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The first DNA barcoding records of three *Evergestis* Hübner, [1825] species in Turkey with molecular evaluations (Lepidoptera: Crambidae, Glaphyriinae)

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Abstract

Turkish populations of *Evergestis nomadalis* Lederer, 1870, *Evergestis boursini* Amsel, 1939, and *Evergestis pazukii* Alipanah, 2018 were barcoded and presented detailed morphologies for the first time herein. Species delimination of the *Evergestis* Hübner, [1825] populations were evaluated based on the mitochondrial sitochrom oxidase I subunit gene. In the consensus tree, which was constructed using the neighbor joining, Bayesian inference, and maximum likelihood algorithms, the molecular relationships of genera/tribus in the subfamily Glaphyriinae were shown with some evaluations.
KEY WORDS: Lepidoptera, Crambidae, Glaphyriinae, *Evergestis nomadalis*, *Evergestis boursini*, *Evergestis pazukii*, barcoding, Turkey.

El primer registro del AND código de barras de tres especies de *Evergestis* Hübner, [1825] en Turquía con evaluación molecular (Lepidoptera: Crambidae, Glaphyriinae)

Resumen

Las poblaciones turcas de *Evergestis nomadalis* Lederer, 1870, *Evergestis boursini* Amsel, 1939 y *Evergestis pazukii* Alipanah, 2018 fueron etiquetadas con el código de barras y presentada su morfología por primera vez aquí. La delimitación de las poblaciones de las especies de *Evergestis* Hübner, [1825] fueron consensuadas basándose sobre el gen subunidad I de la citocromo c oxidasa. El árbol fue construido con algunas evidencias, usando la asociación de proximidad, la inferencia bayesiana y algoritmos de probabilidad máximos, así como las relaciones moleculares de los géneros / tribus en la subfamilia Glaphyriinae.
PALABRAS CLAVE: Lepidoptera, Crambidae, Glaphyriinae, *Evergestis nomadalis*, *Evergestis boursini*, *Evergestis pazukii*, código de barras, Turquía.

Introduction

The subfamily Glaphyriinae is now represented by its new combination of 51 genera and over 300 species (REGIER *et al*., 2012; ALIPANAH *et al*., 2018). Since the Evergestinae (ten genos) and Noordinae (a genus) species are paraphyletic with Glaphyriinae species through molecular phylogeny, these subfamilies are synonyms for Glaphyriinae. Recently, although molecular phylogeny studies between family/subfamily and even lower taxonomic groups have been generally compatible with the morphological systematics, new taxonomic status recommendations have been rapidly increasing for Lepidoptera (BAUM, 1992; HALL, 2003; ADUSE-POKU *et al*., 2009; SILVA-BRANDÃO *et al*.,

2009; BRABY, 2010; BRABY & EASTWOOD, 2019). A main problem has been that some of the genus-containing species have been shown to be more closely related to species containing other genera rather than their congeners (REGIER *et al*., 2009; REGIER *et al*., 2012). It was stated that this mismatch was caused by the lack of a rich molecular dataset containing different species in different geographies and also because the morphological features used in species identification do not reflect enough synapomorphic characters (HALL, 2003; YOUNG, 2006; ADUSE-POKU *et al*., 2009; HAUSMANN *et al*., 2009; BRABY, 2010; KAWAHARA *et al*., 2017; BRABY & EASTWOOD, 2019; KEMAL *et al*., 2019; KIZILDAĞ *et al*., 2019). Therefore, it is highly preferred to test morphologically-identified species with their molecular barcodes in order to represent phylogeny at upper taxonomic levels for their correct systematic studies (SOLIS & MITTER, 1992; ÖUNAP *et al*., 2008; ÖUNAP & VIIDALEPP, 2009; ÖUNAP *et al*., 2011; ÖUNAP *et al*., 2016; MURILLO-RAMOS *et al*., 2019). Traditionally, taxa with correctly identified genera delimination have to be phylogenetically monophyletic groups in the same subfamily (KRISTENSEN *et al*., 2007).

