Taxonomic Position and Status of *Polyommatus (Agrodiaetus) iphigenia* (Lepidoptera, Lycaenidae) from the Peloponnese, Southern Greece*

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Accepted September 10, 2015

SHAPOVAL N., LUKHTANOV V. 2015. Taxonomic position and status of *Polyommatus* (*Agrodiaetus*) *iphigenia* (Lepidoptera, Lycaenidae) from the Peloponnese, southern Greece. Folia Biologica (Kraków) **63**: 295-300.

In our study we use a 690 bp fragment of the COI gene to analyze a taxon from southern Greece, usually treated as *Polyommatus (Agrodiaetus) iphigenia nonacriensis* (Brown, 1977). The previous conclusions on taxonomy and nomenclature of *P. (A.) iphigenia nonacriensis* were not supported by molecular or cytological data, therefore the problem of identity of this taxon has remained unsolved. We found that with respect to *COI* haplotypes, *P. (A.) iphigenia nonacriensis* from Greece is similar to the studied populations of *P. (A.) iphigenia (Armenia, 1847)* from Turkey and Armenia. Thus, we confirm that the only Greek *Agrodiaetus* butterfly with blue wing coloration in males actually belongs to the species *P. (A.) iphigenia*.

Key words: *Agrodiaetus*, butterflies, Lepidoptera, Lycaenidae, molecular marker, Greece, Peloponnese.

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The subgenus Agrodiaetus Hübner, 1822 is a species-rich monophyletic lineage within the genus Polyommatus (Latreille, 1804) (TALAVERA et al. 2013) and one of the most complicated and troublesome groups of the Palaearctic Lepidoptera (COUTSIS 1986; VILA et al. 2010; LUKHTANOV et al. 2015a). It includes numerous species, subspecies, and forms with uncertain taxonomic status, and the majority of these taxa are weakly differentiated with respect to genitalia structure, wing colour and wing pattern (HESSELBARTH et al. 1995; LUKHTANOV et al. 2008, 2014, 2015b). Within this subgenus, FORSTER (1960) (who considered Agrodiaetus as a valid genus) located a polytypic species Agrodiaetus iphigenia (Herrich-Schäffer, 1847) that included the following subspecies: A. iphigenia iphigenia (Herrich-Schäffer, 1847), A. iphigenia barthae (Pfeiffer, 1932), A. iphigenia araratensis de Lesse, 1957, A. iphigenia iphidamon (Staudinger, 1899), A. iphigenia iphigenides (Staudinger, 1886), *A. iphigenia juldusus* (Staudinger, 1886) and *A. iphigenia rueckbeili* Forster, 1960. At a later time, the only Greek *Agrodiaetus* butterfly with blue wing coloration in males was found in northern Peloponnese (Mt. Chelmos, Greece) and provisionally identified as *A. damone* (Eversmann, 1841) (BROWN & DE WORMS 1975). Later it was described as subspecies *A. iphigenia nonacriensis* (Brown, 1977). Recently two new taxa from Turkey were described: *P. (A.) iphigenia iphicarmon* from Taurus mountains (Isparta province) (ECKWEILER & ROSE 1993) and *P. (A.) iphigenia manuelae* from Hakkari province (ECKWEILER & SCHURIAN 2013).

Recent DNA-based phylogenetic analyses and karyotype studies demonstrated that *P*. (*A*.) *iphigenia* sensu lato was an artificial polyphyletic assemblage consisting of several species (WIEMERS 2003; KANDUL *et al.* 2004, 2007; LUKHTANOV *et al.* 2005). In particular, these approaches proved that

^{*}Supported by the grant from the Russian Science Foundation N14-14-00541 to the Zoological Institute of the Russian Academy of Sciences. A postdoctoral fellowship was provided to N. SHAPOVAL from St Petersburg State University.

P. (A.) juldusus, P. (A.) iphigenides and P. (A.) iphidamon are distinct species, not even closely related to P. (A.) iphigenia. Polyommatus (A.) juldusus and P. (A.) iphigenides were recognized as members of the P. (A.) damone species-group which is the sister lineage to the P. (A.) iphigenia species-group. Polyommatus (A.) iphidamon was recognized within the P. (A.) carmon (Herrich-Schäffer, 1851) species complex which is not even distantly related to the P. (A.) iphigenia group. Finally, P. (A.) iphicarmon was recognized as a distinct species within the P. (A.) iphigenia species-group.

