

***Ooencyrtus mirus* (Hymenoptera, Encyrtidae), discovered in Europe parasitizing eggs of *Halyomorpha halys* (Hemiptera, Pentatomidae)**

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Abstract

Ooencyrtus mirus Triapitsyn & Power (Hymenoptera, Encyrtidae) is recorded for the first time in Europe. It was found parasitising eggs of the invasive true bug *Halyomorpha halys* Stål (Hemiptera, Pentatomidae). This parasitoid is part of the *Ooencyrtus telenomicida* species complex where accurate species identification requires molecular data. Using morphology, the identification of the *Ooencyrtus* species parasitising brown marmorated stink bug eggs in Greece is ambiguous, but the sequences of the standard DNA barcode region (*COI*) and *ITS2* place them in *O. mirus*.

Keywords

brown marmorated stink bug, DNA barcoding, egg mass, new record, parasitoid

Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Hemiptera, Pentatomidae), is native to eastern Asia but it was accidentally introduced into the United States in 1996 in Pennsylvania, and several years later in Europe, in 2004 in Switzerland (Hoebeke and Carter 2003; Leskey et al. 2012; Haye et al. 2014; Xu et al. 2014). It was primarily considered as an urban and household pest (Wermelinger et al. 2008; Inkley 2012), however,

H. halys is highly polyphagous, and causes severe damage to a wide range of economically important plants (Lee et al. 2013; Leskey and Nielsen 2018; Andreadis et al. 2022).

In the native range of *H. halys* (north-eastern Asia), substantial control is provided by numerous natural enemies including parasitoids, predators, and entomopathogens (Lee et al. 2013). In China, the egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae) has been identified as the most specialized and efficient agent for classical biological control of *H. halys* populations, while *Trissolcus mitsukurii* (Ashmead) (Hymenoptera, Scelionidae) is the main parasitoid in Japan (Yang et al. 2009; Qiu 2010; Lee et al. 2013; Zhang et al. 2017). Adventive populations of *T. japonicus* and *T. mitsukurii* that followed their host have been reported worldwide (Talamas et al. 2015; Sabbatini Peverieri et al. 2018; Stahl et al. 2019a; Abram et al. 2019; Bout et al. 2021; Dieckhoff et al. 2021; Rot et al. 2021). In the newly invaded areas, mainly the eggs and more rarely the nymphs and adults of *H. halys* are attacked by various predators and a complex of egg parasitoids belonging to the genera *Trissolcus* Ashmead, *Telenomus* Haliday, *Gryon* Haliday (Hymenoptera, Scelionidae), *Anastatus* Motschulsky (Hymenoptera, Eupelmidae) and *Ooencyrtus* Ashmead (Hymenoptera, Encyrtidae) (Abram et al. 2017; Biddinger et al. 2017; Morrison et al. 2017; Costi et al. 2019; Tillman et al. 2020). However, parasitism caused by native egg parasitoids is generally not high enough to effectively reduce the population density of *H. halys* (Dieckhoff et al. 2017; Costi et al. 2019; Stahl et al. 2019b). In Greece, *H. halys* eggs were found to be parasitised by *Anastatus bifasciatus* (Geoffroy) (Hymenoptera, Eupelmidae) and by the species identified here as *Ooencyrtus mirus* Triapitsyn & Power (Hymenoptera, Encyrtidae) (Andreadis et al. 2021, as *O. telenomicida*). The parasitism rate under natural conditions was assessed in Andreadis et al. (2021), while in this study we discuss its identity.

Methods

Host colony maintenance

To obtain large numbers of natural host eggs, captive *H. halys* were reared on green bean (*Phaseolus vulgaris*) pods and green bean plants in a mesh cage (30 × 30 × 30 cm) with vinyl window and zip closure (Raising Butterflies, UT, USA) and maintained at 26 °C, 60% RH under a 16:8 h light dark photoperiod. This colony was initiated in 2019 from mixed-sex adults collected from homes and fields in the area of central Macedonia, Northern Greece. Egg masses were removed carefully every other day, placed in Petri dishes (60 mm diameter), and labelled by date to monitor the age of host eggs. Moistened cottonwool was placed in the dishes to increase humidity.