In this study, relationships between genera in Glaphyriina were investigated using the new taxonomic status advice. The phylogeny estimation of *Evergestis* Hübner, [1825] was presented for the first time herein, with the molecular barcoding of three *Evergestis* species from Turkey.

Methods

The photograps of morphological and genital preparation, materials for this study, the *Evergestis pazukii* specimen (Van-Turkey/Lep-Pyr003), *Evergestis boursini* specimen (Van-Turkey/Lep-Pyr008) and *Evergestis nomadalis* specimen (Van-Turkey/Lep-Pyr024) were obtained from the Centre for Entomological Studies Ankara (Cesa) Collection.

The legs from each sample were washed thoroughly with ethanol and dried. Genomic DNA extraction and polymerase chain reaction (PCR) amplification of the mtCOI gene were performed used the RED Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, Missouri, USA) according to the method of KEMAL *et al*. (2018). The PCR products were purified before being bidirectional sequenced with the universal primers (LepF1/R1) by Macrogen (Macrogen, Amsterdam, Netherlands).

In the present study of the 3 new mtCOI gene sequences, the sequences of another 237 closely-related species/populations were also downloaded from GenBank (https://www.ncbi.nlm.nih.gov/) and the Boldsystem database (http://www.boldsystems.org/index.php/), and used for the analyses. Some species the subfamily Odontiinae were chosen as the outgroup. Multiple sequence alignment was performed using the ClustalW algorithm in MEGA 7.0 software (Pennsylvania State University, Pennsylvania, USA). A total of 243 taxa were used for the phylogeny estimation of the genus *Evergestis*.

Genetic distances based on a 658-bp sequence of the COI subunit gene were calculated using the Kimura 2-parameter distance model (KIMURA, 1980). The neighbor-joining (NJ) tree was constructed used the Kimura 2-Parameter distance model in MEGA 7.0 software. Maximum-likelihood (ML) bootstrapping analyses were achieved with 1000 replicates using RA×ML Blackbox on XSEDE v.8.2.4 (STAMATAKIS *et al*., 2008) on the CIPRES Science Gateway. A Bayesian inference (BI) analysis was performed in MrBayes 3.2.6 (RONQUIST & HUELSENBECK, 2003) with the Markov chain Monte Carlo algorithm. The program JModeltest v.2.1.7 (POSADA, 2008) selected the JC+G evolutionary model as the best model according to the akaike information criterion for Bayesian inference. The program was run for 10,000,000 generations, with a sample frequency of 100 and a burn-in of 25,000.

Results

Description of the Turkish populations

Evergestis nomadalis Lederer, 1870

Material examined: TR - Van Pr. Gürpınar, Ba¸et Mt. 2800 m, 7-VIII-2018, M. Kemal & A. Koçak leg. (Cesa).

Upperside of wings: Forewing ground colouration uniformly yellowish brown. Post-discal band broad. Post discal dark spots well marked at inner side of band. Ciliae uniformly developed, not chequered, darker basally. Hind-wing ground colouration pure white. Submarginal band poorly developed, very light brown. Ciliae uniformly developed, slightly darker basally otherwise whitish (Fig. 1.1a).

Male genitalia (Cesa pre-no/GP3183): Uncus long, broader than *boursini*, slightly narrower than *pazukii*. Gnathos broader, pointed at tip, with a row of spinules dorsally. At base, gnathos protruds shoulder-like (Fig. 1.2a-3a). Dorsal and ventral margins of valva more or less parallel, rounded apically (Fig. 1.2a). Distal end of aedeagus with a broad plate covered by minute spicules. Inside, a row of long teeth well developed (Fig. 1.2a-4a).

Evergestis boursini Amsel, 1939

Material examined: TR-Van Pr. Çatak, Saklıvadi 1-IX-2016, M. Kemal & A. Koçak leg. (Cesa).

