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Research Article

A DNA Barcode Library of Some Neuroptera from Azerbaijan

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Authors' Contributions
IGK performed the experiments. All auhtors analyzed the data. MAA and IGK prepared figures. IGK prepared the tables. LNM and IGK collected samples. IGK wrote the first draft. VAK and IGK revised and reviewed the paper.

Keywords
Antlion, Owlfly, Mantidfly, COI, DNA

Abstract | COI sequences were obtained for 25 species of Neuroptera. It is difficult to recognize the immature antlions of *Palpares libelluloides* (Linnaeus, 1764) and *P. turcicus* with similar brown rings on the last abdominal segments. The specimen that could not be determined was marked as "*Palpares* sp. questionable". The genetic method has finally solved this question; now *Palpares* sp. questionable (IGK15) is surely assigned to *P. turcicus*. *Myrmecaelurus solaris* (Krivokhatsky, 2002) (IGK2) ML. and *M. trigrammus* (Pallas, 1781) (IGK22) differ from each other in dendrogram by more than 10%. The NJ tree shows that the genus *Myrmecaelurus* (Costa, 1855) is supported by 97-100%, and it is connected with another close genus *Nohoveus* (Navás, 1918) (Azerbaijanian *N. zigan*) and Chinese population of *N. atrifrons* (Hölzel, 1970). The sequences of *Bubopsis hamata* (Klug, 1834) (IGK25) and *B. andromache* (Aspöck *et al.*, 1979) (IGK26) turned to be identical. A genetic approach forces us to synonymize these two names: *Ascalaphus hamata* (Klug, 1834) *Bubopsis andromache* (Aspöck *et al.*, 1979) syn. n. A noticeable convergence of the compact cluster of the genus *Bubopsis (*Mac Lachlan, 1898) with the owlfly *Deleproctophylla variegata* (Klug, 1845), which, together belongs to the subfamily (Ascalaphinae Lefèbvre, 1842), indicates a characteristic point of embranchment in the genus *Libelloides* (Schäffer, 1763) inside Libelloidini Pantaleoni, (Loru, 2018). Thus, Libelloidini is a daughter tribe within Ascalaphinae. Although the support between the clades of *L. macaronius kolyvanensis* – *L. hispanicus ustulatus* – *D. variegata* and *B. hamata* + *B. andromache* is not so high (44), it organizes the traditional owlflies of the Ascalaphidae family into one cluster, opposed to the cluster that unites all the studied antlions (Myrmeleontidae). Thus, the proposal to merge the Myrmeleontidae and Ascalaphidae into one family as suggested by Machado *et al.* (2018) is not supported by our data.

Novelty Statement | The article presents the first report on the DNA analysis of the lacewing insects of Azerbaijan. A total of 25 species of antlions, mantidflies and owlflies were DNA barcoded.

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Introduction

To date, up to 6,000 species of net winged insects have been recorded and described worldwide (Engel *et al.*,

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2018). However, there is still a lot of confusion in their taxonomy. Machado *et al.* (2018) recently suggested a new classification for antlions and owlflies united into one family.

Currently, approaches based on DNA sequence have become increasingly important for assessing biodiversity and identifying species (Taberlet *et al.*, 2012). Our objective is to examine the relationships of the Myrmeleontoidea inhabiting Azerbaijan.

The current composition of the fauna of Myrmeleontidae, Ascalaphidae, Mantispidae, and Nemopteridae in Azerbaijan consists of 38 species. Out of the above-mentioned species, 25 belong to antlions, 6 species to owlflies, 4 species to mantidflies, and 3 species to spoontails (Kerimova and Krivokhatsky, 2018; Kerimova, 2020).

We aimed to use Polymerase chain reaction (PCR) technique to study the molecular similarity or difference of some species and subspecific taxa of Azerbaijan's neuropterans, which belonged to different subfamilies and tribes and were collected in different locations.

A molecular phylogeny of the Neuropterida was proposed by Haring and Aspöck (2004). Michel *et al.* (2017) presented a time-calibrated phylogeny of antlions. We repeat this work here on the material collected mainly in Transcaucasia with an emphasis on superfamily Myrmeleontoidea.

