C.haksik_F2	99.925	-			
C.haksik_M1	99.87	99.845	-		
C.haksik_F1	99.905	99.94	99.855	-	
C.haksik_F	99.845	99.86	99.785	99.83	-

Fig. 1. Resulting maximum likelihood reconstruction of *Calicnemia* with focus on *C. haksik* based on 92 loci. Bootstrap support values are indicated at each node, * represents 100 and the value is given if less than 100. Results of genetic similarity matrix for two mated pairs and an additional female of *C. haksik*.

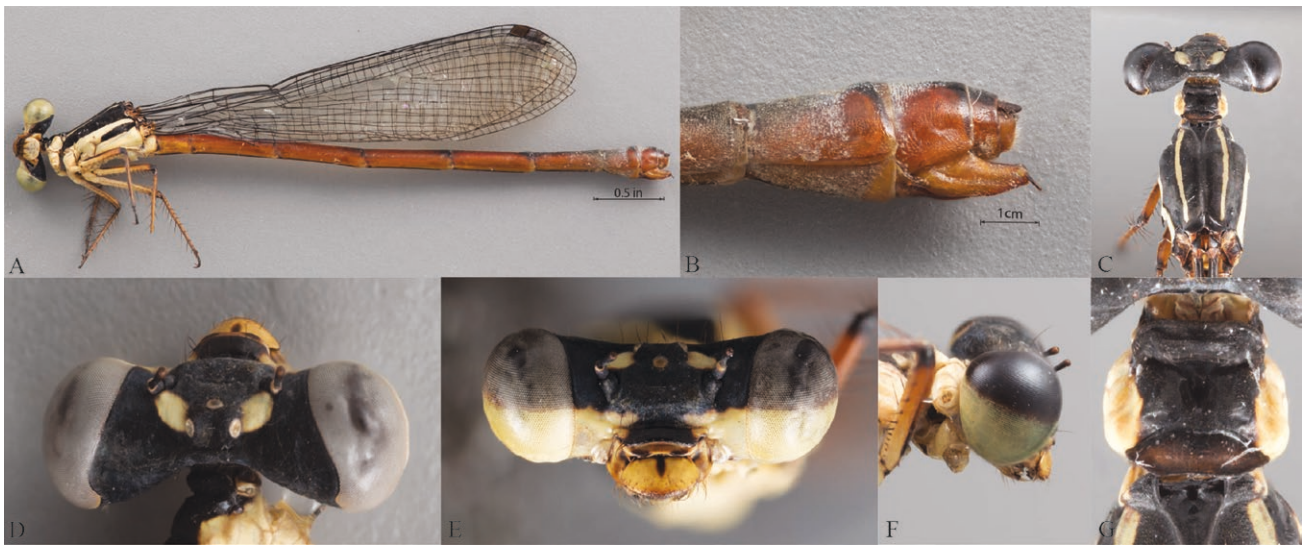


Fig. 2. A) lateral habitus, B) lateral terminalia, C) dorsal thorax, D) dorsal head, E) frontal head, F) lateral head, and G) dorsal pronotum.

two rows of lateral spines long dark reddish brown, dark red tarsal claws that darken apically to black tips, claws with a small tooth located on the basal half of their length (Fig. 2A).

Wings

Hyaline, 17 postnodal crossveins in forewing and 16 in hindwing. Pterostigma brown (Fig. 2A).

Abdomen

S1 pale yellow, S2 to S4 dark red-orange start from proximal to ¾ of the segment, ⅓ orange at distal with black dorsal stripe, S1 to S4 black lines encircling the posterior end, overall all ventral segments are yellow with black ventral stripe, S5 to S7 orange with black dorsal stripe, black lines expanding half way down the posterior end, S8 to S10 orange with black dorsal spot (Fig. 2A). Ovipositor overall dark yellow (Fig. 2B).

Measurements (mm)

Abdomen (including anal appendages): 26.84 mm; Forewing: 23.30 mm; Hindwing: 21.89 mm.

Discussion

There are numerous challenges when studying and associating closely related species (eg DNA concentration and quality, sequencing

success, co-amplification of contaminants, cost, incomplete lineage sorting, etc.) (Moritz et al. 2004, Shokralla et al. 2014, Cheng et al. 2023). DNA barcoding provides an empirical assessment that is valuable when determining conspecifics but also remains imperfect due to many of the aforementioned issues (Bourke et al. 2013, Dowton et al. 2014, Shokralla et al. 2014). The barcoding region of COI has known limitations; it is clear that the inclusion of additional markers may be more effective at species delimitation (Dupuis et al. 2012). By limiting analyses to only one marker, there is the potential to infer incorrect evolutionary patterns and relationships as well as a need to account for additional genetic complexity as a gene tree does not always equate to a species tree (Dowton et al. 2014, Liu et al. 2017). High throughput sequencing is a method that accepts a lower initial DNA concentration, DNA quality, and avoids the difficulties of successful PCR and sequencing costs for multiple loci, and has efficient quality control procedures (Goodman et al. 2023). It has been previously demonstrated that there is a positive relationship between the number of loci incorporated into the analysis and the success of species delimitation by allowing for more robust evolutionary relationships to be inferred which therefore increasing confidence in species identification (Dupuis et al. 2012). Further, multiple loci approaches offer more post hoc phylogenetic research that seeks to place all species in an evolutionary context.

