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Genetic patterns, host use and larval morphology in Tunisian populations of *Orgyia trigotephras*

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Abstract

Orgyia trigotephras Boisduval 1829 (Erebidae Lymantriinae) is a polyphagous moth widely distributed across the Mediterranean Basin. Current taxonomy validates several taxa at subspecies level within this species. Two of them, *Orgyia trigotephras anceps* Oberthur 1884 and *Orgyia trigotephras transiens* Staudinger et Rebel 1901 were found to occur in Tunisia. Although considered a rare species in southern Europe, an extensive outbreak was observed in Tunisia in the last decade. In this paper we present details on genetic patterns (mitochondrial DNA marker CO1), on larval phenotypic traits and on host plant species of Tunisian populations of *O. trigotephras*. Tunisian specimens clearly differentiated into two lineages, restricted to western and eastern Tunisia respectively. Both Tunisian haplotype-lineages strongly diverge from southern Spanish and southern Italian 'conspecifics', questioning current taxonomy. Furthermore, we describe four larval phenotypes occurring in Tunisia and register *Quercus coccifera*, *Quercus suber*, *Erica multiflora* and *Pistacia lentiscus* as the four main host plant species. There was no association of the two genetic lineages with larval phenotypic traits. However, host plant species differed significantly between the two lineages.

Key words: *Orgyia trigotephras*, phylogeography, CO1, host plants, larval phenotypes.

Introduction

Orgyia trigotephras Boisduval 1829 (Erebidae Lymantriinae) is a xerothermophilous tussock moth that feeds on evergreen oaks and other Mediterranean shrub species. The moth is widely distributed across the Mediterranean Basin, from Anatolia (Patočka and Turčáni, 2008) to south-western Europe, France (Berard *et al*., 2010), Spain (Montoya and Masmano, 1993) and North Africa (Villemant and Fraval, 1993). Several subspecies are currently recognized: *Orgyia trigotephras corsica* Boisduval 1834 from Corsica (Bella *et al*., 2011; but validated at species rank in de Freina and Witt, 1987); *Orgyia trigotephras anceps* Oberthur 1884 from Morocco (Daniel and Witt, 1975); *Orgyia trigotephras sicula* Staudinger et Rebel 1901 (=*Orgyia trigotephras calabra* Stauder 1916) with a characteristic dark brown wing and body coloration from southern Italy, Sicily and Malta (Stauder, 1923; Bella *et al*., 2011); *Orgyia trigotephras transiens* Staudinger et Rebel 1901 (=*Orgyia trigotephras panlacroixii* Oberthur 1876) from North Africa (de Freina and Witt, 1987) and *Orgyia trigotephras holli* Oberthur 1916 (=*O. trigotephras panlacroixii*) from Algeria. In Tunisia, two taxa were recorded: in the northwest (Aïn Draham) *O. trigotephras transiens* (Lord Rothschild *et al*., 1917), and *O. trigotephras anceps* in the north (Bizerte) and the northeast (Cap Bon) (Chnéour, 1955). So far, no molecular works were conducted to clarify their taxonomic status.

Life history of *O. trigotephras* has not been studied comprehensively, so far. Nevertheless, the few existing records suggest differences between regions. In Spain, Italy and Algeria the species is reported to have one generation per year overwintering in the egg stage, with a flight period of the adults from May to August (Ortiz and Templado, 1973; Chakali *et al*., 2002; Bella *et al*., 2011). In Tunisia *O. trigotephras* is bivoltine with a first generation from April to June and a second generation from October to December (Chnéour, 1955; Ezzine, 2007). In Corsica the species is bivoltine too (Bella *et al*., 2011). Females have reproductive capacity up to 200 eggs (Ezzine, 2007), they are apterous and thus show no dispersal at the adult stage. Mating and egg deposition take place on the same plant where pupation occurs. Dispersal is larger in the larval stage, early instars larvae are transported by wind, caterpillars of later instars usually move from one plant to another, but this type of dispersal is usually limited to neighbouring plants (Ezzine, personal observation).

O. trigotephras is considered to be polyphagous. In southern Italy, *Orgyia trigotephras etrusca* Verity 1905 (=*O. trigotephras sicula*) is found feeding on and sporadically causing defoliation of *Pistacia lentiscus* (Bella *et al*., 2011), whereas in Sicily and Malta, the subspecies *O. trigotephras calabra* feeds mainly on *Sarothamnus* sp. (Bella *et al*., 2011). In southern Spanish dune habitats (Ayamonte), *O. trigotephras* prefers *Retama monosperma* (Dionisio, 2002), whilst in the north-eastern region of Albacete in Spain *O. trigotephras* is one of the main lepidopteran species found on *Quercus ilex* (Montoya and Masmano, 1993). In Morocco and Algeria the subspecies *O. trigotephras anceps* was found to feed mainly on evergreen oaks, *Quercus suber* and *Q. ilex* (Villemant and Fraval, 1993; Chakali *et al*.,

2002). The subspecies *O. trigotephras transiens* was found to feed on *Calicotome* ssp., *Retama monosperma* and *Q. ilex* (Oberthür, 1916). In Tunisia *O. trigotephras* feeds on *Q. suber*, *Q. ilex*, *Quercus coccifera* and on *P. lentiscus* (Chnéour, 1955; Ezzine *et al*., 2010). Altogether, these host-plant records lead us to hypothesize that some host adaptation or specialization might occur at the regional/species level.