Material and Methods

The Neuroptera specimens were collected during the period from 2012 to 2017 using hand nets and light traps. The field works were carried out in the in the following territories: Nakhchivan Autonomous Republic districts, Gobustan district, Siazan, and Shabran districts in northeastern Azerbaijan and the Caspian Sea coastal at the foot of Mountain Beshbarmak (Figure 1, Table 1). For comparison, samples collected outside of Azerbaijan were also included. Most specimens were stored in 95% ethanol but some species, particularly the mantispids (*Sagittalata perla* (Pallas, 1772), *Mantispa scabricollis* McLachlan, 1875), and owlflies *Bubopsis andromache* (Aspöck *et al.*, 1979) and *B. hamata* (Klug, 1834) were used dry. The species were identified using published keys (Aspöck *et al.*, 1980; Krivokhatsky, 2011). Molecular identifications were carried out by sequencing the DNA barcode region of mitochondrial gene COI. All materials are kept in the Institute of Zoology of the Ministry of Science and Education of the Azerbaijan Republic (Baku).

Table 1: Locations where Neuroptera species were collected.

Family	Species	Species code	GenBank accession number	Location of collection
Myrmeleontidae	*Palpares libelluloides* (Linnaeus, 1764)	IGK1	MT621181	Nakhchivan, Ordubad,
	Palpares turcicus Koçak, 1976	IGK 4	MT621190	Nakhchivan, Ordubad
	Palpares sp. questionable	IGK 15	MT621171	Nakhchivan, Ordubad
	Myrmecaelurus solaris Krivokhatsky, 2002	IGK2	MT621178	Lerik, Gosmolian
	Myrmecaelurus trigrammus (Pallas, 1781)	IGK 22	MT621179	Fizuli
	Acanthaclisis occitanica (Villers, 1789)	IGK 5	MT621180	Siazan, Saadan
	Acanthaclisis occitanica (Villers, 1789)	IGK 6	MT621193	Lerik, Gosmolian
	Acanthaclisis occitanica (Villers, 1789)	IGK 7	MT621182	Nakhchivan, Ordubad,
	Nohoveus zigan (Aspöck, Aspöck et Hölzel, 1980)	IGK 8	MT621186	Siazan, Caspian Sea shore
	Creoleon plumbeus (Olivier, 1811)	IGK 9	MT621187	Siazan, Caspian Sea shore
	Myrmeleon hyalinus hyalinus Olivier, 1811	IGK12	MT621174	Turkey
	Myrmeleon hyalinus distinguendus Rambur, 1842	IGK 17	MT621176	Sumgait, Caspian Seashore
	Myrmeleon hyalinus distinguendus Rambur, 1842	IGK 18	MT621177	Turkey
	Neuroleon (*Ganussa*) *tenellus* (Klug, 1834)	IGK 13	MT621170	Gobustan
	Distoleon tetragrammicus (Fabricius, 1798)	IGK 19	MT621188	Siazan, Sadaan
	Macronemurus bilineatus Brauer, 1868	IGK 21	MT621194	Nakhchivan, Ordubad
	Euroleon nostras (Geoffroy in Fourcroy, 1785)	IGK 23	MT621191	Siazan, Sadaan
Ascalaphidae	*Deleproctophylla variegata* (Klug, 1845)	IGK 3	MT621185	Nakhchivan, Ordubad
	Libelloides hispanicus ustulatus (Eversmann, 1850)	IGK 11	MT621173	Shabran, Galaalty
	Libelloides macaronius kolyvanensis (Laxmann,1842)	IGK 10	MT621183	Nakhchivan, Ordubad
	Bubopsis hamatus (Klug, 1834)	IGK 25	MT621192	Siazan, Beshbarmak
	Bubopsis andromache (Aspöck *et al.*, 1979)	IGK 26	MT621184	Gobustan
Mantispidae	*Sagittalata perla* (Pallas, 1772)	IGK 14	MT621175	Nakhchivan, Ordubad
	Mantispa scabricollis McLachlan, 1875	IGK 24	MT621189	Nakhchivan, Ordubad,
Nemopteridae	*Lertha ledereri* (Sélys-Longchamps, 1866)	IGK 16	MT621172	Lerik, Gosmolian

