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MOLECULAR TOXONOMY OF CAELIFERA, ORTHOPTERA FROM MOROCCO

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ABSTRACT

The taxonomic analysis of Orthoptera relies on phenotypic, genotypic, and biological traits. However, outdated data hinders the accuracy of morphological identification. Creation of a genomic database focusing on the controversial Caelifera species that cause difficulty in identification is required. This study identified six species from the Moroccan Middle Atlas using both morphological and molecular traits. Analysis of two subspecies of the species *viz., Dociostaurus jagoi occidentalis* from the high altitudes of the Middle Atlas has led to suspension of countermeasures against these.

Key words: Caelifera, classification, conservation, environment, molecular identification, middle atlas, insects, Orthoptera, phylogeny, taxonomy

The North African Orthoptera fauna (Chopard 1943), although old, remains a valuable reference for the determination of species. Several genera have been revised and the classification has undergone rearrangements and new species have been described (Louveaux et al., 1987) from Morocco and more specifically in the Middle Atlas Morocco. So far, studies on these have primarily focused on the bioecology, census, and population dynamics (El Ghadraoui et al., 2010; El Ghadraoui et al., 2003; Errabhi et al., 2017; Essakhi et al., 2015). Status of certain species is far from clear and a greater diversity is expected, particularly among morphologically close genera. The use of molecular tools for identifying Orthoptera is becoming the most reliable method. However, a major limitation is the lack of reference sequences for many groups, highlighting the need for regional genomic databases. Despite constraints like difficulty detecting hybrids and pseudo-gene issues (Moussi et al., 2018), molecular tools remain the only objective way to address morphological ambiguities and authentically identifying species. The present study explores the systematic classification of orthopterans in the Middle Atlas of Morocco through molecular methods. Focus is on the molecular identification of species considered doubtful in five stations of the Middle Atlas, namely Mezdou (Sefrou region), Tijma (Guigou region), Ajaabou (El-Hajeb-Ifrane region), Serghina (Boulmane region), and Ain Kehla (Timhddit region).

MATERIALS AND METHODS

The studied region is divided into five stations in the Middle Atlas of Morocco, characterized by mosaic vegetation, different coverage rate, and especially a well-marked altitudinal gradient. These include the following: Imhdit site, Aine Kehlla- South-33°, 85’72.7" N, -5°,53’50.6"W; Guigou site, Tjma- South-33°, 47’57.5"N, -4°,86’65.7"W; El Hajeb-Ifrane site, Ajaabou- South-east, 33°, 58’89.6"N -5°, 26’13.9"W; Sefrou site- Mezdou North 33°.47’59.4"N -4°.86’75.9"W; Boulmane site, Serghina South 33°, 34’11.0"N -4°, 49’10.8"W. These are based in the west of the basin of Sebou, the climate is wet with significant rainfall on the Causse Moyen-Atlasique from Ifrane to Timhdit. However, and under the effect of ecological barriers such as Tichikout and Ait khbach, Sefrou, Guigou, and Boulmane are under the semi-arid influence with low annual rainfall (Qadem, 2018). A random sampling technique was used with collection

carried out, at the beginning of the morning, using an entomological net at places where the vegetation is low with >70% coverage, or by hand when there are no factors agitating the individuals. The captured individuals were stored in tubes to the laboratory for observations under microscope.

Verification of identities was done using molecular taxonomy. Specimens were preserved after identification, in absolute ethanol and stored at 4°C. Total DNA extraction was performed using the Bioneer AccuPrep® Genomic DNA Extraction Kit to obtain high-quality DNA from insect legs. This method involves the homogenization of insect legs and the addition of a lysis buffer to release the DNA. The lysate is then mixed with a binding buffer and loaded onto a silica membrane spin column, where the DNA binds to the membrane while impurities are removed by washing with appropriate buffers. Finally, the DNA is eluted in a small volume of elution buffer. The resulting DNA is of high quality and purity, suitable for use in various downstream molecular biology applications. Primers were designed to amplify the entire length of the COI gene, The PCR reaction mixture was prepared by combining 5U of hot-start DNA polymerase, 10 µl of 10× HotStar buffer premixed with MgCl2 and dNTPs, 1ul of forward and reverse primers (10 um), and 2ul of template DNA in double distilled water to make a final volume of 25 µl. The PCR cycle involved an initial denaturation at 92°C for 2 min, followed by 10 cycles of denaturation at 92°C for 20 sec, reannealing at 52°C for 30 sec, and elongation at 60°C for 180 sec. This was followed by 30 cycles using the same steps but with an increase of 20 sec/ cycle to the elongation step's duration. The PCR products were purified and sequenced immediately from the two DNA strands. The PCR products were then sequenced, edited and aligned using BioEdit software (version 7.0.5.3), then they were checked to find out their degree of similarity to GenBank (https://blast.ncbi.nlm.nih.gov) followed by using Bioedit software. These sequences were then analyzed using the nucleotide BLAST program and ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) and the resulting sequences were submitted to the GenBank database. Sequences were used to generate a phylogenetic tree using the MEGA 11 software (Stecher at al., 2020) Maximum likelihood analysis was used to estimate phylogenetic relationships and inferred trees were evaluated by 1000 bootstrap replicates (Zahri et al., 2021).

