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Molecular taxonomy of *Tomares* hairstreaks (Lepidoptera, Lycaenidae, Theclinae)

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Abstract

Tomares hairstreaks comprise about 10 species distributed from Europe and North Africa to Central Asia. The taxonomy of the genus is hampered by the absence of diagnostic characters by which specimens can be unambiguously assigned to species. Our investigation of morphology and DNA barcode variations within and between *Tomares* species shows that while well-defined species (*T. ballus*, *T. mauritanicus*, *T. callimachus*, *T. desinens* and *T. fedtschenkoi*) diverge, poorly characterized taxa (*T. nogelii*, *T. nesimachus*, *T. dobrogensis*, *T. romanovi* and *T. telemachus*) show very little to no differentiation in mtDNA. We reinstate *Tomares callimachus* spp. *hafis* (Kollar, 1849) as a valid subspecies (**stat. rev.**) and propose taxa *telemachus* Zhdanko, 2000 and *uighurica* Koçak, Seven & Kemal, 2000 as synonyms of *T. romanovi* and *T. nogelii nogelii* respectively (**syn. nov.**). We relegate *Polyommatus epiphania* Boisduval, 1848, recently revived as a valid subspecies of *T. callimachus*, back to synonymy under the latter, and reconsider the status of *T. nogelii dobrogensis* (Caradja, 1895) in the light of new molecular data. We use a nuclear gene (EF-1α) in addition to COI barcodes to reconstruct the phylogeny of the group.

Key Words

biogeography, butterflies, DNA, hybridization, introgression, phylogenetics

Introduction

Over the last decade, lycaenid butterflies have been a popular model group in studies of hybridization (Mallet et al. 2011; Gillespie et al. 2013; Nice et al. 2013; Sakamoto and Yago 2017), sympatric and cryptic speciation (Dincă et al. 2011; Vodă et al. 2015; Lukhtanov et al. 2015; Busby et al. 2017; Bereczki et al. 2018), population genomics (Gompert et al. 2014; Vanden Broeck et al. 2017; Chaturvedi et al. 2018), chromosome evolution (Lukhtanov and Dantchenko 2017), ecological specialization (Downey and Nice 2013; Schär et al. 2018) and conservation genetics (Sielezniew et al. 2012; Frye and Robbins 2015; Takeuchi et al. 2015; Koubínová et al. 2017; Roitman et al. 2017; Matthews et al. 2018). Part of this popularity maybe due to the fact that lycaenids have the highest rate of protein-coding sequence evolution among butterflies (Pellissier et al. 2017). Nevertheless, lycaenid taxonomy is still riddled with cases of uncertainty. Ranking is often disputed in geologically young species-complexes with limited phenotypic or genetic differentiation, or where geographical clines, hybridization, and sympatric or cryptic speciation are involved.

The ~10 species in Palaearctic hairstreak genus *Tomares* Rambur 1840 (*sensu* Weidenhoffer and Bozano 2007) present such a case. These butterflies are characterized by having 11 veins on the forewings (10, 11 or 12 in other Theclinae Swainson 1831), tailless hindwings with vestigial tornal lobe, bright red-orange patches on otherwise dark brown upperside of both wings, and tibiae with large projections at the tarsal end. These characteristics have granted them a tribe of their own (Tomarini Eliot 1973). Despite being generally rare, all *Tomares* species show individual and local variability in adult size as well

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as ground color intensity and the shade and size of the orange patches on their wings, which can sometimes be completely absent. Some *Tomares* are better characterized than others: *Tomares fedtschenkoi* is a large, phenotypically distinct species with a disjunct distribution in Central Asia (Tuzov et al. 2000; Weidenhoffer and Bozano 2007). *Tomares ballus*, a myrmecophilous species ranging from France to North Africa, and *T. mauritanicus*, a variable butterfly with an almost continuous distribution along the Atlas Mountains, are also easily distinguishable (Tennent 1996; Tolman and Lewington 1997; Tarrier and Delacre 2008). The remaining species share a common range from southeastern Europe to Jordan (Larsen 1974; Benyamini 1990) and Central Asia (Lukhtanov and Lukhtanov 1994; Toropov and Zhdanko 2009) and present several cases of poorly understood taxonomy.

Among these, the closely related *T. callimachus* and *T. desinens* are both distinguished by the absence of orange coloration within the transverse bands on the underside of the hind wings (UNH). They both fly in sympatry in Azerbaijan and Iran (Nekrutenko and Effendi 1980; Nazari 2003). Despite some geographic variability among disjunct populations, recognition of subspecies in *T. callimachus* has been discouraged (Hesselbarth et al. 1995; van Oorschot and Wagener 2000). *Tomares desinens* was described in 1980 from a series collected in the semi-arid zone of Talysh mountains in Azerbaijan, and was later found also in northern Iran (Nazari 2003) and southeastern Turkey (Kemal and Koçak 2005). Beside being the smallest species, *T. desinens* is also characterised by chequered fringes as well as complete development of UNH elements without any trace of green scales.

The eastern species *T. romanovi*, often readily identifiable by its striking bluish-green UNH and the reduction or absence of maculae, is found from southeastern Turkey to the Kopet Dagh Mountains where it is sympatric with *telemachus*, a poorly described taxon based on undulated wing margins, light grey UNH and alleged differences in female genitalia, all variable characters interchangeable with the sympatric *T. romanovi*. Specimens with reduced green scales and prominent maculae on their UNH, approaching that of *T. nogelii*, occur also in Caucasus and southeastern Turkey.

The most difficult problem however concerns the taxonomic identity of the remaining three taxa, *T. nogelii*, *T. nesimachus* and *T. dobrogensis*. The issue has been addressed extensively in the past (Larsen 1974; Hesselbarth and Schurian 1984; Hesselbarth et al. 1995; Koçak 2000; van Oorschot and Wagener 2000). In summary, lack of unique external morphological characters, the nearly identical male genitalia, presence of local and clinal variation, and co-occurrence of distinct yet similar phenotypes in sympatry and synchrony, particularly in Turkey, presents serious challenges in interpretation of species or definition of subspecies in this group. Two distinct phenotypes exist within *T. nogelii*, connected by a bewildering array of intermediates (van Oorschot and Wagener 2000; Weidenhoffer and Bozano 2007). The often smaller *T. nesimachus* is known from Anatolia to Jordan, and is considered endangered in Israel (Pe'er and Settele 2008). The often larger *dobrogensis*, presumed extinct in its type locality in Romania until recently (Dincă et al. 2009; Rákosy and Craioveanu 2015) but common in disjunct populations in Ukraine, Crimea and xerothermic localities north of the Crimean peninsula (Nekrutenko and Tshikolovets 2005), was elevated to species due to its presumed "nearly sympatric" occurrence with the smaller *T. nogelii* in Turkey (Koçak 2000), creating an odd distribution pattern that is unique among butterflies in the region (Hesselbarth et al. 1995).

The documented variation and overlap of species characters and ranges between the taxa in the *T. nogelii* complex continues to be a serious problem in their interpretation. In their comprehensive investigation, van Oorschot and Wagener (2000) found no single character that could be used to distinguish these taxa, and advocated use of various character combinations in conjunction with ecological characters (such as larval hosts) to achieve species identification. Perhaps out of desperation, Koçak (2000) suggested the rank of 'semi-species' for *nogelii*, *nesimachus* and *dobrogensis* under the 'superspecies' *T. nogelii*. The need for a genetic analysis has been expressed before (van Oorschot and Wagener 2000). We tested the usefulness of mtDNA COI barcodes in combination with ecological and morphological characters to reassess the taxonomy proposed by van Oorschot and Wagener (2000) and Weidenhoffer and Bozano (2007), and reconstructed a phylogeny for *Tomares* using an additional nuclear gene (EF-1α) in conjunction with COI barcode data.