Parasitoid colony

Ooencyrtus mirus (Chalcidoidea: Encyrtidae) adults were obtained in the summers of 2020 and 2021 from parasitized *H. halys* eggs collected from the underside of apricot

tree leaves and of green bean plant leaves, respectively, in Themi, Thessaloniki, Northern Greece (40°32'17"N, 23°00'04"E and 40°32'12"N, 23°00'01"E, respectively). A colony of *O. mirus* was established for multiple generations at the Institute of Plant Breeding and Genetic Resources, Laboratory of Entomology at 26 °C, 60% RH and under a 16:8 h L:D photoperiod. The colony reported here is not exactly the same as in Andreadis et al. (2021) because it additionally has the specimens from the 2021 collecting event. Parasitoids were reared in the laboratory using fresh *H. halys* egg masses that were placed in the lid of a plastic Petri dish (3.5 cm in diameter) at the bottom of 460 ml round clear plastic cups (9 cm diameter × 7 cm high). Cups were closed with a plastic lid fitted with a 250-mesh net for air circulation. Pure honey drops *ad libitum* and a cotton wick saturated in honey water were placed on the bottom of a small plastic Petri dish (3.5 cm in diameter). Moistened cottonwool was placed inside the plastic cups to increase humidity. Two egg masses were left in the parasitoid rearing cups for 24 h, after which they were removed and transferred to new cups. Adult wasps that emerged from these cups were returned to the parasitoid rearing cups.

Sex ratio (proportion of males) of *O. mirus* was measured on 1- and 3-days old eggs of *H. halys*. Freshly emerged male and female parasitoids from the colony were placed together in single pairs for 48 h to ensure mating. After this period, each female was offered 10 *H. halys* eggs, on the lid of a plastic Petri dish (3.5 cm in diameter) inside the plastic cups. After 24 h, the parent parasitoids were removed and the parasitized eggs were reared at 26 °C, 60% RH under a photoperiod of 16:8 h L:D until adult emergence for a total period of 30 days, to ensure all adults are accounted for. Emergent wasps were counted and identified by sex. Only replicates in which parasitoid attack by *O. mirus* occurred on at least one egg were included in the data analysis.

Morphological characterisation

For this study specimens were sampled at two different moments from the laboratory colony, in February 2021 and January 2022. Live parasitoids were killed in 80% ethanol and kept at –20 °C until preparation. For examination, specimens were chemically dried using hexamethyldisilazane (HMDS) (Heraty and Hawks 1998), mounted on rectangular cards or point mounted on black points for photography. Images were taken as described in Fusu et al. (2018). The use of diffuse light is paramount not only for imaging but also for the correct interpretation of colour and sculpture when comparing and identifying specimens (Gibson and Fusu 2016). Imaged specimens, except for the DNA vouchers, were labelled with a unique number “Fusu PHOTO 2023-NN” to ensure their future recognition.

Identification of the specimens was done by comparing them with specimens from the same rearing event as the neotype of *O. telenomicida* (Vassiliev) (see Triapitsyn et al. 2020), specimens of *O. telenomicida* from Italy from the same locality (Tuscany) and host but not the same rearing event as in Roversi et al. (2018), and specimens from the same lab grown colony that served for the description of *O. mirus* (Triapitsyn et al. 2020). All the above specimens, the DNA barcoded specimens, and specimens

in ethanol are deposited in the Lucian Fusu Collection at the “Alexandru Ioan Cuza” University, Iasi, Romania. Further specimens of *O. mirus* from Greece are stored in ethanol at the Institute of Plant Breeding and Genetic Resources, Thermi, Greece. We additionally used for identification the keys published by Ferrière and Voegelé (1961), Triapitzin (1989), Huang and Noyes (1994), Hayat and Mehrnejad (2016) and Samra et al. (2018).

Gastral tergites are abbreviated as Gt1 to Gt3.

Molecular methods

DNA was individually extracted from four whole specimens using a non-destructive method as described in Cruaud et al. (2019). After extraction the exoskeleton was mounted as described above. Two molecular markers (*COI* and *ITS2*), used before for the delimitation of *Ooencyrtus* species (Samra et al. 2018; Triapitsyn et al. 2020, 2021) were amplified by PCR. For *COI* we used the standard primer pair for the animal DNA barcode, LCO1490/HCO2198 (Folmer et al. 1994) while the *ITS2* region was amplified with the primers ITS2-F and ITS2-R2 (Yara 2006). Standard 25 µl PCRs were performed as described in Triapitsyn et al. (2020), the sequences were assembled as in Fusu and Polaszek (2017) and deposited on GenBank under the accession numbers OQ870212–OQ870215 (*COI*) and OQ877248–OQ877251 (*ITS2*).