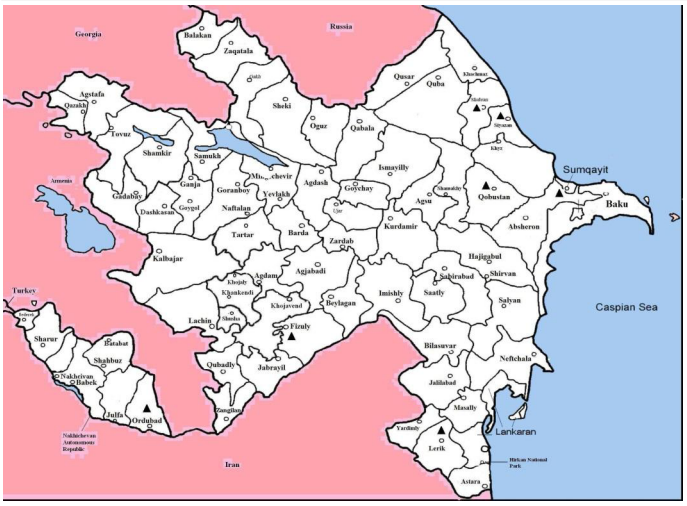

Figure 1: Map of locations.

DNA extraction and PCR amplification were performed at the Department of Research and Collections of the University of Oslo; Natural History Museum of Norway. The DNA was isolated from legs using the Qiagen DNeasy Blood and Tissue Kit™, following the protocol for Animal Tissue.

The barcoding fragments were amplified by PCR using the following primers: Forward LCO1490 (5'–GGTCAACAAATCATAAAGATATTGG–3') and reverse HCO2198 (5'–TAAACTTCAGGGTGACCAAAAAATCA–3') (Folmer *et al.*, 1994). The PCR amplifications were performed using master cycler gradient- eppendorf (Pro S model, Germany) with 10µM of each primer (LCO 1490F and HCO 2198R), 1, 25 mM dNTPs for each tube, 1.5 mM $MgCl_2$, 1U Taq polymerase, and 2.5 µl of 10x PCR buffer, 200µg of extracted DNA in a final volume of 25 µl. The PCR thermal cycling parameters were: 1 cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 1 min annealing at 54°C and extension at 72°C for 1:15 min followed by a final extension of 10 min at 72°C. Labeled PCR strips were kept at -20°C until used. The PCR products were subjected to electrophoresis in 1% agarose gel and stained with gold view (1ng/mL) to confirm amplification (Figure 2). Amplicons were sequenced by StarSEQ GmbH (Mainz, Germany).

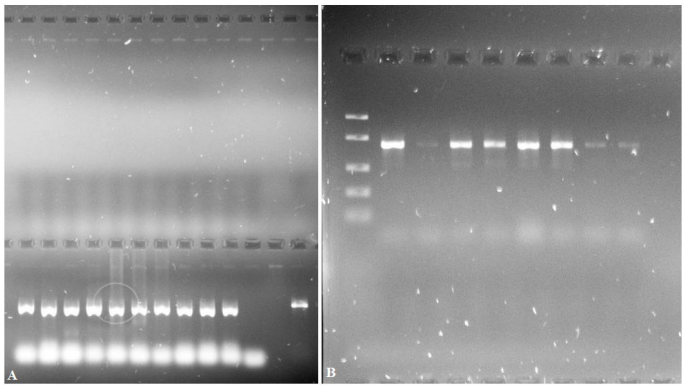

Figure 2: a, b. Electrophoresis of PCR products in 1% agarose gel.

The final consensus COI sequences were obtained after overlapping both forward and reverse sequences using contig express. All sequences were analyzed using NJ cluster analysis (Saitou and Nei, 1987).

Data assembly and alignment

Forward and reverse DNA sequences were produced as electropherograms (ab1 files). First, low-quality motifs at both ends of the sequences were trimmed, and the sequences were assembled and manually edited in codon code Aligner v8.0.1 (Codon Code Corporation, Dedham, MA, USA); subsequently, consensus sequences (contigs) were generated. Consensus sequences were entered into the BLAST to determine the closest sequence identity in GenBank. The COI sequences obtained by us for 25 species of lacewings of Azerbaijan fauna have been deposited to GenBank with accession numbers presented in Table 1.