RESULTS AND DISCUSSION

Of all the collected species, seven species from the family Acrididae caused confusions in identification. These include- I_2: *Ramburiella hispanica* I_4: *Dociostaurus jagoi jagoi*; I_5: *Dociostaurus jagoi occidentalis*; I_7: *Calliptamus wattenwylianus*; I_8: *Oedipoda fuscocincta* and I_{10}: *Aiolopus thalassinus tamulus*. GenBank accession numbers being: OP390818 (for *Ramburiella hispanica*), OP391707 (for *Dociostaurus jagoi jagoi*), OP391725 (*Dociostaurus jagoi occidentalis*), OP412414 (for *Calliptamus wattenwylianus*), OP391786 (*Oedipoda fuscocincta*), and OP392008 (*Aiolopus thalassinus tamulus*) (Fig. 1). The phylogenetic tree allowed characterized six major phylogenetic classes. The two subspecies I_4: *D. jagoi jagoi* and I_5: *D. jagoi occidentalis*, are distributed on the two branches of the first clade with extremely high bootstrap values, up to 96%. This result is in perfect agreement with their remarkably close relationship, which is consistent with traditional taxonomic results (Pomares and Fernández 2018). Based on classic taxonomy, the I_2 clade: *R. hispanica* should separate from the I_4 clade: *D. jagoi jagoi* and I_5: *D. jagoi occidentalis*, but this is a parallel evolution with the latter. This may be related to the elongated body's unique origin and these species' narrow vertex. This observation has allowed us to classify these two species as full-fledged species, despite their close relationship, but they are all part of the Gomphocerinae Fieber, 1853 (Krauss and Burmeister, 2010). Prior to this study, the species *Dociostaurus jagoi* whose presence in Morocco in general and in the Middle Atlas in particular has been controversial for a long time.

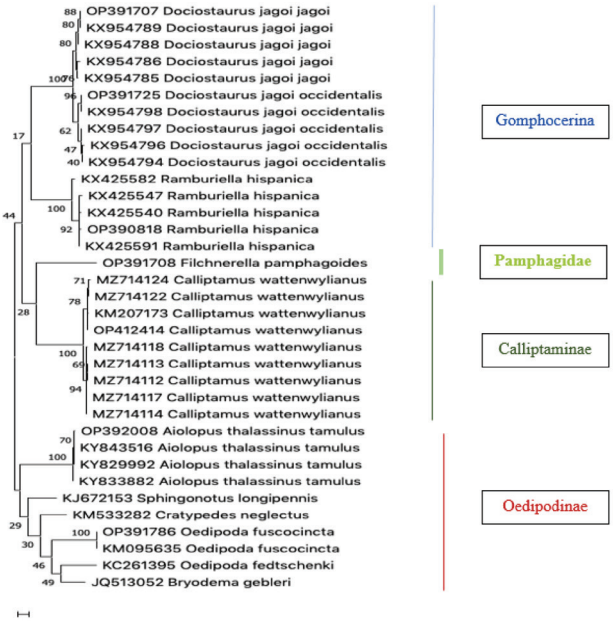

Fig. 1. Phylogenetic tree, DNA nucleotide sequences (using the maximum likelihood method)

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AUTHOR CONTRIBUTION STATEMENT

AZ and LEG conceived and designed research. AZ and NR conducted experiments. NR contributed new reagents and/or analytical tools. HN, AL and AZ analyzed data. AZ and NR wrote the manuscript. All authors read and approved the manuscript.

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CONFLICT OF INTEREST

No conflict of interest.

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