To our sequences we added those of *Ooencyrtus mevalbelus* Guerrieri & Samra, *Ooencyrtus pistaciae* Hayat & Mehrnejad, and *Ooencyrtus zoeae* Guerrieri & Samra (from Samra et al. 2018). For *O. telenomicida* we included sequences from the type locality, from the East Mediterranean populations (*sensu* Triapitsyn et al. 2020), and from the Italian populations (from Roversi et al. 2018). Sequences of *Ooencyrtus pityocampae* (Mercet) from Samra et al. (2015, 2018) were used as outgroup. The *COI* sequences were trivial to align while the *ITS2* sequences were aligned using MAFFT v.7.475 with the E-INS-i algorithm (Kato and Standley 2013).

Separate phylogenetic analyses were performed for the two genes because the goal was to check congruence between mitochondrial and nuclear gene trees. The *ITS2* sequences were analysed in an unpartitioned analysis while the *COI* sequences were partitioned by codon position. The best partitioning scheme and substitution models were selected during the phylogenetic reconstruction using IQTree v.1.6.12 (Nguyen et al. 2015; Kalyaanamoorthy et al. 2017); support was assessed based on 1,000 bootstrap pseudoreplicates. Phylogenetic analyses were also conducted in MrBayes V.3.2.7 (Ronquist et al. 2012) as described in Dascălu et al. (2022). Distances were calculated in MEGA X v.10.0.5 (Kumar et al. 2018) using the *p* distance and the Kimura 2 parameters model to facilitate comparison with Triapitsyn et al. (2020) and Samra et al. (2018).

The nuclear and mitochondrial sequences were not concatenated for a partitioned phylogenetic analysis because most of the published sequences for the two genes cannot be correlated. This analysis was performed by Triapitsyn et al. (2020) by concatenating one randomly selected exemplar sequence for each gene and species.

Results

Sex ratio

In the investigated Greek population of *O. mirus*, a strongly female-biased eclosion sex ratio (proportion of males 0.39 ± 0.05) is observed (Independent Student's t-test, $p = 0.004$).

Morphology

Using Ferrière and Voegelé (1961) and Trijapitzin (1989) the specimens from Greece are identified as *O. telenomicida*. However, in the more recent key of Huang and Noyes (1994) they will not run smoothly to this species because of conflicting character states in the first couplet: hind coxa is brown (though not exactly concolorous with mesoscutum it is not yellow either) in combination with a basally orange gaster (Fig. 1C, E). If the colour of the hind coxa is disregarded, it will run to *O. telenomicida* but again the colour of the scutellum will not fit because it is not bright metallic green.

In Hayat and Mehrnejad (2016) they will run to *O. telenomicida*, but again the hind coxa is not yellow, F1 is not shorter than F2, while F2 to F5 are not more than 2× as long as broad (they are less than 2× as long as broad and F1 is of the same length as F2). In the key provided by Samra et al. (2018) they will not run to *O. telenomicida*, again because of the dark hind coxa.

Following the designation of a neotype for *O. telenomicida* by Triapitsyn et al. (2020) the hind coxa is brown in this species in all specimens from the type locality situated in East Romania and in most specimens from Ukraine and European Russia (Triapitsyn et al. 2020). A dark hind coxa would hence agree with the specimens from Greece. However, when comparing them (Table 1), they differ in the colour of the head and mesosoma which in topotypical *O. telenomicida* have a bluish-green luster (Triapitsyn et al. 2020, figs 10A, 14), while these are almost completely black in the Greek specimens (Fig. 1). On the other hand, the dark and mostly non-metallic colour of the head and mesosomal dorsum is characteristic for the Mediterranean *O. telenomicida* and *O. mirus*.

The extent of the yellow coloration on the base of the gaster varies in the Greek *Ooencyrtus*, from only the basal half of Gt1 to Gt1–Gt3 being yellow (basal half of the gaster up to the cercal plates). The least yellow specimens are thus similar in this respect to typical *O. telenomicida*, while the more yellow ones to *O. mirus* and the Mediterranean *O. telenomicida* (Table 1). Also, in the Greek population the anterior surface of the mid coxa can be variably extensively brown.

One must take into consideration when comparing *Ooencyrtus* specimens that the yellow colour of the legs and base of the gaster is influenced by the treatment received by the specimen. Air dried specimens that have the gaster strongly collapsed will exhibit a darker base of the gaster compared to uncollapsed specimens in alcohol or specimens dried with hexamethyldisilazane. Specimens that had their DNA extracted through lysis, will have the basal tergites of the gaster and the legs of a pale brown colour instead

Table 1. Colour characteristics of *O. telenomicida* and *O. mirus*.