Thus, the taxonomic significance of the morphological features is low in *Agrodiaetus*. Therefore, descriptions which were based mainly on external morphological characters alone (e.g. FORSTER 1960), did not clarify the phylogenetic position and identity of taxa from the *P*. (*A*.) *iphigenia* sensu FORSTER 1960 assemblage. Unlike the abovementioned taxa, *P*. (*A*.) *iphigenia nonacriensis* has never been studied genetically, therefore its identification, taxonomic status and phylogenetic position have remained unverified. Our paper addresses a detailed analysis of this taxon, which is apparently present only in Greece (Peloponnese peninsula).

Material and Methods

Two females of *P*. (*A*.) *iphigenia nonacriensis* were collected in 2008 in southern Greece (see Table 1 and Fig. 1). A fragment of the mitochondrial *cyto-chrome oxidase I* gene (*COI*) (first 690 positions) was used as a molecular marker. For DNA amplification for *COI* we used primers K698 and Nancy (CATERINO & SPERLING 1999). Sequencing of the double-stranded product was carried out at the Research Resource Center for Molecular and Cell Technologies (Saint-Petersburg State University). Collected specimens are kept in the Zoological Institute of the Russian Academy of Science (St. Petersburg).

Table 1

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Sample ID or Genbank number	Taxon	COI haplotype group	Country	Locality and coordinates	Altitude	Date	Collec- tors	References
LR08D163	iphigenia nonacriensis	iphigenia	Greece	Kalavrita: N 38°01'37"; E 22°13'23"	1700 m	17 July 2008	NS, LR, VL	current study
LR08D164	iphigenia nonacriensis	iphigenia	Greece	Kalavrita: N 38°01'37"; E 22°13'23"	1700 m	17 July 2008	NS, LR, VL	current study
AY496756	iphigenia araratensis	iphigenia	Turkey	Van Province, Çatak	-	July 2001	NK, VL	KANDUL <i>et al.</i> 2004
AY496755	iphigenia araratensis	iphigenia	Armenia	Aiodzor Mts., Gnyshik village	-	June 2000	AD	KANDUL <i>et al.</i> 2004
AY556849	iphigenia	iphigenia	Armenia	Aiodzor Mts., Gnyshik village	1800-2200 m	20 July 1998	AD	WIEMERS 2003
EF104609	iphigenia	iphigenia	Turkey	Isparta Province, Yenişarbademli	-	July 2004	_	KANDUL et al. 2007
AY496757	iphigenia	iphigenia	Turkey	Gümüshane Province, Gümüshane	_	July 2001	VL, AD	KANDUL et al. 2004
AY556991	iphigenia	iphigenia	Turkey	Isparta Province, Dedegöl Geçidi west. Kurucuova	1700 m	21 July 1998	MW	WIEMERS 2003
AY556984	iphigenia	iphigenia	Turkey	Fethiye, Salur Dagi, west. Elmali	1700-1900 m	21 July 1998	MW	WIEMERS 2003
AY557027	iphigenia	iphigenia	Turkey	Erzincan Province, Çaglayan	1500 m	05 July 1999	MW	WIEMERS 2003
AY557061	iphigenia	iphigenia	Turkey	Erzincan Province, Çaglayan	1500 m	15 July 1999	MW	WIEMERS 2003
AY557079	baytopi	iphigenia	Turkey	Van Province, 25-32 km N Çatak	2000-2200 m	18 July 1999	MW	WIEMERS 2003
AY557084		iphigenia	Turkey	Van Province, Güzeldere Geçidi, Baskale	2500 m	19 July 1999		WIEMERS 2003
AY557087	baytopi	iphigenia	Turkey	Van Province, Güzeldere Geçidi, Baskale	2500 m	19 July 1999	MW	WIEMERS 2003
AY496720	baytopi	iphigenia	Turkey	Van Province, Güzeldere Geçidi, Baskale	2500 m	July 2001	VL	LUKHTANOV <i>et al.</i> 2005
AY556897	rovshani	iphigenia	Iran	Azarbayjan-e Sharqi Province, Dugijan, 30 rm NE Marand	2000 m	15 July 2000	MW	WIEMERS 2003
AY557149	rovshani	iphigenia	Iran	Azarbayjan-e Sharqi Province, Mahmutabad, W Kaleybar	2200-2400 m	29 July 2002	WE	WIEMERS 2003