Materials and methods

Taxon sampling

A total of 274 specimens representing all species and many subspecies of *Tomares* were sampled, of which 240 produced usable barcode sequences (Suppl. material 1: SI1). In addition, 15 public barcode records from BOLD and two GenBank sequences of *Tomares* from previous studies (KT286572, KF647240) were included in our dataset. Two other Genbank records (FN601323, KJ020235) were excluded due to suspicion of contamination. Sister-group relationships in Theclini is not yet fully resolved; however, following Espeland et al. (2018) we included Genbank COI and EF-1α sequences for one member of Theclini (*Artopoetes metamuta*, GU372569, GU372660) and one member of Arhopalini (*Semanga superba*, KT286525, KT286218) as putative outgroups. Fresh material could not be found for a few populations of *Tomares*, including the rare *T. ballus cyrenaica* known from Libya and Egypt, although our specimens from Tunisia (DNAwthTomares 025, 026 and 125) seem to be related. The voucher data are publicly available through the BOLD dataset "DS-TOMARES", accessible at https://doi.org/10.5883/DS-TOMARES.

Molecular techniques

Two dry legs from each adult specimen were detached and stored in individual vials. The extraction of total genomic DNA, amplification and sequencing were performed in the Centre for Biodiversity Genomics (Guelph, Ontario, Canada) using previously described protocols (Hajibabaei et al. 2005). Initially, full-length mtDNA barcode sequences (658 bp) were obtained for nearly all specimens, and based on results from sequence similarity (neighbour-joining) analyses and the quality of DNA, a subset was selected for additional gene sequencing. Failed samples were targeted for smaller overlapping fragments of COI (132 bp) using mini-barcode primers and protocols described previously (Meusnier et al. 2008). Elongation factor 1 alpha (EF-1α) sequences were also obtained for all 10 species using primers and protocols described previously (Brower and DeSalle 1994; Aubert et al. 1999). This nuclear marker was chosen due to its relative ease of amplification and its proven usefulness in genus- and subfamily-level phylogenetic studies in Lepidoptera (e.g. see Nazari et al. 2007; Todisco et al. 2018). Amplified DNA from all specimens was sequenced in both directions for each gene, and final sequencing products were run on an ABI 3730XL DNA analyzer (Life Technologies, Foster City, CA). Complementary strands were assembled into contigs and edited manually, and primers were removed using SEQUENCHER 4.5 (Gene Codes Corporation, Ann Arbor, MI). Sequences were aligned using CLUSTALX 2.0 (Thompson et al. 1997), evaluated by eye and converted to Nexus using SE-AL 2.0a11 (Rambault 2002). New sequences were deposited in GenBank, and accession numbers are given in Suppl. material 1: SI1. COI barcode sequences are also available publicly through the BOLD dataset "DS-TOMARES", accessible at https://doi.org/10.5883/DS-TOMARES.

Morphological characters

The widespread mtDNA haplotype sharing observed among five species (*T. nogelii*, *T. nesimachus*, *T. dobrogensis*, *T. romanovi*, *T. telemachus*) did not help in resolving the long standing problem of species identities in this complex. To remedy this, we examined morphological characters and re-evaluated the taxonomic status and geographical boundaries of the available names under this complex specifically looking for cases of sympatry and synchrony. The problem of correct identification of specimens in this group however makes past records in the literature difficult to verify.

Dissections of male and female specimens of *Tomares* were carried out by WtH. Some of the dissected specimens were also included in the molecular analysis. Male and female genitalia were prepared using standard protocols and fixed in Euparal glycerin. Male genitalia were photographed in dorsal and ventral view. In a few cases, the aedeagus was damaged proximally. Female genitalia preparations included the last two tergites, but components often had to be fixed and photographed separately in dorsal view. Photographs were taken under a standardized condition and digitally processed. Females of *T. telemachus* and *T. desinens* were not dissected due to lack of sufficient material (Suppl. material 2: SI2). To find additional diagnostic characters, male androconial patches, antennae, and fringes of upperside and underside of the wings in the *T. nogelii* species-group, as well as *T. callimachus* from various localities, were examined and photographed under microscope (Suppl. material 3: SI3).

Sequence data analysis

Neighbour-joining (NJ) trees for barcode data were constructed initially using the QUICKTREE algorithm (Howe et al. 2002) and under the Kimura two-parameter (K2P) model (Kimura 1980). Additional NJ and Maximum Parsimony (MP) analyses was conducted in PAUP* 4.0a164 (Swofford 2003); Maximum Likelihood (ML) trees were generated using PHYML online (Guindon and Gascuel 2003) under AIC criterion and 100 bootstrap replicates (Suppl. material 4: SI4). The best-fit model selected by PHYML for the combined dataset (GTR + G + I) was further corroborated by IQ-TREE (Nguyen et al. 2015), and parameters from this model were used to conduct a Bayesian analysis in MRBAYES 3.2.6 (Ronquist et al. 2011). The MCMC analysis was allowed to run for 10,000,000 generations until stationary was reached. Convergence of parameters after the exclusion of the burnin phase was tested using TRACER 1.7.1 (Rambaut et al. 2018). The haplotype diagram was constructed in TCS 1.21 (Clement et al. 2000), with a 95% confidence limit for parsimony. Shorter barcode fragments or those with ambiguous bases were excluded from haplotype analyses. Trees were edited using FIGTREE 1.4.4 (Rambault 2018).

Results

Morphology

Genitalia of both sexes in all *Tomares* species differed in size in accordance with the specimen wingspan. Female genitalia were relatively uniform, with triangular papillae anales, sclerotized ductus bursae and doctus seminalis, and round and membranous corpus bursae with no signa (Suppl. material 2: SI2). The spine on the proximal part of the valva in male genitalia showed consistent variation: it was reduced or absent in *T. mauritanicus* and *T. ballus*, small and projecting backward in *T. fedtschenkoi*, and small and projecting forward in *T. desinens* and *T. callimachus callimachus* . In the southern population of *T. callimachus*, the spine was needle-shaped and proportionally longer than the northern populations. The remaining five species (the *nogelii*-complex) showed very similar male genitalia with a distinct, forward-looking and needle-shaped spine, with

Syrian *nesimachus* having proportionally the shortest spine in this group (Fig. 1). The male androconial patch on the UPF in *Tomares* species was larger in *dobrogensis* and *nogelii* and corresponded with the specimen size, but otherwise it was not very useful in discriminating between the "difficult" taxa (Suppl. material 3: SI3). A summary of variable morphological and ecological characters in the *nogelii*-complex is presented in Table 2.

Molecules

Despite a wide geographic coverage, various populations of *T. ballus*, *T. mauritanicus* and *T. fedtschenkoi* formed well-supported clusters with small internal variation. We observed a gap in DNA barcodes (1.00 ± 0.24%), as well as EF-1α sequences, between the "northern" (Kazakhstan, Ukraine, Russia and N. Azerbaijan) and "southern" (S. Azerbaijan, Armenia, Iran and Turkey) populations of *T. callimachus*. The disjunct Kazakh population of *callimachus* showed identical mtDNA haplotypes with specimens from Ukraine and southern Russia. Further subdivisions were evident within the southern cluster (Fig. 2). Minor variation observed in the male genitalia of *T. callimachus* (e.g. in the length of spines on proximal part of valvae; not shown) appeared to be independent of geographical origin and did not correspond to the N-S split in DNA barcodes.