Body part	<i>O. telenomicida</i> (type locality)	<i>O. telenomicida</i> (Mediterranean)	<i>O. mirus</i> (type locality)	<i>O. mirus</i> (Greece)
Frontovortex	Dark with comparatively strong greenish-blue lustre	Black with weak bronze-green reflections	Black with weak bronze-green reflections	Black with weak bronze-green reflections
Mesoscutum	Dark with comparatively strong greenish-blue lustre	Black with bronze-green reflections	Black with weak bronze-green reflections	Black with weak bronze-green reflections
Scutellum	Dark with comparatively strong greenish-blue lustre apically	Black with weak bronze-green reflections apically	Black with weak bronze-green reflections apically	Black with weak bronze-green reflections apically
Base of gaster	From only base of Gt1 yellow to Gt1 and Gt2 yellow	Gt1–Gt3 yellow	Gt1–Gt3 yellow	From only base of Gt1 yellow to Gt1–Gt3 completely yellow
Hind coxa	Brown	Yellow	Yellow	Brown

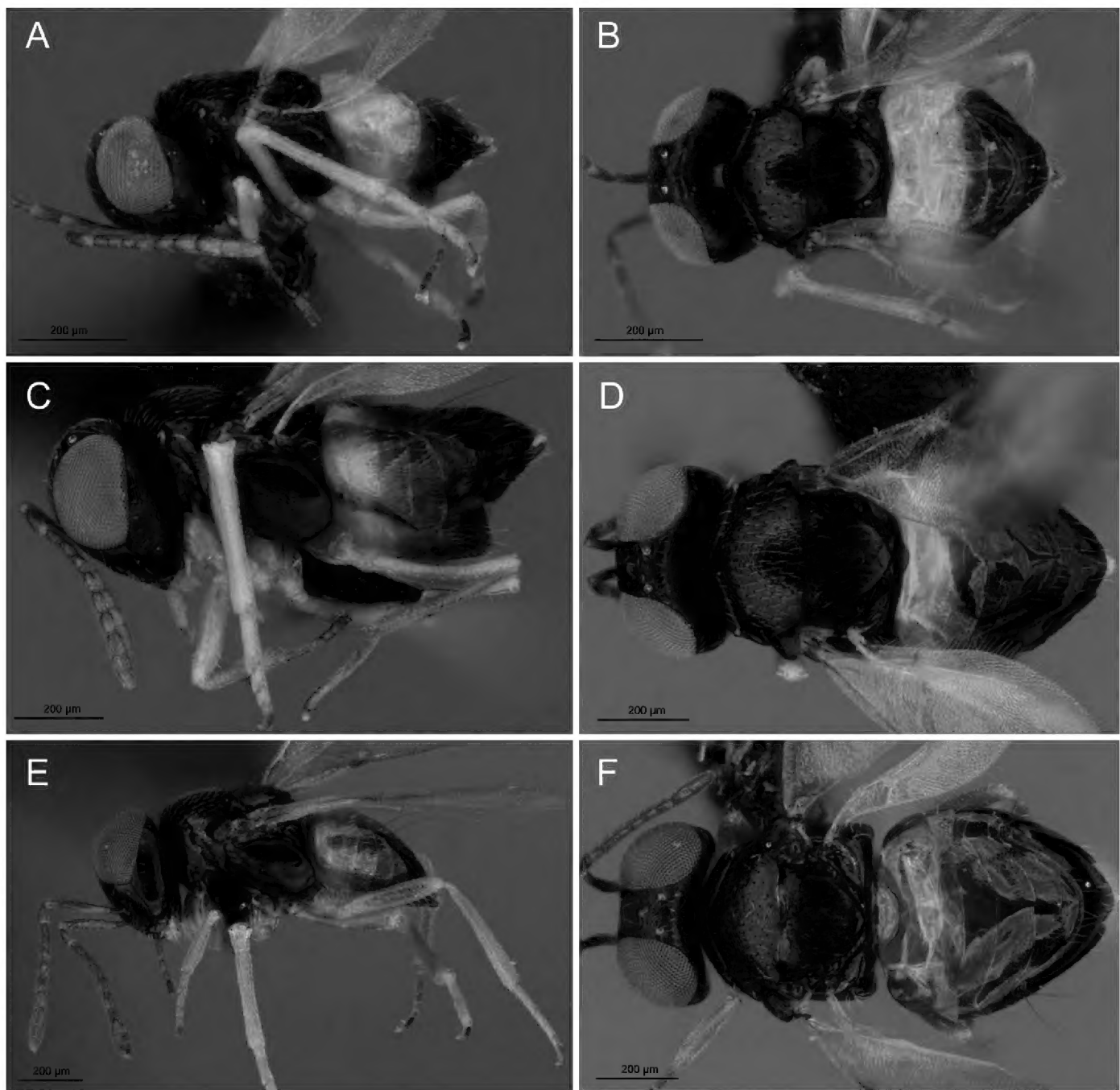


Figure 1. *Ooencyrtus mirus*, lateral and dorsal habitus **A, B** *O. mirus* from Pakistan, specimen 2023-02 **C, D** *O. mirus* from Greece, specimen 2023-03 **E** *O. mirus* from Greece, specimen OoGr04 **F** *O. mirus* from Greece, specimen OoGr03.

of a saturated yellow colour (cf. Fig. 1C with Fig. 1E). This is presumably because the yellow colour is in part generated by the internal tissues seen through the thin cuticle; removing the tissues during DNA extraction changes the colour. The metallic colour of the dark body parts is not affected by the DNA extraction process, unless the specimens are examined in transmitted light instead of, or combined with, reflected light.

Molecular markers

The divergence calculated using either the p-distance or the K2P distance was very similar, hence we discuss further only the p-distance. On both molecular markers the specimens from Greece are most similar to *O. mirus*. On the *COI* sequences the divergence between the Pakistani (type locality) and Greek *Ooencyrtus* is 1.3%, while on *ITS2* it is 0.7%. The closest species to *O. mirus* in terms of genetic divergence is *O. pistaciae* with 6.2% on *COI* and 4.5% on *ITS2* when compared to the sequences of *O. mirus* of Pakistani origin. On both ML phylogenetic trees (Figs 2, 3) the specimens from Greece and Pakistan are grouped together with 100% bootstrap. The species is