Upperside of wings: Forewing bi-coloured light brown and greyish-brown. Veins whitish, especially at outer margin well-developed. Discal and post-discal whitish lines parallel and well marked. Spindle-shaped discal marking distinct, bordered by dark brown scales. Ciliae chequered by white and brown. Hind-wing ground colouration dirty cream. Submarginal band unsharp, at anal region indistinct. Marginal line dark brown. Ciliae weakly chequered by dark brown, at anal region almostt whitish (Fig. 1.1b).

Male genitalia (Cesa pre-no/GP2555): Uncus long, slender. Remarkably narrower than those of *nomadalis* and *pazukii*. Gnathos broader at base, shorter, with a row of spinules dorsally (Fig. 1.2b-3b). Dorsal and ventral margins of valva not parallel, more pointed apically (Fig. 1.2b). Distal end of aedeagus with a plate covered by minute spicules. Inside, three minute teeth rows well distinguished (Fig. 1.2b-4b).

Evergestis pazukii Alipanah, 2018

Material examined: TR–Van Pr. Başkale, Ziyanis 9 7 2015, M. Kemal & A. Koçak leg. (Cesa)

Upperside of wings: Forewing general colouration dirty grey-brown. Markings rather weak. Post-discal area paler. Marginal line slightly undulated, dark brown. Ciliae chequered. Hind-wing dirty creamy, suffused with dark brown scales, submarginal area dark brown with partly developedcreamy marginal lunules. Ciliae chequered especially at apical region (Fig. 1.1c).

Male genitalia (Cesa pre-no/GP2537): Uncus long, straight, well chitinized. Comparing with nomadalis and boursini, broader than those of others. Gnathos shorter, slender, with a row of spinules dorsally (Fig. 1.2c-3c). Dorsal and ventral margins of valva almost parallel, roundish apically (Fig. 1.2c). Distal end of aedeagus with a broad plate covered by minute spicules. Inside, two parallel spindle-shaped minute teeth rows well marked (Fig. 1.2c-4c).

MTCOI GENE-BASED MOLECULAR EVALUATION OF *EVERGESTIS* SPECIES

The newly characterized mtCOI DNA gene sequences were deposited in GenBank with GC contents and accession numbers as follows: *Evergestis pazukii* 29.78%, MN259518; *Evergestis boursini* 30.09%, MN259521; *Evergestis nomadalis* 29.93%, MN259519.

Subfamily Glaphyriinae contains 51 genera, according to the new combination and only the COI barcode (658 bp) belonging to the species/populations of 24 genera, which are available in the Boldsystem/Genbank. With the new data presented, phylogeny estimates were therefore performed with the existing barcode records. The topologies of the three trees were quite compatible with each other. Therefore, three support values were shown in the NJ tree. In the presented phylogenetic tree, the populations of some species were narrowed; respectively, *E. pallidata* (Hufnagel, 1767) 32 populations, *E. limbata* (Linnaeus, 1767) 11 populations, *E. caesialis* (Herrich-Schäffer, 1849) 10 populations, *E. unimacula* (Grote & Robinson, 1867) 7 populations, *E. aenealis* ([Denis & Schiffermüller], 1775) 10 populations, *E. extimalis* (Scopoli, 1763) 10 populations, *E. simulatilis* (Grote, 1880) 4 populations, *E.*

dumerlei Leraut, 2003 5 populations, *E. sophialis* (Fabricius, 1787) 8 populations, *E. rimosalis* Guenée, 1854 13 populations, *E. forficalis* (Linnaeus, 1758) 26 populations, *E. subterminalis* Barnes & McDunnough, 1914 17 populations, and *E. isatidalis* (Duponchel, [1833]) 3 populations.