While average K2P distances between five *Tomares* taxa (*ballus*, *mauritanicus*, *callimachus*, *desinens* and *fedtschenkoi*) ranged between 1.6–3.0% (Table 1), the taxa *nogelii*, *nesimachus*, *dobrogensis*, *romanovi* and *telemachus* formed a large unresolved cluster with very little to no differentiation but with a high internal diversity (0.36 ± 1.38%). The haplotype network analysis in TCS identified 30 haplotypes in this group, six of which were shared between two or three species (Fig. 3). The haplotype-sharing appeared both in sympatry and allopatry, but geographically constrained, unique haplotypes were also common. All five species shared haplotypes with one another except *romanovi* and *dobrogensis*, and *telemachus* only shared haplotypes with *romanovi*. To

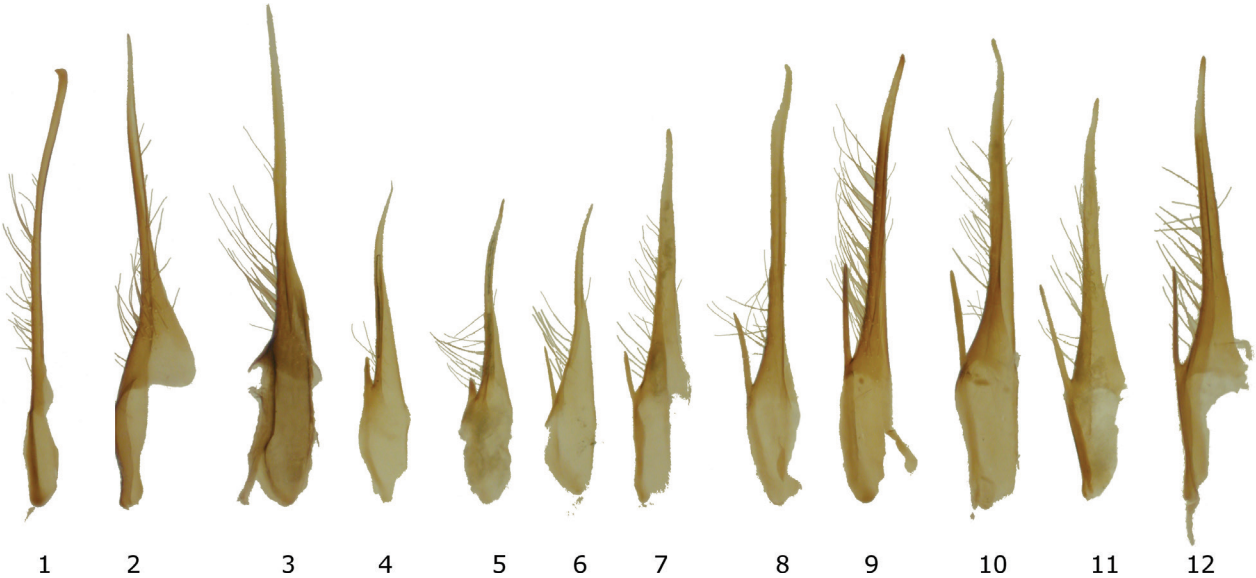

Figure 1. Right valvae in male genitalia of *Tomares* species. **1.** *T. mauritanicus* GP76 (Morocco); **2.** *T. ballus* GP77 (Morocco); **3.** *T. fedtschenkoi* GP78 (Tajikstan); **4.** *T. desinens* GP86 (Qazvin, Iran); **5.** *T. callimachus callimachus* GP75 (Crimea); **6.** *T. callimachus hafis* GP86 (Zanjan, Iran); **7.** *T. nesimachus* GP84 (Damascus, Syria); **8.** *T. "telemachus"* GP79 (Turkmenistan); **9.** *T. romanovi* GP74 (Lorestan, Iran); **10.** *T. nogelii nogelii* GP88 (Nevshehir, Turkey); **11.** *T. nogelii nogelii* GP85 (Sivas, Turkey); **12.** *T. nogelii dobrogensis* GP83 (Ukraine). All dissections and images by WtH.

Table 1. Average K2P distances and standard deviation of COI barcodes between *Tomares* taxa.

	ballus	***mauritanicus***	***callimachus***	***desinens***	***fedtschenkoi***	***nogelii***	***nesimachus***	***dobrogensis***	***romanovi***	***telemachus***
ballus	0.3 ± 0.2									
mauritanicus	1.6 ± 0.2	0.2 ± 0.2								
callimachus	3.0 ± 0.3	3.0 ± 0.2	0.6 ± 0.4							
desinens	2.5 ± 0.2	2.5 ± 0.1	2.4 ± 0.2	0.2 ± 0.2						
fedtschenkoi	2.4 ± 0.2	3.0 ± 0.2	2.5 ± 0.3	2.1 ± 0.2	0.2 ± 0.2					
nogelii	2.2 ± 0.2	2.2 ± 0.2	2.3 ± 0.2	1.6 ± 0.1	2.4 ± 0.2	0.2 ± 0.2				
nesimachus	2.2 ± 0.3	2.1 ± 0.2	2.3 ± 0.2	1.6 ± 0.2	2.3 ± 0.2	0.3 ± 0.3	0.3 ± 0.3			
dobrogensis	2.3 ± 0.3	2.2 ± 0.2	2.3 ± 0.3	1.7 ± 0.2	2.4 ± 0.3	0.4 ± 0.2	0.4 ± 0.3	0.5 ± 0.3		
romanovi	2.1 ± 0.3	2.0 ± 0.2	2.2 ± 0.2	1.5 ± 0.2	2.3 ± 0.2	0.4 ± 0.1	0.3 ± 0.2	0.5 ± 0.2	0.3 ± 0.2	
telemachus	2.3 ± 0.3	2.2 ± 0.2	2.3 ± 0.2	1.6 ± 0.1	2.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1

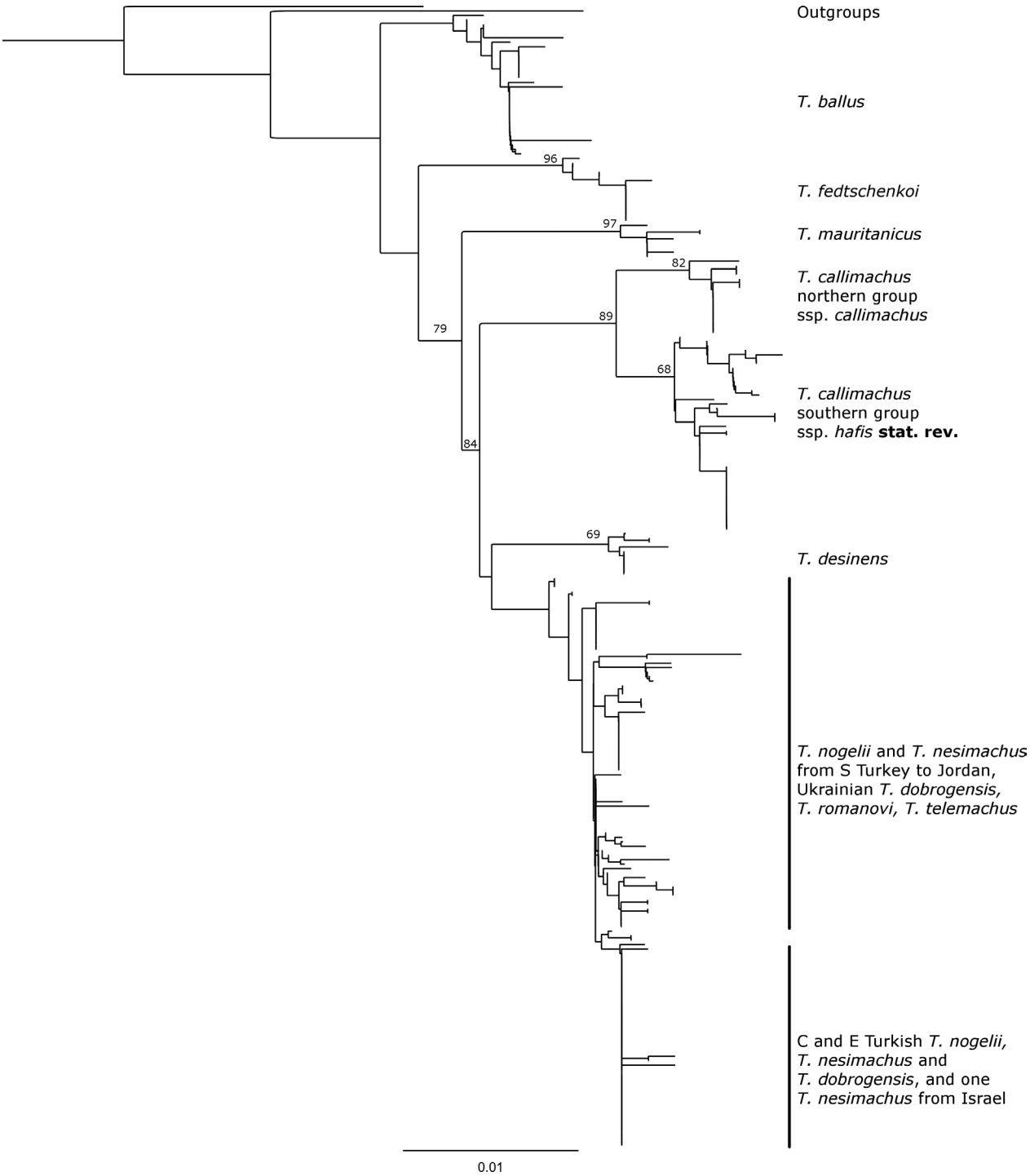

Figure 2. Neighbour-Joining tree of 271 barcode sequences of *Tomares*. Values are bootstrap of 100 replicates for supported nodes.