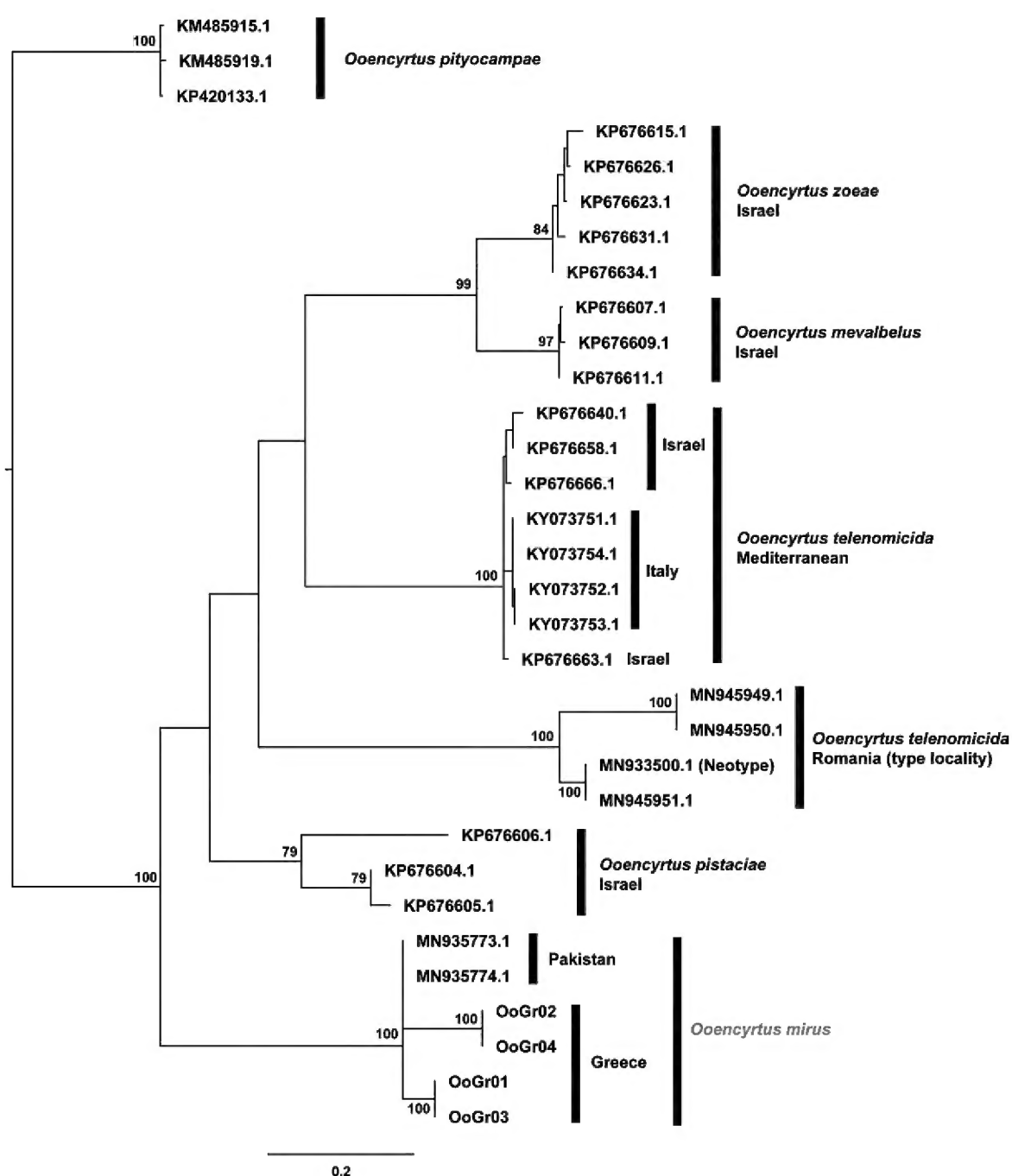


Figure 2. ML tree of the *Ooencyrtus telenomicida* species complex inferred from the analysis of *COI* sequences.