List of studied material (49 specimens). Collectors: V. Lukhtanov (VL), N. Shapoval (NS), N. Kandul (NK), A. Dantchenko (AD), L. Riepel (LR), M. Wiemers (MW), D. Sobanin (DS) and W. Eckweiler (WE)

Table	l cont.
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Sample ID or Genbank number	Taxon	COI haplo- type group	Country	Locality and coordinates	Altitude	Date	Collec- tors	References
AY954023	rovshani	iphigenia	Azerbaijan	Talysh, Zuvand	_	July 2003	VL	LUKHTANOV et al. 2005
AY496788	rovshani	iphigenia	Iran	Sabalan	_	July 2001	VL	KANDUL <i>et al.</i> 2004
EF104626	rovshani	iphigenia	Azerbaijan	Talysh, Zuvand	_	July 2003	VL	KANDUL <i>et al.</i> 2007
AY557124	tankeri	iphigenia		Bayburt Province, Kop Geçidi south. Maden	2350 m	29 July 1999	MW	WIEMERS 2003
AY557125	tankeri	iphigenia	Turkey	Bayburt Province, Kop Geçidi south. Maden	2350 m	29 July 1999	MW	WIEMERS 2003
AY496794	tankeri	iphigenia	Turkey	Erzurum Province, Erzurum	_	July 2001	VL	KANDUL et al. 2004
AY556990	iphi carmon	iphigenia	Turkey	Isparta Province, Dedegöl Geçidi west. Kurucuova	1700 m	21 July 1998	MW	WIEMERS 2003
EF104608	iphicarmon	iphigenia	Turkey	Isparta Province, Yenişarbademli	_	July 2004	-	KANDUL et al. 2007
AY557117	turcicus	iphigenia	Turkey	Erzurum Province, Köskköy, 25 km N Erzurum	1900 m	28 July 1999	MW	WIEMERS 2003
AY557065	turcicus	iphigenia	Turkey	Erzincan Province, 5 km SE Çaglayan	1500 m	15 July 1999	MW	WIEMERS 2003
AY557054		iphigenia	Turkey	Iğdir Province, Badilli, Tuzluca	1800-2000 m	12 July 1999	MW	WIEMERS 2003
AY496798	turcicus	iphigenia	Armenia	Pambak mts.	_	June 2000	AD	KANDUL et al. 2004
AY496770	phyllides			Kirgizski range	_	June 2000	NK, VL	KANDUL et al. 2004
AY496771	F	damone	Kazakhstan	Karzhantau mts	_	June 2002	NK, VL	KANDUL et al. 2004
AY496769	phyllides kentauensis	damone	Kazakhstan	Karatau Mts., Turpan Pass	-	June 2000	NK	KANDUL <i>et al.</i> 2004
AY954011	phyllides askhabadicus	damone	Iran	Khorosan, Chakane		July 2003	VL	LUKHTANOV et al. 2005
FJ663239	phyllides	damone	Tadzhikista n	Iskanderkul, N 39°04'01"; E 68°22'01"		20 July 1994	VL	LUKHTANOV et al. 2009
FJ663229	damone altaicus	damone	Kazakhstan	12 km S of Zajsan, N 47°22'01"; E 84°51'36"		23 June 1997	VL	LUKHTANOV et al. 2009
FJ663228	damone altaicus	damone	Kazakhstan	12 km S of Zajsan, N 47°22'01"; E 84°51'36"		23 June 1997	VL	KANDUL <i>et al.</i> 2007
AY496734	damone altaicus	damone	Russia	Altai, Kuraiski Mts., Aktash	_	August 2000	NK	KANDUL <i>et al.</i> 2004
AY496735	damone damone	damone	Russia	South Urals, Guberli Mts., Adaevo	-	July 2001	VL	KANDUL <i>et al.</i> 2004
AY496736	damone irinae	damone	Russia	Volgograd Reg., Kamyshinsky	-	July 2000	AD	KANDUL <i>et al.</i> 2004
AY556853	1 0	damone		Kitabsky national reserve Zeravshansky Mts	1500-2500 m	15 June 2000		WIEMERS 2003
AY557155	1 0	damone	Kirgizia	25 km S Song Kul, Molto Tau	_	22 July 1998	WE	WIEMERS 2003
AY496758	iphigenides	damone	Kazakhstan		-	July 2000	NK, VL	KANDUL <i>et al.</i> 2004
AY496759	juldusa ka- sachstanus		Kazakhstan	Dzhungarian Alatau Mts. Kysylagash	-	June 2000	NK	KANDUL <i>et al.</i> 2004
	karatavicus			Shymkent Reg., Karatau Mts	_	June 2000	VL	KANDUL et al. 2004
AY496774		damone	Ukraine	Crimea, Ai-Petri Mt.		July 2000	NK	KANDUL <i>et al</i> . 2004
AY953988	iphidamon	carmon	Iran	Gorgan, Schahkuh		July 2002	AD	KANDUL et al. 2004
AY556919	iphidamon	carmon	Iran	Gorgan, Schahkuh N 36°32'25"; E 54°25'57"		21 July 2000	MW	WIEMERS 2003
AY954000	stempffer i	outgroup	Iran	Esfahan, Khansar		July 2002	VL	KANDUL et al. 2004
AY556927	icarus	outgroup	Iran	Golestan Province, Hadjiabad, 25 km SSW Gorgan		23 July 200	MW	WIEMERS 2003