better understand the extent of haplotype variation within this group, we separated the records and re-evaluated the haplotype network based on geographical localities and morphological identifications. Two main haplogroups were observed, one of which consisted exclusively of *nogelii*, *nesimachus* and *dobrogensis* from central and eastern Turkey together with a single *nesimachus* specimen from Israel (Fig. 3). We found 10 sites with multiple haplotypes in southern Turkey (Konya, Niğde, Adana), Israel (Dalyya), Syria, Azerbaijan, Turkmenistan (KopetDagh) and Ukraine (Fig. 4), although records from these sites were never in synchrony.

Our phylogenetic reconstruction of combined sequence data strongly supports monophyly of *Tomares* and five species within the genus (*ballus*, *mauritanicus*, *fedtchenkoi*, *callimachus* and *desinens*). However, throughout all analyses, the taxa *nogelii*, *nesimachus*, *romanovi*, *dobrogensis* and *telemachus* formed a well-supported clade, within which they were paraphyletic with respect to each other (Fig. 5).

Table 2. Summary of characters that show variation among taxa in the *nogelii* complex.

Character	***nogelii, dobrogensis***	***nesimachus***	***romanovi, telemachus***
collection dates	25 April–30 May	5 April–31 May	15 April–31 May
elevation (m)	85–2075	250–2000	600–1300
habitat	hygric habitats	xeric rocky habitats with sparse vegetation	usually xeric rocky habitats with sparse vegetation; rarely other
zoogeographic zone	Pontomediterranean – Armenian	Syrian – Palaeoeremic	Iranian – Caspian
larval host plant (primary, secondary)	*Astragalus*, *Asteracantha*	*Astracantha*, *Astragalus*	*Astragalus*
orange patch on UPF	absent in 40% of specimens	always present	always present
dark patch at the tip of UPF	continuous along costal and outer margins	nearly triangular	continuous along costal and outer margins
submarginal black spots on UPF	connected, forming an undulated dark band	variable; usually a series of disjunct spots, sometimes connected to form a deeply serrated band	connected, forming an undulated or serrated dark band
marginal black border on UPF	always wide, equally or wider than costal border	always narrow	always wide, equally or wider than costal border
orange patch on UPH	reduced or absent in nearly 30% of specimens, if present always narrow and nearly rectangular	always present, wide, nearly rectangular basally, with both sides of the angle more or less equal in length	always present, variable in size and shape
UNH pattern (see Suppl. material 3: SI3)	usually gray-brown with prominent maculae	usually gray-brown with prominent maculae	usually uniform bluish-green with no maculae; varies in peripheral populations
needle-shape spine in male genitalia (see Fig. 1)	long	short	long

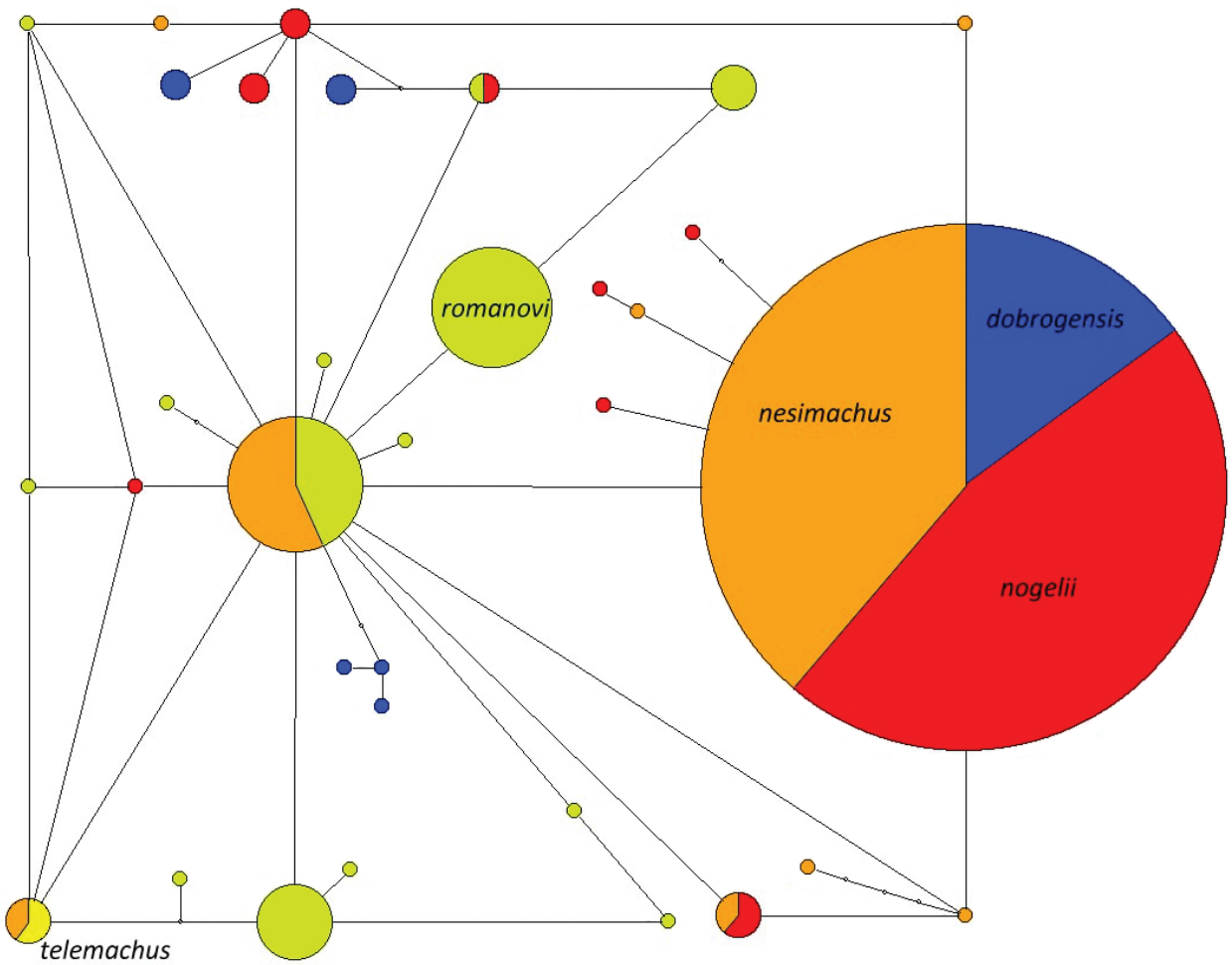

Figure 3. TCS Haplotype Network of the *nogelii* complex. Colors indicate morphological identifications (red = *nogelii*, blue = *dobrogensis*, orange = *nesimachus*, green = *romanovi*, yellow = *telemachus*). The most common haplotype (large circle) comprises central and eastern Turkish individuals of *nogelii*, '*nesimachus*' and '*dobrogensis*', as well as a single *nesimachus* from Israel.