In the presented phylogenetic tree, the inner group was divided into two main clades (A1 and A2 nodes with red points). The A1 node lineage was divided into two subclades (B1 and B2 nodes with blue points). The B1 clade included all *Evergestis* species and species of *Prorasea* Grote, 1878, *Crocidolomia* Zeller, 1952, *Orenaia* Duponchel, [1845], *Cylindrifrons succandidalis* (Hulst, 1886), *Dichogoma prognealis* (Druce, 1895), and *Trischistognatha* Warren, 1892. The B2 clade included all *Glaphyria* Hübner, [1823] species and species of *Nephrogramma* Munroe, 1964, *Scybalistodes* Munroe, 1964, *Lipocosma* Lederer, 1863, *Schacontia themis* Solis & Goldstein, 2013, *Lipocosmodes fuliginosalis (*Fernald, 1888), *Dicymolomia* Zeller, 1872, *Stegea salutalis* (Hulst, 1886), *Chalcoela* Zeller, 1872, *Abegesta* Munroe, 1964, *Xanthophysa psychialis* (Hulst, 1886), and *Aethiophysa* Munroe, 1964. The A2 node lineage contained *Noorda blitealis* (Walker, 1859), *Alatuncusia bergii* (Möschler, 1890), *Dichogama* Lederer, 1863, and *Hellula* Guenée, 1854 (Figure 2).

The Turkish population of *E. pazukii* was closest to the *E. dumerlei* Leraut, 2003 clade, with high support values (91/1.00/92), and the two species had a sister position to *E. lupalis* Zerny, 1928, *E. frumentalis* (Linnaeus, 1761), and *E. sophialis* (Fabricius, 1787). The sister groups clustered *E. aridalis* Barnes & McDunnough, 1914 and *E. extimalis* (Scopoli, 1763) in the basal position. The species *Prorasea* Grote, 1878 was the closest to the *E. simulatilis* (Grote, 1880) group and both were also basal to the *E. pazukii* clade. The species *Crocidolomia* Zeller, 1852 was also the closest to *E. boursini* (Turkish population), following the basal *E. nomadalis* (Turkish population) and *Orenaia* Duponchel, 1845 species, which had a sister position to this clade and low support values. In the presented phylogenetic tree, the genetic distances between the species presented in the same clade with the other species of the genus are shown in the K2P model (Table 1).

Table 1.– K2P-genetic distances between the species in same clade with species of the presented *Evergestis*.

ID	Taxa	Accession Numbers	K2P-genetic distance %		
–	*Evergestis pazukii*	MN259518			
–	*Evergestis boursini*	MN259521	10.70		
–	*Evergestis nomadalis*	MN259519	7.90	9.20	
PHLAB721-10	*Evergestis dumerlei*	HQ968734	5.20	10.50	9.40
PHLAH487-12	*Evergestis lupalis*	–	7.60	10.00	8.30
LEFIL642-10	*Evergestis frumentalis*	KX041562	7.00	8.10	6.90
LEFIG593-10	*Evergestis sophialis*	HM876264	7.10	8.30	8.30
ODOPE530-11	*Evergestis extimalis*	KX045338	7.40	10.90	8.50
LBCG203-80	*Evergestis simulatilis*	–	6.50	7.00	6.50

Discussion and conclusions

In this study, it was determined that three populations evaluated morphologically were typical *nomadalis* / *boursini* / *pazukii* members. For the first time detailed genital characteristics of the species are presented. Today, with the understanding of the importance of genetic barcoding, a significant number of European insects have been barcoding, and this process is rapidly ongoing (HAUSMANN *et al*., 2013; HENDRICH *et al*., 2014; KUMAR *et al*., 2019). The genus *Evergestis* Hübner, [1825], which is mostly distributed in the Holarctic region, is globally known as having 79 species. Today, only a quarter of the species have been barcoded and almost all of the records have been obtained from USA and Canada. In this study, new barcode records, obtained from different geographies and different species, were presented for the first time.

The species of *Evergestis* were spread out with the species of the other genera in the clade represented by the B1 node. *E. pazukii* had the closest relationship with *E. dumerlei* Leraut, 2003.