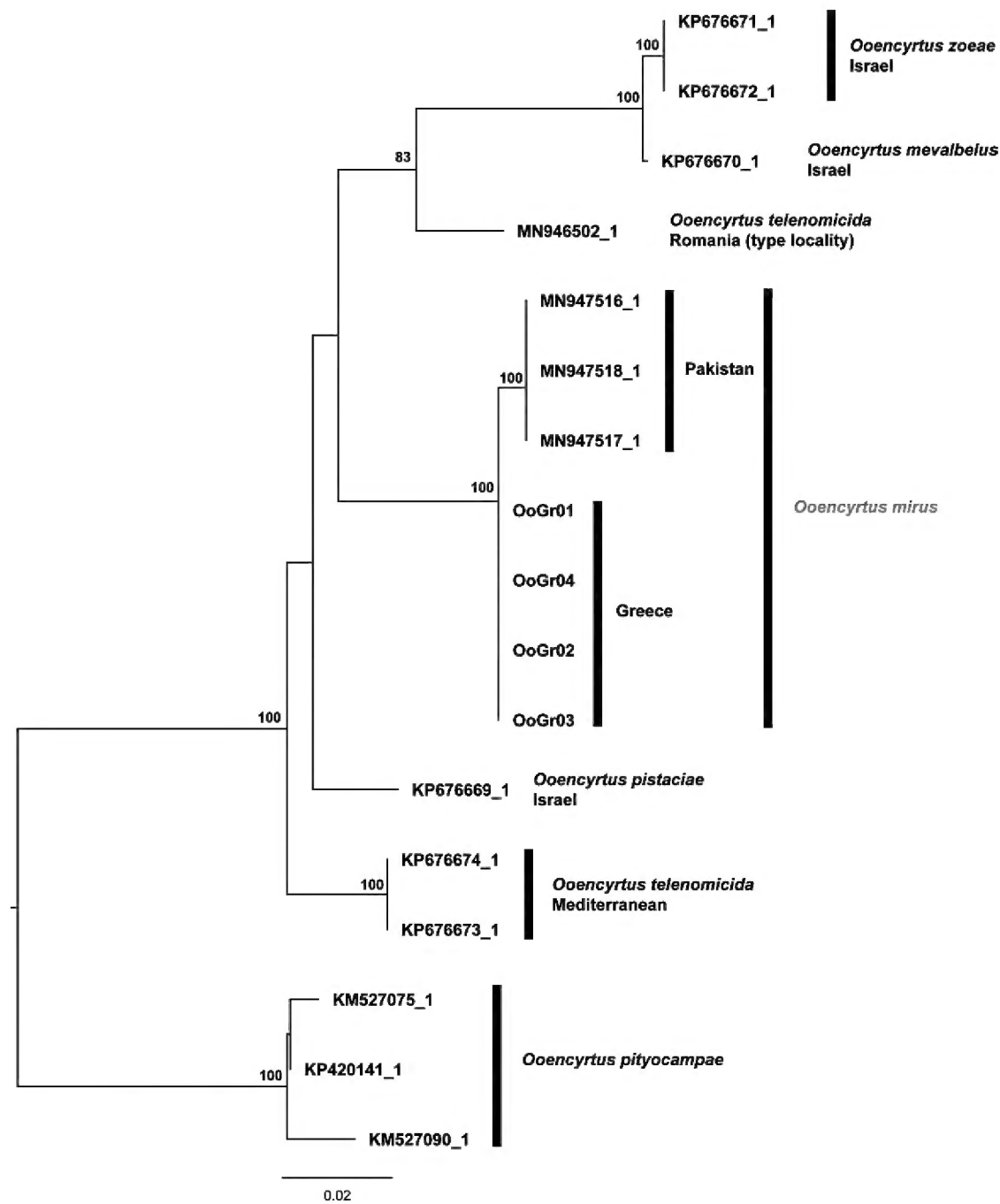


Figure 3. ML tree of the *Ooencyrtus telenomicida* species complex inferred from the analysis of *ITS2* sequences.

basal to all of the other ingroup species on *COI* but sister to a group formed by *O. telenomicida*, *O. zoeae* and *O. mevalbelus* on *ITS2*, though in both cases with low support. The two trees are concordant in the sense that the included species are distinct both on the nuclear and the mitochondrial marker. The trees obtained in MrBayes were similar to those obtained in IQTree (data not presented).

Two distinct *COI* haplotypes were identified for *O. mirus* in Greece. Because mitochondria are maternally transmitted in most animals, it means that the colony was founded by a minimum of two females, perhaps one from the 2021 and the other from the 2022 collecting events.

Discussion

Ooencyrtus mirus is part of the *O. telenomicida* species group as first proposed by Trjapitzin (1967) and then defined by Hayat et al. (2014). These species are characterised by

an antenna with elongated antennomeres, all funiculars being longer than broad and the clava about as long as the three preceding funiculars combined, the mandible with one to two teeth and a broad truncation, and the gaster with at least the first tergite yellow to brown, lighter than the following tergites. Hayat et al. (2014) additionally characterised the group as having all coxae and most of the femora dark brown, however, in *O. telenomicida* (Mediterranean populations) all legs including coxae are yellow (Samra et al. 2018), while in *O. telenomicida* from the type locality the legs are similarly light coloured, only the hind coxa being darkened (Triapitsyn et al. 2020). *Ooencyrtus mirus* likewise has all legs completely yellow including coxae (Pakistan population) (Triapitsyn et al. 2020) or the hind coxa is partly brown (Greek population).