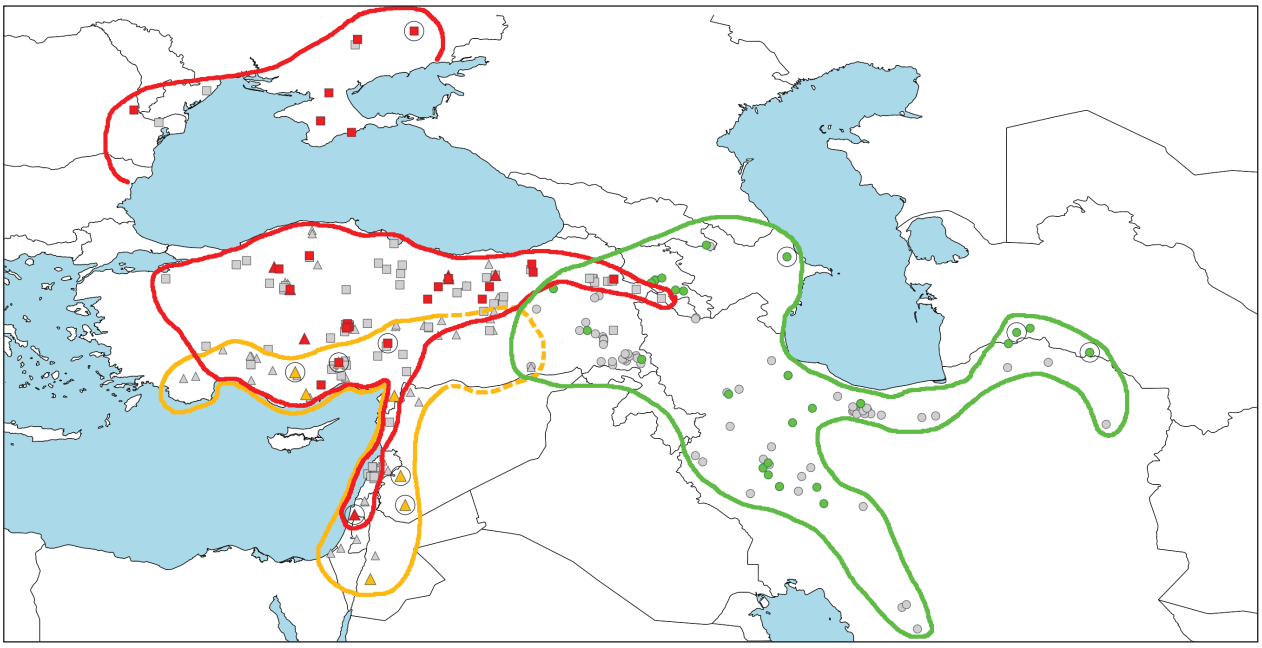

Figure 4. Distribution of taxa in the *nogelii* complex. Shapes represent morphological identifications (□ = *nogelii*, Δ = *nesimachus*, ○ = *romanovi*), colors represent COI barcode haplotypes (red = *nogelii* haplotypes, orange = *nesimachus* haplotypes, green = *romanovi* haplotypes). Sites with shared or more than one haplotypes are circled. Records in gray are concatenated from literature. Approximate taxon boundaries are inferred from represented haplotypes. For haplotype network, see Figure 3.

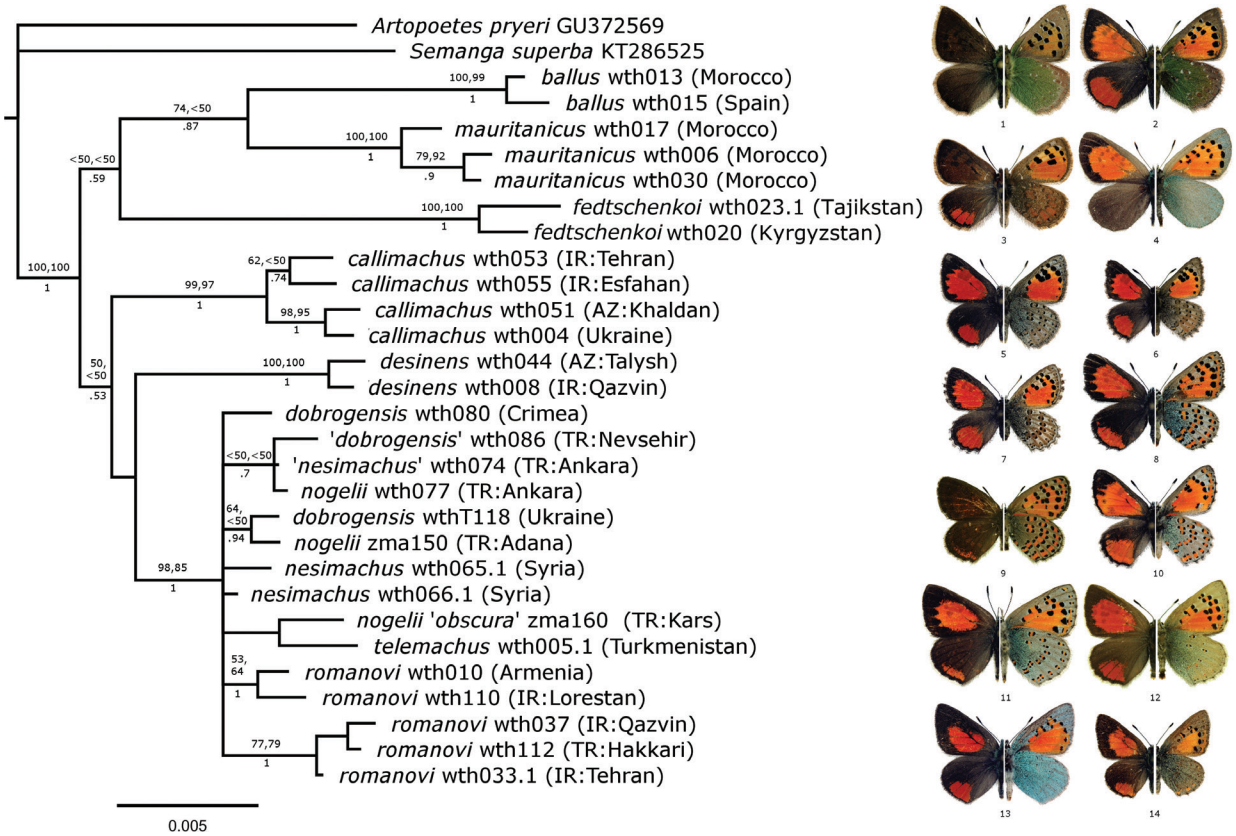

Figure 5. Bayesian phylogeny of selected *Tomares* sequences based on combined data (COI + EF-1α). Values above branches are bootstrap support obtained under Parsimony and Likelihood criteria for each node, and values below branches are Bayesian posterior probabilities. Images: 1) *ballus* wth013 Morocco, 2) *ballus* wth055 Spain, 3) *mauritanicus* wth017 Morocco, 4) *fedtchenkoi* wth020 Kyrgyzstan, 5) *callimachus callimachus* wth051 Azerbaijan, 6) *callimachus hafis* wth053 Iran, 7) *desinens* wth042 Iran, 8) *dobrogensis* wth080 Crimea, 9) *nogelii* zma153 Turkey, 10) *nesimachus* wth065 Syria, 11) *romanovi obscura* zma161 Turkey, 12) *romanovi cachetinus* zma146 Azerbaijan, 13) *romanovi romanovi* wth010 Armenia, 14) *telemachus* wth005 Turkmenistan.