When *E. pazukii* was described by ALIPANAH *et al*. (2018), it was suspected to be a morphologically similar species to *E. russulatalis* (Hampson, 1900). The identity of this species remains controversial, as no molecular data has been obtained. Therefore, no molecular comparison was made between them.

The *E. boursini* Amsel, 1939 and *E. nomadalis* Lederer, 1870 populations were closer to the *Crocidolomia* Zeller, 1852 and *Orenaia* Duponchel, 1845 than to the other *Evergestis* species. The genus *Orenaia* is known to be closely related to *Evergestis*, like *Cornifrons* Lederer, 1858, but the distinction of the genera has been based on strong sinapomorphic characters at the morphological level. AMSEL (1939) reported that *E. boursini* was close to *E. serratalis* Staudinger, 1870 (forewing pattern), and it was reported to be more similar to *E. spiniferalis* (Staudinger, 1900) (valve shape and size) (ALIPANAH *et al*., 2018). However, since neither species has molecular data, the genetic distance and phylogeny between them could not be estimated. Equivalent data should be presented for both evaluations in order to evaluate the morphological systematic and molecular taxonomy of *Evergestis* together. However, due to the lack of molecular data of this genus, the relationships between both intragenus and within intergenera remains unsolved. Hence, it seems difficult to test whether conventional taxonomy reflects phylogeny in light of molecular data.

In the presented phylogeny tree, the *Orenaia* and *Crocidolomia* species were monophyletic, but the phylogenetic relationships of the genera were unsolved because of their low support values. The COI barcode length of *Cornifrons* was less than 658 bp, and thus was not evaluated in this study.

The new combinations of the subfamily Glaphyriinae have been reported in several studies, and three synonymic (Dichogominae, Evergestinae, and Noordinae) subfamily members were evaluated in subtaxonomic categories. According to the results of the present study, two tribus were recommended in Glaphyriinae, where the A1 node represents Glaphyriini and the A2 node represents Noordiini. Glaphyriini was divided into two2 subtribus, where the B1 node represents Evergestiina and the B2 node represents Glaphyriina. The genus *Dichogama* was not monopyletic like *Evergestis*. It was aimed to analyze the phylogeny of this subfamily via a cladistic analysis and different gene sequences (SOLIS & MITTER, 2008; REGIER *et al*., 2012). However, one of the biggest problems was the lack of morphological and molecular data of the different species from different geographies. Although the distribution areas of these three species have been reported in this study, there are no molecular records for any of them (GOATER, 2005; KOÇAK & KEMAL, 2014; KEMAL & KOÇAK, 2017; ALIPANAH *et al*., 2018). Moreover, there are no current molecular data for any member of *Upiga* Capps, 1964, *Paregesta* Munroe, 1964, *Plumegesta* Munroe, 1972, *Ennomosia* Amsel, 1956, or *Cornifrons* (length less 658 bp). In this study, three new barcode records and generic/tribus-level phylogeny estimations are presented. The phylogenetic analyses reveal that the genus *Evergestis* is not monophyletic. The new molecular data of the three species obtained in the presented study provides the chance to understand the evolutionary relationships of *Evergestis*. Further studies will require a large number of barcoding to determine the limits of the genera and tribes of Glaphyriinae.