The group includes *O. telenomicida* (the first described species), *Ooencyrtus acastus* described by Trjapitzin from the Russian Far East as part of the group (Trjapitzin 1967) and *Ooencyrtus gonoceri* described by Viggiani from Italy (Viggiani 1971), both stated as being very close to *O. telenomicida* by Huang and Noyes (1994); *Ooencyrtus brunneipes* described by Noyes (1978) from England as close to *O. telenomicida*; *Ooencyrtus nigerrimus* and *Ooencyrtus fecundus* described by Ferrière and Voegelé (1961) from Morocco and included in the group by Trjapitzin (1967); *O. exallus* described from Zimbabwe by Prinsloo (1987) and *Ooencyrtus seronis* Hayat from Saudi Arabia, included in the first definition of the group by Hayat et al. (2014); *O. zoeae* and *O. mevalbelus* described from Israel as very close to *O. telenomicida* (Samra et al. 2018); and finally *O. mirus* described from specimens reared in quarantine in USA and originating in Pakistan (Triapitsyn et al. 2020).

Ooencyrtus acastes Trjapitzin, though originally included in the group by Trjapitzin (1967) was not mentioned by subsequent authors, probably because it has a uniformly dark gaster. Hayat and Mehrnejad (2016) broaden the sense of the *O. telenomicida* group to also include species with a uniformly dark gaster and exclude the colour of the legs from the definition. Thus, they include more species such as *O. pistaciae* that has a uniformly dark brown gaster. Judging from the genetic closeness of this species to *O. mirus*, broadening the concept of the species group is phylogenetically justified.