In this study, there were several issues that rendered barcoding impractical. While initial extractions were successful, traditional Sanger sequencing was consistently unsuccessful and became costly. Numerous PCR protocols and primer combinations (8) were attempted for regions in COI and COII with limited success. As the PCR protocols became less specific to capture the sequence, significant double banding issues began to arise. After several months, enough PCR products were generated and acceptable for sequencing. Sequencing was then attempted, with very limited success. Consensuses sequences were not able to be generated for the majority of the samples either due to failed sequencing of the forward or reverse or a significant amount of variation between the two sequences. These issues were consistent regardless of the primers, and there was not enough overlap between the sequences. All of these issues were negated once AHE sequencing was performed.

There continues to be questions around the minimum number of loci necessary to identify and delineate species (Dupuis et al. 2012, Dellicour and Flot 2018). High throughput sequencing minimizes the problems associated with barcoding by capturing many loci simultaneously, resulting in many reads for each locus, allowing for rigorous quality control and even the identification of known genetic issues with current COI barcodes (eg NUMTs). However, high throughput sequencing could be considered excessive for species associations if the data is not generated more broadly within the focal group where associations are being attempted. High throughput sequencing is typically generated for higher-level phylogenomic hypothesis testing, such as updated classifications and/or evolutionary histories of lineages/traits (eg Bybee et al. 2021, 2022, Kohli et al. 2021). Here, we expand the utility of previously generated AHE data originally intended for higher-level phylogenetic research to answer more narrow questions, such as associations of life stages and sexes used for traditional taxonomic descriptions, which help form the backbone of research focused at higher levels. We were able to confirm the association of *C. haksik* females and provide a description of the female increasing the amount of female taxonomic literature available. The odonate diversity in Vietnam is high (eg Phan et al. 2018, Toan and Phu 2019) and being able to identify females on sight will assist in biodiversity and ecological studies in the future.

Conclusion

Here we demonstrate additional uses of AHE data previously designed to answer higher-level phylogenomic questions to be an efficient way to also answer narrow taxonomic questions such as male-female associations with high confidence. We acknowledge that the initial cost for high throughput sequencing is high (eg cost of genome sequencing to support probe design, probe design, probes, and sequencing in large batches of at least 96 taxa), as well as high costs associated with the subsequent required bioinformatics (eg pipeline development, supercomputer resources). Simply, we aim to show that high throughput data is robust and can be used to explore questions beyond the intended use of the original data. This method is not a feasible option, nor should it be required for all association or 'barcode' projects. However, AHE data could be used to complement ongoing barcoding efforts (eg Nguyen 2022, Putt et al. 2023, Hu et al. 2024) if the AHE loci include the barcode region and it is publicly available. High throughput sequencing is an established method and has become an approach to explore higher-level phylogenetics in many groups (eg Coleoptera (Haddad et al. 2017), Diptera (Young et al. 2016), Hymenoptera (Klopfstein et al. 2019), Lepidoptera (Toussiant et al. 2018), Trichoptera (Frandsen et al. 2024)). With large amounts of data continuing to be generated and published, it is becoming more realistic for researchers to work together in extensive data exploration with narrow-focused questions. We accomplished this by confirming the association of *C. haksik* females caught with identifiable males in Vietnam, allowing for the description of the previously unknown female.

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Author contributions

Cordon Wade (Conceptualization [equal], Data curation [lead], Investigation [equal], Writing—original draft [equal], Writing—review & editing [equal]), Laura Sutherland (Data curation [supporting], Formal analysis [equal], Supervision [supporting], Writing—original draft [supporting], Writing—review & editing [equal]), Pungki Lupiyaningdyah (Data curation [equal], Formal analysis [equal], Methodology [equal]), Sam Bennett (Data curation [supporting], Investigation [equal]), Hong Pham (Investigation [equal], Project administration [equal]), Gareth Powell (Conceptualization [equal], Methodology [equal], Project administration [supporting], Supervision [equal], Writing—review & editing [equal]), and Seth Bybee (Conceptualization [equal], Funding acquisition [lead], Methodology [equal], Project administration [equal], Resources [equal], Supervision [equal], Writing—review & editing [equal])

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Conflicts of interest. None declared.

Data availability

The data underlying this article are available in the Dryad Digital Repository at https://doi.org/10.5061/dryad.tb2rbp094

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