Sequences for representatives of myrmeleontidae and ascalaphidae species were aligned and compared to confirm the BLAST results. COI sequences, obtained here, were aligned using the multiple alignment model of Clustal W (Thompson *et al.*, 1994) as implemented in MEGA v 7.0 (Kumar *et al.*, 2016) with their corresponding sequences of Neuroptera and *Parainocellia bicolor* (Raphidioptera, EU839733), as the outgroup, retrieved from GenBank (species list, retrieved from GenBank and used in constructed phylogenetic trees, was given in Table 1). Additionally, it was confirmed by translating the sequence to the amino acid sequence that it did not have a slipped open reading frame. Sequences were checked, and those containing the premature stop codon were reassembled.

Each sequence was checked, and species names, determined by morphological descriptions, were verified via BOLD system v.3 (http://v3.boldsystems.org/) after characterization of COI.

Phylogenetic tree construction

For phylogenetic analyses, the optimum partitioning scheme and the nucleotide substitution model were estimated in partition finder v1.1.1 (Lanfear *et al.*, 2012) written in Phyton v2.7.14. The analysis was conducted to estimate the best partitioning scheme. The best-fit partitioning scheme with its respective substitution model was selected according to the Akaike Information Criterion (AIC). The final dataset and file containing the inferred partition scheme and model selection results are provided as **Supplementary File 1**. Phylogenetic trees were generated for maximum likelihood (ML) under the GTR+I+G model (the best-fitting substitution model was a general time-reversible substitution model (Tavaré, 1986) with rate heterogeneity described by a gamma distribution discretized into four bins (Yang, 1993) and a proportion of invariant sites (Fitch and Margoliash, 1967). We determined the number of generations necessary to be

run and the burn-in by examining the log-likelihood (lnL) plots through Tracer v1.5 (Rambaut and Drummond, 2007). We ran four Markov chains (one cold and three heated) with $7x10^6$ generations, sampling every 200 generations. Phylogenetic trees were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/ software/figtree/).

Results and Discussion

There was strong node support the upper branches of the ML tree. The bases of trees were poorly supported. All Azerbaijani neuropteran specimens clustered with congeners where duplicates were present. Three sequences obtained for specimens of *Myrmeleon hyalinus* (Olivier, 1811) and presented in the ML tree belong to the same species. The genetic difference between it and its nearest neighbor was 5% (0.05). The differences between the three sequences in the tree do not exceed two percent, and in the dark (*distinguendus*) and light (*hyalinus*) subspecies, it is generally less than one percent. We found the same result in the NJ tree. The subspecies relationship of *Myrmeleon hyalinus hyalinus* Olivier, 1811 and M. *hyalinus distinguendus* Rambur, 1842 was substantiated by Hölzel (1987). However, recently, geographical subspecies and mixed populations in sympatrical zones were discussed using morphological features (Kerimova and Krivokhatsky, 2018) and confirmed by genetic data in the given study.

Also, in both dendrograms, a closer interpopulation relationship of individuals (sequences IGK 12 and IGK 18 from Turkey) is more evident than for a subspecies relationship, which indicates a heterogeneous composition of populations.

The specimen *Palpares* sp. undertermined (Table 1, IGK15) belongs to *P. turcicus* Koçak, 1976. The two species from *Palpares libelluloides* species group divided morphologically with high levels of support. Previously, we described the problem of difficult recognition of immature forms of adult antlions *Palpares libelluloides* and *P. turcicus* with similar brown rings on the last abdominal segments (Krivokhatsky *et al.*, 2017). For *libelluloides*, another characteristic feature was the small spots on the cubital forks of the hindwing against the extensive brown spots in *turcicus*. The specimen that could not be determined was marked as questionable. The genetic method finally solved this question now: *Palpares* sp. questionable (IGK15) is now surely assigned to a known species, *P. turcicus*.