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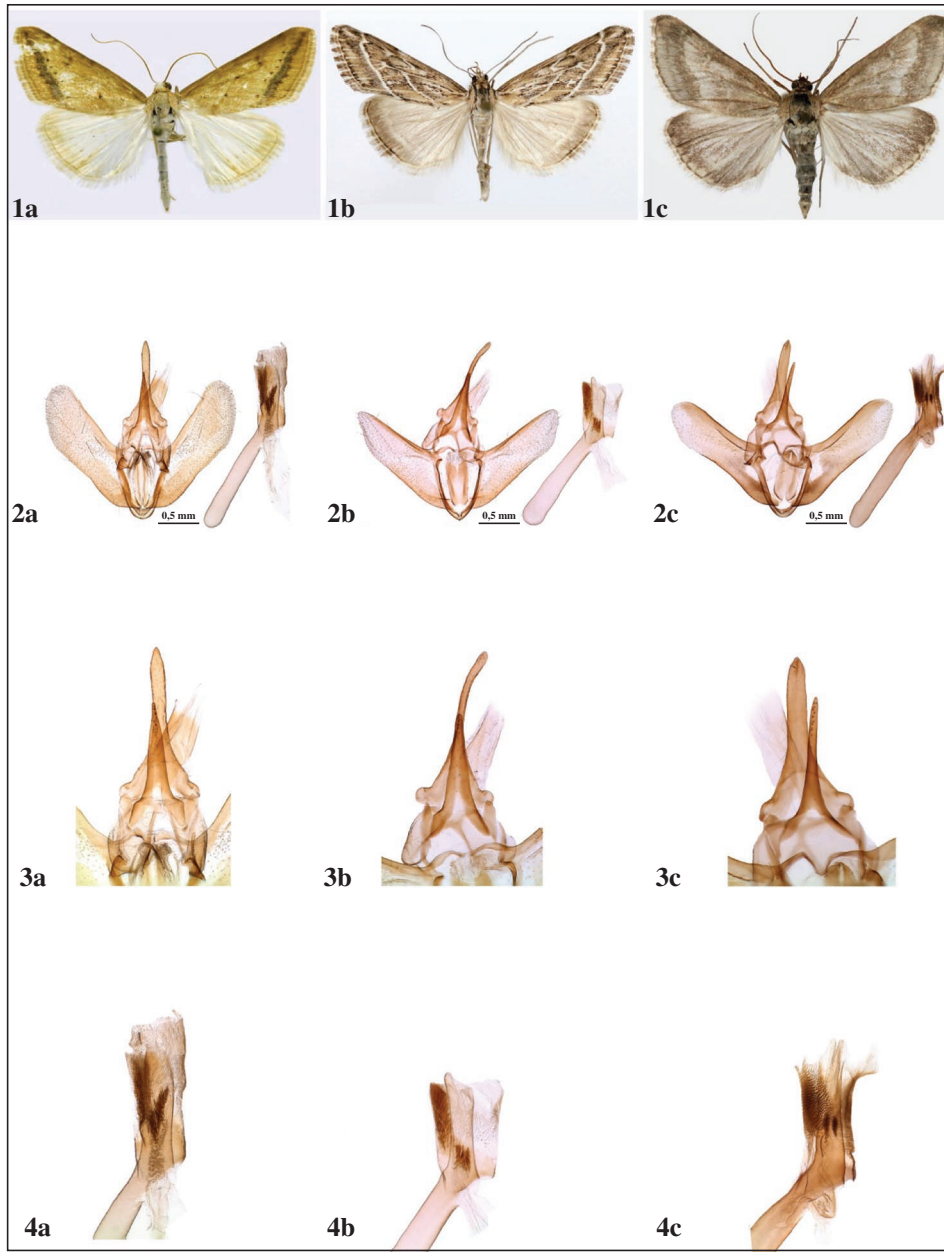

Fig. 1.– Diagnostic characters of three *Evergestis* species based upon the wing markings and male genitalia. Line 1: Upperside of the males of *Evergestis nomadalis* (**a**), *Evergestis boursini* (**b**), *Evergestis pazukii* (**c**). Line 2: total view of the male genitalia. Line 3: ventral view of uncus and gnathos (enlarged). Line 4: distal end of aedeagus (enlarged).