Within this species group we define a subset of morphologically very close species that we name “*O. telenomicida* species complex”. These species are characterized by the sculpture of the scutellum consisting of elongate cells, cells more obviously elongate mesally and especially on sides (Ferrière and Voegelé 1961, fig. IG; Hayat and Mehrnejad 2016, fig. 38; Hunag and Noyes 1994, fig. 309) in combination with the presence of conspicuous white setae on the mesoscutum and a bare band behind parastigma, between the linea calva and basal fold (Ferrière and Voegelé 1961, fig. IIA; Hayat and Mehrnejad 2016, fig. 37; Hunag and Noyes 1994, fig. 309). In the west Palaearctic the complex contains *O. telenomicida*, *O. brunneipes*, *O. mevalbelus*, *O. zoeae* and *O. mirus*.

The species within this complex differ only in the colour of the legs, the intensity and hue of the metallic shine on head and thorax (Samra et al. 2018) and propodeum size (Noyes 1978). However, the genetic divergence between species on *COI* is very high, ranging from 5 to 9% (Samra et al. 2018; Triapitsyn et al. 2020) being above the largest intraspecific distance of 4.8% recorded in Chalcidoidea (Al Khatib et al. 2014;

Viciriuc et al. 2021). The presence of very divergent *COI* sequences is not necessarily an indication of specific status because this could have been generated by mitochondrial capture from another more distantly related species (e.g. Dascălu et al. 2022), or by geographic isolation (e.g. Fusu 2017: 536) in combination with extreme philopatry of females. It could also be an artefact generated by the preferential amplification of NUMTS in selected populations (Cruaud et al. 2017). However mitochondrial capture and NUMTS can be excluded because of the similar divergence seen on a nuclear marker (*ITS2*), while geographic isolation is an unlikely explanation in this case because the species are fully winged and presumably good dispersers. Crossing experiments by Samra et al. (2018) showed that even less divergent *Ooencyrtus* species (5% on *COI*) are reproductively isolated, while Pollmann et al. (2023) found that in *Lariophagus* Crawford (Hymenoptera, Pteromalidae) there is strong to complete reproductive isolation above 7.2%.

In the case of *O. mirus* the divergence between the specimens from Pakistan and those from Greece is very small on both genes (1.3 and 0.7% on *COI* and *ITS2*, respectively). To put this into perspective, the smallest interspecific K2P distance on *COI* in the *Ooencyrtus telenomicida* species group is 5% (between *O. mevalbelus* and *O. zoeae*) (Samra et al. 2018) while 4.6% is the *p*-distance between the most divergent sequences from the type locality of *O. telenomicida*. On *ITS2* the divergence is less clearcut interspecific or intraspecific because *O. mevalbelus* and *O. zoeae* differ by 0.4%, while the next smallest interspecific *p*-distance of 3.4% is between *O. telenomicida* (Mediterranean) and *O. pistaciae*.

When comparing *O. mirus* with *O. telenomicida*, Triapitsyn et al. (2020) used the colour of the legs and the extent of yellow colour on the base of the gaster: in *O. mirus* the legs, including coxae and at least the proximal half of the gaster are yellow while in *O. telenomicida* (in the narrow sense as defined by their neotype designation) the hind coxa is brown and the gaster has a much narrower yellow basal band, never extending to the cercal plates. According to these colour characteristics, the specimens from Greece are *O. telenomicida*, as published already by Andreadis et al. (2021). However, the colour of the head and mesosoma are very similar between the specimens of *O. mirus* irrespective of their origin (Fig. 1). The frontovertex, scutellum and mesoscutum have only a faint dark green, bronze and copper luster, visible under certain angles of light. In this, the species differs from *O. telenomicida* which has a much stronger metallic shine on the frontovertex, mesoscutum and especially the apex of scutellum. Furthermore, the metallic colour is blue-green in *O. telenomicida* instead of bronze-green (Triapitsyn et al. 2020, figs 10A, 12A, 14). The blue colour is present both in the type locality according to the neotype (Triapitsyn et al. 2020, figs 10A, 14) and in Ukraine, the original type locality (Triapitsyn et al. 2020, fig. 12A). A violet to blue scutellum is also mentioned by Ferrière and Voegelé (1961) who examined specimens from Morocco, Spain, and Turkey but also from the south of European Russia.

In conclusion, because of the high genetic similarity, we include the Greek *Ooencyrtus* in *O. mirus* even though the colour of the hind coxa disagrees and the gaster is

as yellow as in typical *O. mirus* only in few specimens. The colour differences could be due to the geographic distance and to the thelytokous parthenogenetic reproduction of the Pakistan population used to describe the species. It is infected with a strain of *Wolbachia* and thelytoky appears to be irreversible: even if males are produced after curing the infection, the females are not receptive to their courtship behaviour (Power et al. 2022). The newly discovered Greek population has both males and females.

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