Myrmecaelurus solaris (Krivokhatsky, 2002) (IGK2) and *M. trigrammus* (Pallas, 1781) (IGK22) differ from each other in dendrogram by more than 10%. The same result we see in the NJ tree. In Azerbaijan, these two pale yellow species, close in opinion, are found together only on the Caspian coast, where they inhabit multiple biotopes (Kerimova and Krivokhatsky, 2018). In the second dendrogram, with the involvement of the additional species *Myrmecaelurus major* McLachlan, 1875 from the Genbank, it becomes clear that the species of the same larger size category are more closely related, while the smaller one, *M. trigrammus*, is located at a considerable distance from the larger pair.

As we see from the NJ tree, the genus *Myrmecaelurus* Costa, 1855 is supported by 97-100% (3 species: *M. major, M. trigrammus*, and *M. solaris*), and it is connected with another close genus *Nohoveus* Navás, 1918 (Azerbaijanian population of *N. zigan* (Aspöck *et al.*, 1980) and Chinese population of *N. atrifrons* Hölzel, 1970, taken from Genbank; 100 %). In the last catalog of antlions of the world fauna (Stange, 2004), these two genera were synonymized; however, we recognize their independence in the tribe *Myrmecaelurini*, confirmed by absolute differences in the structure of male genitalia (Krivokhatsky, 2011).

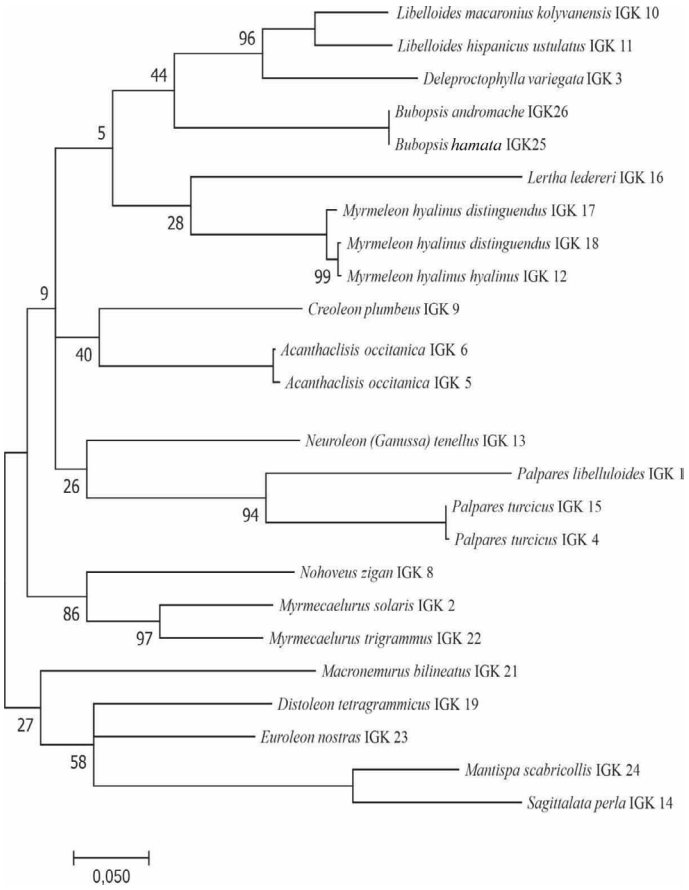

Figure 3: ML tree based on COI barcodes.

As can be seen from the ML tree, the sequences of *Bubopsis hamata* Klug, 1834 (IGK25) and *B. andromache* (Aspöck *et al.*, 1979) (IGK26) are identical (Figure 3). The same result occurs in NJ (Figure 4). A genetic approach forces us to synonymize these two names: *Ascalaphus hamatus* Klug, 1834 = *B. andromache*. syn. n. However, two nomenclature types are corresponding to the presence of discrete subspecific ranks of the species. We propose that sympatric (Aspöck *et al.*, 2001) morphological forms of sole East-Mediterranean-Iran-Arabian species actually

coexist in nature and belong to infrasubspecific morphs. Thus, *B. hamata* (Klug, 1834) includes *B. hamata* morpha *typica*, and *B. hamata* morpha *andromache*, that coexist geographically and are not divided genetically with the COI barcode method.