Our analysis included, besides P. (A.) *iphigenia* nonacriensis, all the principal members of the P. (A.) *iphigenia* species-group and P. (A.) damone species-complex available from GenBank database (WIEMERS 2003; KANDUL *et al.* 2004, 2007; LUKHTANOV *et al.* 2005, 2009). We also included P. (A.) *iphidamon* sequences in our analysis, since this taxon was treated by FORSTER (1960) as a subspecies of *P*. (*A*.) *iphigenia*. Since *Polyommatus icarus* (Rottemburg, 1775) and *P. stempfferi* (Brandt, 1938) were earlier inferred as outgroups to the subgenus *Agrodiaetus* (TALAVERA *et al.* 2013), we used them to root the phylogram. A complete list of specimens included in this study is given in Table 1. A Bayesian approach was used for estimating the phylogeny. Bayesian analyses were

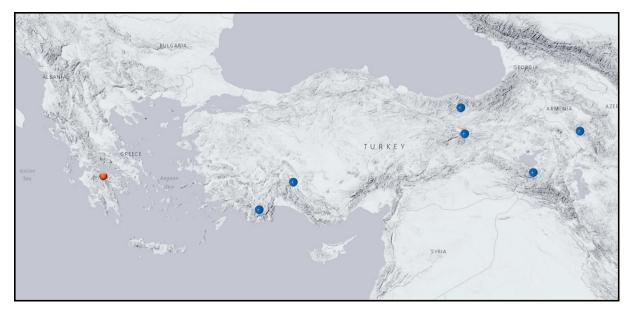


Fig. 1. Sampling localities of specimens used in present study: *P*. (*A*.) *iphigenia nonacriensis* (red circle) and *P*. (*A*.) *iphigenia* s. str. (blue circles).

performed using the program MrBayes 3.1.2 with the nucleotide substitution model GTR+G+I. jModelTest was used to determine optimal substitution models for Bayesian inference (BI) analysis (POSADA 2008). TRACER, version 1.4 was used for summarizing the results of the Bayesian phylogenetic analyses (http://beast.bio.ed.ac.uk/Tracer).

Results and Discussion

Analysis of a dataset of 49 specimens recognized P.(A.) *iphidamon* as a highly differentiated lineage with a basal position. All other specimens constituted two major clades: the P.(A.) *damone* lineage, and the P.(A.) *iphigenia* lineage (Fig. 2).