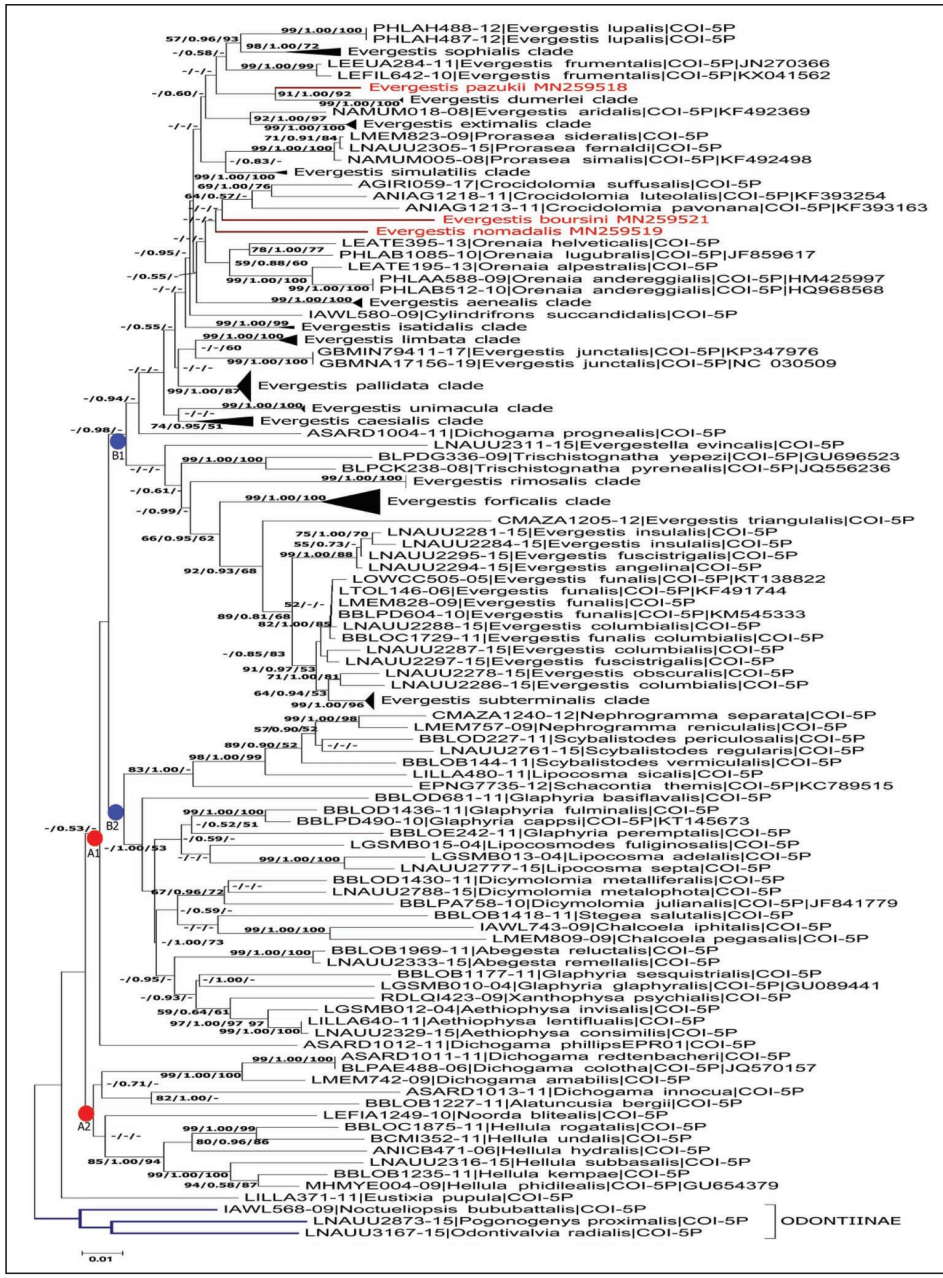

Fig. 2.– The phlogenetic tree of Glaphyriinae populations constucted with NJ, BI and ML algorhitims. Numbers at the nodes indicate the NJ bootstrap values, the BI posterior probability and the ML bootstrap values. A dash indicates a value less than 0.50 or 50%. Bar, 1 substitutions per 100 nucleotide positions.