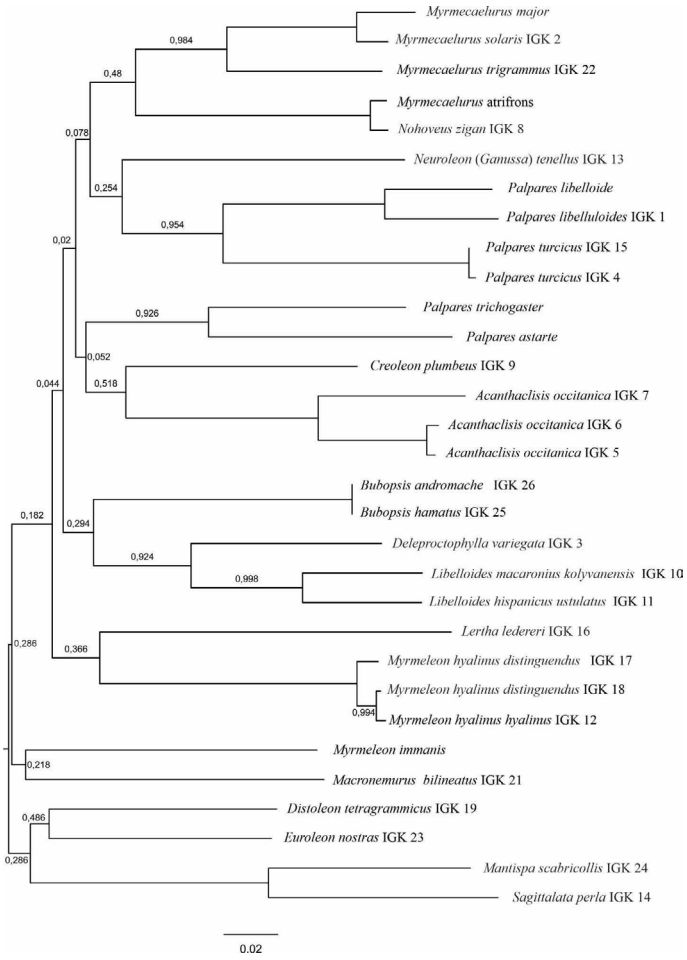

Figure 4: Neighbor-joining tree with GenBank data and result of molecular species delimitation based on COI barcodes.

A noticeable convergence of the compact cluster of the genus *Bubopsis* Mac Lachlan, 1898 with the owlfly *Deleproctophylla variegata* (Klug, 1845), which, together belongs to the subfamily Ascalaphinae Lefèbvre, 1842, indicates a characteristic point of embranchment in the genus *Libelloides* Schäffer, 1763 inside Libelloidini Pantaleoni, (Pantaleoni and Loru, 2018). Thus, Libelloidini is a daughter tribe within Ascalaphinae.

We should note that although the support between the clades of *L. macaronius kolyvanensis – L. hispanicus ustulatus – D. variegata* and *B. hamata – B. andromache* is not so high (44), it organizes the traditional owlflies of the Ascalaphidae family into one cluster, opposed to the cluster that unites all the studied antlions (Myrmeleontidae). Thus, the proposal to merge the Myrmeleontidae and Ascalaphidae (Machado *et al.*, 2018) is not supported by our data. Although the research material is not sufficient for a final conclusion.

The position of the only species of the family Nemopteridae, *Lertha ledereri*, was recovered within the Myrmeleontidae, which on both dendrograms (forms a single, poorly supported cluster with the genus) joined the cluster of the genus *Myrmeleon*. This is definitely an artifact since a well-supported separate position of the cluster of the superfamily Nemopteroidae from the Myrmeleontoidae on the phylogenetic tree involving 3 species of spoontails with 100% support is reliable (Michel *et al.*, 2017).

To the next artifact in the second dendrogram, we assign the sequence *Acanthaclisis occitanica* IGK 7 from Ordubad, which is far apart from two closely related sequences (IGK6 and IGK5) from different areas (Figures 3, 4).

Our study provided the first DNA barcode library of Neuroptera from Azerbaijan, including 25 species. The present dataset will be the first step toward the DNA barcoding of Azerbaijan Neuroptera.

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Conflict of interest

The authors have declared no conflict of interest.

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