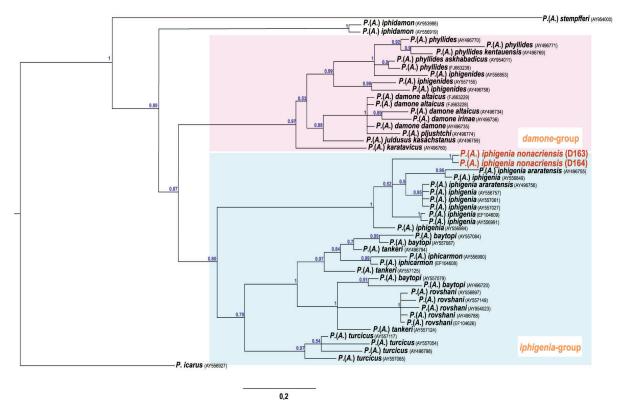


Fig. 2. The Bayesian tree of the *Polyommatus (Agrodiaetus) damone* group (highlighted in pink) and *Polyommatus (Agrodiaetus) iphigenia* group (highlighted in blue) based on analysis of *COI* gene from 49 specimens. Numbers at nodes indicate Bayesian posterior probability. The position of *P*. (A.) *iphigenia nonacriensis* is shown in red.

This agrees with the results of other phylogenetic studies (WIEMERS 2003; KANDUL *et al.* 2004, 2007; LUKHTANOV *et al.* 2005; VILA *et al.* 2010). The *P.* (*A.*) *damone* lineage included the taxa *P.* (*A.*) *phyllides*, *P.* (*A.*) *iphigenides*, *P.* (*A.*) *karata-vicus*, *P.* (*A.*) *damone altaicus*, *P.* (*A.*) *damone damone*, *P.*(*A.*) *damone irinae*, *P.* (*A.*) *juldusus* and *P.* (*A.*) *pljushtchi.* The *P.* (*A.*) *iphigenia* group included the taxa *P.* (*A.*) *baytopi*, *P.* (*A.*) *rovshani*, *P.* (*A.*) *turcicus*, *P.* (*A.*) *iphicarmon* and *P.* (*A.*) *iphigenia P.* (*A.*) *turcicus*, *P.* (*A.*) *iphicarmon* and *P.* (*A.*) *iphigenia P.* (*A.*) *tankeri.*

We also found that in our previous studies (KAN-DUL et al. 2004, 2007; LUKHTANOV & BUDASH-KIN 2007), an error was made during reassembling 4 separate sequence reads of P. (A.) pljushtchi (the representative of the P. (A.) damone speciesgroup) into one contig. Three of them were assembled correctly, while the second fragment was erroneously taken from the other species P. (A.) damon (GenBank accession number AY496733). Thus, the contig of P. (A.) pljushtchi available from Gen-Bank under accession number AY496774 actually represents a chimeric nucleotide sequence. This resulted in erroneous phylogenetic reconstructions where P. (A.) pljushtchi occupied an isolated basal position within the complex of *P*. (*A*.) damone s.l. (KANDUL et al. 2004, 2007; LUKHTANOV & BUDASHKIN 2007). Here, in our analysis, we used the corrected sequence of the taxon *pljushtchi*. We demonstrate that this taxon is closely related to P. (A.) damone and should be considered as a subspecies P. (A.) damone pljushtchi, as it is already treated by TSHIKOLOVETS (2011).

In our phylogenetic reconstruction, two Greek specimens, which were identified in the field as females of *P*. (*A*.) *iphigenia nonacriensis*, formed a well-supported cluster with other sequences of *P*. (*A*.) *iphigenia* s.str. (Fig. 2). Genetic divergence of the Greek samples as compared with other specimens of *P*. (*A*.) *iphigenia* from Turkey and Armenia is low (0.86-1.01%) and is based on seven nucleotide substitutions in the studied COI fragment. Interestingly, specimens of *P*. (*A*.) *iphigenia* s.str. by fixed nucleotide substitutions and by having a unique chromosome number n=15 (WIEMERS 2003; KANDUL et al. 2007).

In the light of the data obtained, and taking into account the genetic and morphological similarity of the taxa *nonacriensis* and *iphigenia*, we consider *nonacriensis* unlikely to be a separate species. It should be synonymized with *P*. (*A*.) *iphigenia* or, at most, considered as a weakly differentiated subspecies of the latter. Thus, our work clarifies the taxonomic status of the Greek *Agrodiaetus* butterfly, which is considered in the literature as *P*. (*A*.) *iphige*- *nia nonacriensis*, and the position of which was under debate.

Acknowledgements

We are grateful to M. WIEMERS for noticing the mistake in assembling the sequence AY496774. We thank N.E. PIERCE and L. RIEPPEL for providing the opportunity to collect in Greece.

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