Discussion

No fossils of *Tomares* are known, and the only fossil attributable to Theclinae is a geologically very young larva (Sohn et al. 2012). The most recent common ancestor (MRCA) of Tomarini and Theclini + Arhopalini seems to have split in Late Eocene around 34 million years ago, giving rise to Deudorigini and Eumaeini later in Oligocene (Espeland et al. 2018). Our phylogenitic reconstruction for the genus shows that the first split within ancestral *Tomares* occurred between the MRCA of (*ballus* + *T. mauritanicus*) + *fedtchenkoi* and the MRCA of the remaining species. The low inter-species divergence in DNA barcodes (1.6–3%) suggest that *Tomares*, much like *Agrodiaetus*, is a geologically young genus that probably arose in Pleistocene (Vila et al. 2010). Pleistocene dispersal between Africa and Europe has been suggested in a wide range of plants and animals, including butterflies (Leestmans 2005; Schmitt et al. 2006; Weingartner et al. 2006; Nazari et al. 2007, 2009; Nazari and Sperling 2008; ten Hagen and Miller 2010; Dincă et al. 2011; Vodă et al. 2016). The maculated UNH pattern in *Tomares* appears to be a plesiomorphic character substituted several times by a carpet of uniform green scales. This trait likely has some survival value: Species with green UNH (e.g. *romanovi*) feel safe and camouflaged resting on large green leaves even in bright sunshine, while species with maculated and brown UNH (e.g. *nesimachus*) normally hide by sitting on the ground with their wings closed and are easily frightened (WtH personal observation).

While morphology and DNA barcodes unequivocally demonstrate separate species status for *T. ballus*, *T. mauritanicus* and *T. fedtchenkoi*, they do not support recognition of subspecies within them. Separating populations into subspecies in the highly variable *T. mauritanicus* has been dismissed before (Tennent 1996). Lack of genetic differentiation or consistent morphological characters to discriminate between North African (e.g. ssp. *cyrenica* Turati, 1924) and European populations of *T. ballus* suggest a recent range expansion or vicariance event. For *T. desinens*, we found the subspecific diagnostic characters suggested by Weidenhoffer and Bozano (2007) inefficient as we observed character gradients and intermediate states between populations from eastern Albors Mountains to Talysh and western Iran. Therefore we do not recognize subspecies boundaries within these four species.

The split in the range of *T. callimachus*, supported by both COI and EF-1α genes, suggests a long period of lack of genetic exchange between the northern and the southern populations. The male genitalia in southern populations show a distinctly narrow and needle-shaped spine that is very different from the northern group (Fig. 1). Other subtle differences between these two groups exist: northern populations generally fly in low elevations (sea level to 1400 m), have duller UNH, fringes that are not (or are barely) chequered, and a smoothly-indented inner edge of the black marginal band on the UPF, while the southern populations fly at higher elevations (400–2600 m), show higher contrast in UNH pattern, distinctly chequered fringes, and an often deeply serrated inner edge of the UPF black marginal band. A separate taxonomic status, at least at subspecies level, is thus warranted. The type locality of *T. callimachus* is "Helenendorf" (previously Khanlar, now Goygol, Azerbaijan), a border area between the two populations and approximately 50 km from the locality of our specimen wth051, which is part of the northern group. Although it is impossible to ascertain the exact locality in the vicinity of Helenendorf where the type series were collected, the lectotype (high quality photos examined courtesy of V. Tshikolovets) shows some characteristics of the northern group (dull UNS, barely chequered fringes, and a weakly-serrated inner edge of the UPF marginal band). Zolotuhin and Anikin (2017) interpreted the illegible lectotype label as "*calmuuc*", referring to the city of Kalmukov in the Uralsk district, Kazakhstan. We reject this interpretation as the label seems to simply read "calimac[us]"; however, even if this interpretation is correct, the lectotype unambiguously belongs to the northern group. We therefore regard the northern populations as ssp. *callimachus* (Eversmann 1848), distributed from Ukraine to Central Asia and northern Azerbaijan (Greater Caucasus Mountains). We disagree with Zolotuhin and Anikin (2017) in recognizing the Georgian population as a distinct subspecies (ssp. *epiphania*, type locality: Odessa; = *callimachus* stat. rev.). This taxon, first mentioned by Boisduval (1848) in comparison to *T. ballus* and subsequently described by Herrich-Schäffer ([1850]), clearly refers to the nominal *T. callimachus*. The type material of *epiphania* is lost, and this taxon has been in synonymy with *T. callimachus* for at least 120 years (Staudinger and Rebel 1901). The oldest available name for the southern population is *hafis* Kollar, 1849, described from "Farsistan" (= Shiraz, southern Iran; type in NHMW, Vienna), and currently in synonymy with *T. callimachus* (Hesselbarth et al. 1995). The original description of *hafis* matches well with our examined material from the southern cluster. Therefore, the name *T. callimachus* ssp. *hafis* (stat. rev.) is here revived to represent the southern subspecies, distributed in Lesser Caucasus, Armenia, southern and southeastern Turkey, northeastern Iraq, and western, southwestern, northern and northeastern Iran to the Kopet Dagh range. The polyphagous larvae of ssp. *callimachus* feeds on several species of *Astragalus*, *Hedysarum* and *Onobrychis* (Weidenhoffer and Vanek 1977; Tuzov et al. 2000; Stradomsky and Fomina 2013; Bury and Savchuk 2015), but no confirmed records exist for the southern populations. If the two subspecies are later discovered in sympatry, the status of *hafis* should be revised to a distinct species. We could not examine specimens from the Pakistani Baluchistan recently described as ssp. *huertasae* (Tshikolovets and Pagès 2016); however, considering the striking morphology of this population and absence of *Tomares* in the large gap between Zagros mountains and Pakistan, this taxon may represent a distinct species.

The remaining five taxa (*nogelii*, *nesimachus*, *dobrogensis*, *romanovi* and *telemachus*) form a clade of closely-related haplotypes with no apparent distinction between taxa. The concordance between mitochondrial COI and nuclear EF-1α genes rules out selective sweeps caused by endosymbiotic bacteria (Toews and Brelsford 2012). *Tomares romanovi* has been generally excluded from this complex or only referred to for its curious similarities with *nogelii* in genitalia and pattern on the underside of the forewing (UNF). Indeed, *romanovi* is often easily distinguishable by its uniform bluish-green UNH and complete lack of maculae; however, peripheral populations within the range of *romanovi* (e.g. those from the Kopet Dagh range, Georgia, Azerbaijan and southeastern Turkey) often demonstrate a reduction or absence of these bluish-green scales and presence of maculae on the UNH, approaching some forms of *nogelii*. The range of *romanovi* is to the east of *nogelii*, and they are parapatric in eastern Turkey (Van and Agri; van Oorschot and Wagener 2000), and although no sympatric records are known, we observed shared haplotypes between *romanovi* and *nogelii* from Agri and Erzincan. Several 'subspecies' described from the boundary of these two species (e.g. *T. nogelii obscura*, *T. nogelii cesa*, *T. romanovi cachetinus*) demonstrate such intermediate states in their morphology. We suggest that these may represent hybrid specimens between *romanovi* and *nogelii* in eastern Turkey and the Caucasus. The range of this hybrid zone, as far as evident from our data, extends probably from Azerbaijan in the east to Elaziğ in the west (Fig. 4). The taxon *telemachus*, described from Karachaudan (Turkmenistan; type in ZISP, Saint Petersburg) based on minor differences with the sympatric *romanovi*, appears to be part of a larger range of variation within the heterogeneous *romanovi* populations in the Kopet Dagh range. With the exception of the examined *telemachus* paratypes, we could not conclusively assign identities to specimens originating from this region due to the intermediate or overlapping character states. Considering also the identical male and female genitalia and shared COI haplotypes, we synonymize *telemachus* with *romanovi* (syn. nov.)

While Oberthür's original (1893) description and illustration of *nesimachus* from "Akbès" (Hatay, southern Turkey) matched very well with our examined material from southern Turkey and the Levant, the central and eastern Turkish specimens generally matched better with *T. nogelii*. We did not detect presence of any of the '*nesimachus*' haplotypes among central and eastern Turkish populations, where various 'ecotypes' of *nogelii* all share a different haplotype. We did not find character combinations proposed by van Oorschot and Wagener (2000) accurate or useful in separating individuals of *nogelii* and *nesimachus*. In our opinion, *nesimachus*-like phenotypes reported as far north as Çankiri and Gümüşhane (van Oorschot and Wagener 2000) are not true *nesimachus.* The diagnostic characters of the genuine *nesimachus* include: *a*) a nearly triangular dark patch at the tip of UPF; *b*) orange patch on UPH nearly rectangular basally, with both sides of the angle more or less equal in length; *c*) marginal black line on UPF always narrow; *d*) considerable variation in submarginal black spots on UPF; sometimes reduced, sometimes complete and connected with marginal line, but the marginal line remains narrow; *e*) no specimens with darkened or reduced orange patch of UPF are known. All reports of *nesimachus* and *nogelii* in central and eastern Turkey, particularly those in sympatry and synchrony, should thus be regarded with skepticism. The *nesimachus* from Syria have a proportionally shorter needle-shape spine in male genitalia (Fig. 1). Our data show that *nogelii* and *nesimachus* overlap only along a narrow range in southern Turkey and the Levant, the exact boundaries of which is yet to be determined. We observed increased haplotype diversity in Adana and Konya and shared haplotypes in Niğde, Mersin and Dalia (Israel), although the two taxa were never synchronous at these localities. Populations of *nogelii* from Mersin and Adana belong to a different haplogroup that seems to be limited in range to the Taurus Mountains and is shared in Niğde with the common haplotype from central and eastern Turkey as well as with the southern *nesimachus* (Fig. 4), and potentially represent hybrid populations between *nogelii* and *nesimachus*. Our *nesimachus* specimens from Syria (Damascus and As-Suwayda), collected in sympatry and synchrony, show multiple haplotypes, one of which is shared with a specimen from Jordan. Lebanese populations of *nesimachus* and *nogelii* are also not sympatric (*nogelii* flies in western slopes and near the coast, *nesimachus* in Antilebanon and eastern slopes) (Larsen 1974) and can be easily told apart. Only *nesimachus* extends as far south as Jordan (Larsen and Nakamura 1983). Adult flight period is correlated with the flowering time of their larval host: *nesimachus* adults in general appear 2–4 weeks earlier than those of *nogelii*, fly in xeric rocky habitats with sparse vegetation, and their larvae only feed on yellow-flowered *Astracantha*, whereas *nogelii* adults emerge later, usually prefer hygric habitats, and their larvae feed on *Astragalus* (Hesselbarth et al. 1995; van Oorschot and Wagener 2000) (Fig. 6). We consider all available evidence to conclude that *nesimachus* is a Levantine species that hybridizes with its northern sister-species *T. nogelii* along a contact zone that extends from southern Turkey to the Levant (Fig. 4). The name *aurantiaca* may refer to hybrid populations from Gaziantep, but an examination of the type series (in ZMHB, Berlin) is pending. In southern Turkey, *nesimachus* and *romanovi* are parapatric but show identical haplotypes across a wide geographic range including, remarkably, between Iran and Jordan (Fig. 3). Two old specimens from Mardin (Hesselbarth et al. 1995: pl. 92, figs 41, 54; ITZA, Amsterdam) show *nesimachus*-like development of maculae as well as a *romanovi*-like green suffusion on the UNH, suggesting hybridization between the two taxa.

All other records of *nogelii*, *nesimachus* and *dobrogensis* from central and eastern Turkey represent various populations of *T. nogelii* ssp. *nogelii* with different larval hosts that share a common, widespread haplotype across

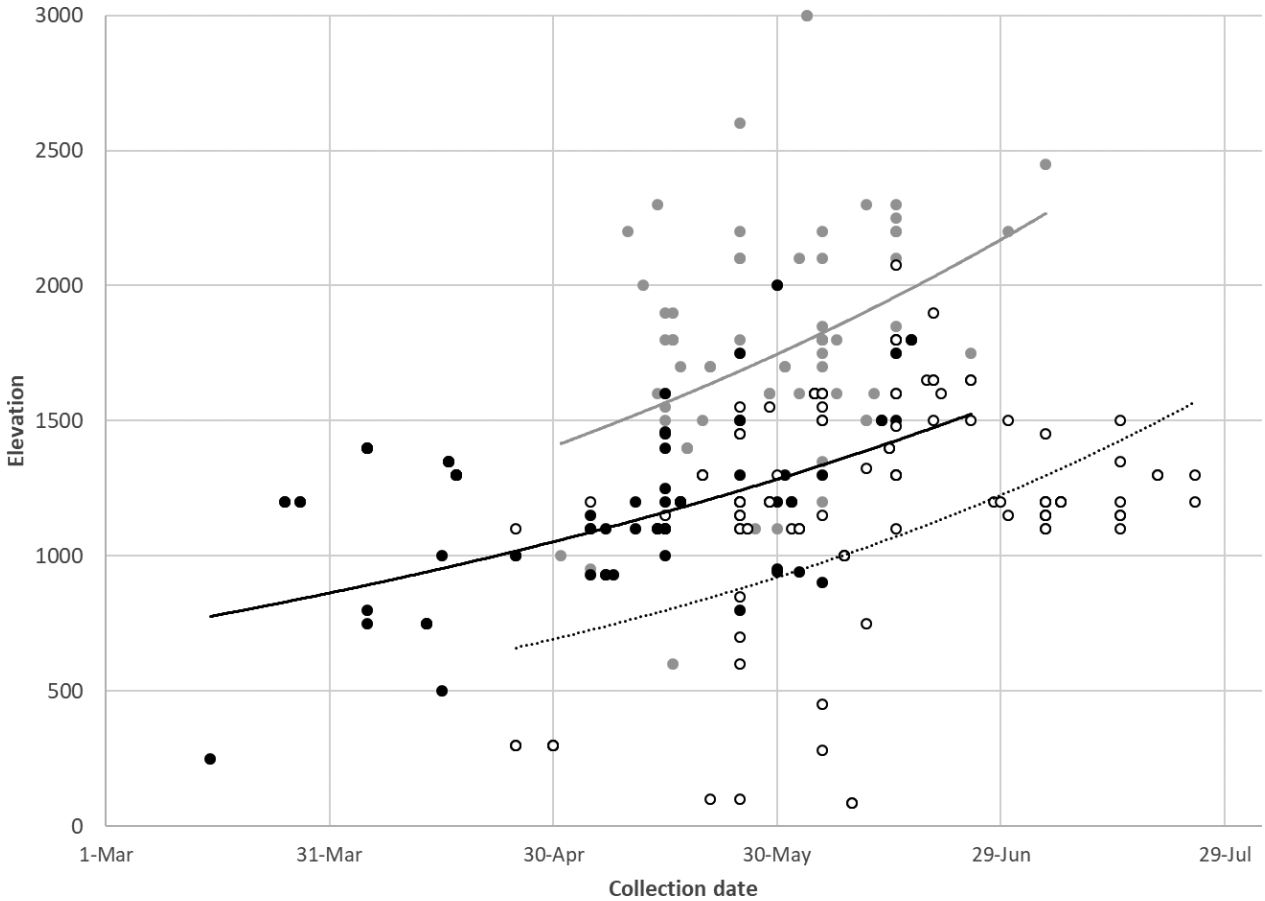

Figure 6. Collection dates vs. elevation in *nogelii* (white), *nesimachus* (black) *and romanovi* (gray).

central to northeastern Turkey (Fig. 4). Small, early-flying *nogelii* feed on smaller *Astracantha* or *Astragalus*, while larger, late-flying *nogelii* feed on the large *Astragalus ponticus*. The forewing length of specimens from Cappadocia and adjacent areas may be twice that of other specimens, but no other consistent differences exist. The taxon *uighurica* Koçak, Seven & Kemal, 2000 (type in CESA, Ankara) was described from Ankara based on these large specimens occurring in June "almost" sympatrically with worn specimens of *nogelii* in April and early June (Koçak 2000). A correlation between adult wingspan and larval host has been demonstrated before (Hesselbarth et al. 1995; van Oorschot and Wagener 2000). All *Tomares* larvae feed exclusively hiding in flower buds, flowers and young seeds inside the umbel (Weidenhoffer and Vanek 1977, WtH personal observation). Large spherical flower stands of *Astragalus ponticus* likely provide more nutrients than the smaller *Astracantha*, contributing to development of larger adults. Here we consider *uighurica* an infra-subspecific name representing an ecotype of *nogelii* (syn. nov.). Individuals from central Turkey attributed to *dobrogensis* examined in our study also did not show any significant phenotypic or molecular differences from *nogelii* collected elsewhere in Anatolia and shared haplotypes with them, while the populations from Ukraine, Crimea and Romania were distinct, showed several unique haplotypes, and were recorded exclusively feeding on *Astragalus ponticus*. We, therefore, recognize ssp. *dobrogensis* representing the isolated populations of *T. nogelli* in Romania and north of the Black Sea, and conclude that it does not occur in Turkey.

Conclusion

Hybridization is not rare in butterflies, and any slight overlap in morphology, behaviour and ecology are likely to allow it to occur (Descimon et al. 1989; Descimon and Mallet 2009). Comprehensive investigations into pre-zygotic isolating mechanisms, post-zygotic hybridization barriers and hybrid viability are required before it can be conclusively demonstrated whether the 'intermediate' specimens from the periphery of species ranges, or different ecotypes co-occurring syntopically in Turkey, are hybrids or reflect natural variation within a single species. Lack of differences in genitalia, overlap in geographic ranges, presence of intermediate phenotypes, low divergence between taxa and widespread haplotype sharing point to either conspecificity of *nogelii*, *nesimachus* and *romanovi*, or presence of extensive introgression between these closely related taxa. On the other hand, accrued and consistent differences in host plant usage, habitat types, elevation, behavior, flight time, and certain wing pattern elements (e.g. the green UNH in *romanovi*) support continued recognition of these taxa as young sister species, in the process of lineage sorting, that co-occur, and occasionally interbreed,

in contact zones at the periphery of their ranges. The three taxa occupy different zoogeographic zones (*nogelii*: Pontomediterranean – Armenian; *nesimachus*: Syrian – Palaeo-eremic, *romanovi*: Iranian – Caspian) (Uvarov 1921; Larsen 1974; Por 1975; Schintlmeister 2008). We prefer to maintain these taxa as separate species for now until genome-wide analyses and new data on karyotypic diversity and symbiosis with ants shed more light on the evolution of these fascinating butterflies.

Revised classification of *Tomares* species

For additional synonymy, see Hesselbarth et al. (1995) and Weidenhoffer and Bozano (2010).

***Tomares ballus* (Fabricius, 1787)**

Distribution. Southwest France to southern Spain and Portugal, Gibraltar, Morocco, Algeria, north Libya, south Tunisia and north Egypt.

Larval host. *Lotus hispidus*, *Boujeania hispida* (?), *Anthyllis vulneraria*, *A. cyticoides*, *Heliatheum* sp. and *Medicago* sp. in Spain (Korb 1924; Higgins and Riley 1970; Muñoz Sariot 2011); *Anthyllis tetraphylla*, *Erophaca boetica*, and *Medicago* cf. *turbinata* in Morocco (Tennent 1996).

***Tomares mauritanicus* (Lucas, 1849)**

Distribution. Algeria and Morocco.
Larval host. *Hedysarum pallidum*, *Hippocrepis multisiliquosa*, *H. minor*, *Astragalus epiglottis*, and *A. pentaglottis* (Higgins and Riley 1970, Tennent 1996).

***Tomares callimachus* (Eversmann, 1848)**
ssp. *callimachus* (Eversmann, 1848)
= *Polyommatus epiphania* Boisduval, 1848 stat. rev.

Distribution. From Ukraine to Central Asia and N Azerbaijan.

Larval host. Recorded on a number of *Astragalus* species from Alatau Mountains and NW Kazakhstan to South Russia, Crimea and Georgia: *Astragalus leptostachys*, *A. macropterus*, *A. physodes*, *A. suprapilosus*, *A. utriger* and *A. vulpinus*, as well as *Hedysarum candidum* in Crimea and *Onobrychis radiate* in Georgia (Weidenhoffer and Vanek 1977; Zhdanko 1997; Tuzov et al. 2000; Stradomsky and Fomina 2013; Bury and Savchuk 2015).

ssp. *hafis* (Kollar, 1849) stat. rev.

Distribution. Lesser Caucasus, Armenia, south and southeast Turkey, north Iraq, west, southwest, north and northeast Iran to Kopet Dagh.

Larval host. Not recorded. The record of *Astragalus physodes* from "Kulp" (Diyarbakir, Turkey) by Korb (1924) is erroneous as the plant does not occur in Turkey (Hesselbarth et al. 1995).

ssp. *huertasae* Tshikilovets & Pagès, 2016

Distribution. Pakistan: Baluchistan.

Larval host. Not recorded.

***Tomares desinens* Nekrutenko & Effendi, 1980**

Distribution. Southeast Azerbaijan, east Turkey (Van), north and northwest Iran.

Larval host. Not recorded.

***Tomares fedtschenkoi* (Erschoff, 1874)**

Distribution. South Turkmenistan, Uzbekistan, Kyrgyzstan, south Kazakhstan and Tajikistan. Records from Afghanistan and Pakistan are questionable (Tshikolovets and Pagès 2016; Tshikolovets et al. 2018).

Larval host. *Astragalus chlorodontus* and *Astragalus agameticus* (Zhdanko 1997).

***Tomares nogelii* (Herrich-Schäffer, [1851])**
ssp. *nogelii* (Herrich-Schäffer, [1851])
=*uighurica* Koçak, Seven and Kemal in Koçak, 2000 syn. nov.

Distribution. Northeast to central Anatolia, and south to the Levant.

Larval host. *Asteracantha* spp. (early fliers); *Astragalus ponticus* and *A. micropterus* (late fliers) in Turkey (Hesselbarth et al. 1995).

ssp. *dobrogensis* (Caradja, 1895)

Distribution. Romania, Crimea, Ukraine. Does not occur in Turkey.

Larval host. *Astragalus ponticus* in Ukraine and Romania (Tuzov et al. 2000; Bury and Savchuk 2015; Rákosy and Craioveanu 2015).

***Tomares nesimachus* (Oberthür, 1893)**

Distribution. Southern Turkey (Mersin, Adana, Hatay to Mardin) to Lebanon, Israel and Jordan.

Larval host. *Astracantha* spp. (Oorschot and Wagner 2000); *Astragalus macrocarpus* in Israel and Jordan (Larsen and Nakamura 1983); *A. densifolius* in Mersin, Turkey (Leestmans et al. 1986).

***Tomares romanovi* (Christoph, 1882)**
= *Tomares telemachus* Zhdanko *in* Tuzov et al. 2000 syn. nov.

Distribution. East Turkey, Georgia, Armenia, Azerbaijan, Iran, and Kopet Dagh range in Turkmenistan.

Larval host. *Astragalus finitimus* in Kopet Dagh and in Armenia (Yerevan)(Weidenhoffer and Vanek 1977;

Hesselbarth et al. 1995; Tuzov et al. 2000); *Astragalus schachrudensis* in Kopet Dagh, Azerbaijan (Ordubad) and Armenia (Ockschaberd) (Christoph 1882; Korb 1924; Zhdanko 1997).

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Supplementary material 1

SI 1. Material examined and Genbank accessions.

Authors: Vazrick Nazari, Wolfgang ten Hagen
Data type: Microsoft Excel Spreadsheet
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Link: https://doi.org/10.3897/dez.67.50252.suppl1

Supplementary material 2

SI 2. Male and female genitalia dissections of *Tomares* species.

Authors: Vazrick Nazari, Wolfgang ten Hagen
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Supplementary material 3

SI 3. Androconia, forewing upperside and hindwing underside in select *Tomares* species.

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Supplementary material 4

SI 4. Phylogenetic trees resulting from Maximum Parsimony (MP, PAUP) and Maximum Likelihood (ML, PHYML) analyses of COI, EF-1a and Combined datasets with bootstrap support values.

Authors: Vazrick Nazari, Wolfgang ten Hagen
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