Abundance of *O. trigotephras* varies strongly across regions, from rare and endangered (Dionisio, 2002) to a common defoliator or even to pest status (Villemant and Fraval, 1993; Chakali *et al*., 2002; Ezzine *et al*., 2010). Besides the habitat and climate we hypothesize that host use might be a cause of the different abundance of the populations across regions.

In this work we aim at (1) investigating genetic patterns of Tunisian populations of *O. trigotephras*, (2) testing association of genetic patterns with different morphological phenotypes of larvae, (3) testing association of genetic patterns with host use and (4) investigating taxonomical implications by comparing the Tunisian DNA barcodes with those from other populations in the Mediterranean basin (Spain and Italy). For this purpose, we investigated moths from several locations along the coast between north-western and north-eastern Tunisia using the mitochondrial DNA CO1 barcode as a marker well differentiating at species and subspecies level (Hebert *et al*., 2003).

Materials and methods

Study area

The study area includes cork oak forests and Mediterranean maquis, distributed along the coast between north-western and north-eastern Tunisia: Tabarka (36°56'N 8°48'E), Amdoun (36°51'N 9°0'E), Nefza (37°1'N 9°5'E), Sejnane (37°11'N 9°11'E), Jebel Ben Oulid (36°52'N 10°48'E) and Delhiza (36°51'N 10°47'E). The three first locations are characterized by dense cork oak forest and the three last by degraded cork oak forests and Mediterranean maquis (figure 1).

Sampling

Larvae in the L5 stage were collected by hand from the host-plants and preserved in 96% ethanol. Egg batches and pupae, which are spun between two or three leaves of the host tree; they were collected by cutting branches using scissors. In the laboratory, eggs and pupae were conserved in plastic boxes for further analysis. Pupae were placed in plastic boxes (21 × 10 × 10 cm) at a temperature of 25 °C, awaiting adult emergence. Emerged adults were killed with ether. Voucher specimens are conserved in the Lepidoptera section of the ZSM (Bavarian State Collection of Zoology, Munich, Germany).

DNA data analysis

Dry legs, fragments of adults, first segments of larval thorax and cremaster part of pupae were sampled into lysis plates for DNA barcoding. In total 59 individuals were sampled (figure 2). DNA extraction, PCR and DNA sequencing were performed at the Canadian Centre for DNA Barcoding, Guelph, Canada (CCDB) following standard high-throughput protocols (Ivanova *et al*., 2006), that can be accessed under http://ccdb.ca/resources.php. PCR amplification with a single pair of primers (Ivanova *et al*., 2006) consistently recovered a 658 bp region near the 5' terminus of the mitochondrial cytochrome c oxidase 1 (CO1), gene that included the standard 648 bp barcode region for the animal kingdom (Hebert *et al*., 2003).

DNA extracts are stored at the CCDB, with aliquots being deposited in the DNA-Bank facility of the ZSM (see http://www.zsm.mwn.de/dnabank/). Sequences and metadata are hosted in BOLD (Barcode of Life Data Systems, project INRGR "Global Geometridae/Lepidoptera of Tunisia-cork oak defoliators-INRGREF"). All sequences are deposited also in GenBank according to the

Figure 1. Geographic distribution of samples in Tunisia. (In colour at www.bulletinofinsectology.org)

iBOL data release policy, GenBank accession numbers are provided in figure 2. Images, GPS coordinates and sequence trace files for each specimen as well as details on host institution can be obtained from the Barcode of Life Data System (BOLD; Ratnasingham and Hebert, 2007). A Maximum Likelihood (ML) Tree was constructed with the software MEGA6 (Tamura *et al.*, 2013), bootstrap method, 500 replicates, Tamura-Nei model, complete deletion, bootstrap values indicated when >50% (cf. figure 3), genetic distances are reported as minimum pairwise distances. A German sequence of the holarctic species *Orgyia antiqua* L. 1758 was used as outgroup. Two sequences of Spanish specimens of *O. trigotephras* from Murcia (by the courtesy of A. Ortiz), and one of a southern Italian specimen (A. Hausmann) were included into the analysis.

stage	total of collected	total of DNA barcodes	ID sample	accession number in Genebank	Location	host-plant
egg-mass	3	2	INRGREF add 0007	GWOSP956-11	Sejnane	*Quercus coccifera*
			INRGREF add 0005	GWOSP954-11		
	3	2	INRGREF add 0008	GWOSP957-11	Tabarka	*Eucalyptus camaldulensis*
			INRGREF add 0009	GWOSP958-11		
larva	4	2	INRGREF add 0016	GWOSP965-11	Nefza	*Quercus suber*
			INRGREF add 0057	GWOSP1006-11	Amdoun	
	13	9	INRGREF add 0045	GWOSP994-11	Nabeul (Jebel Ben Oulid)	*Pistacia lentiscus*
			INRGREF add 0038	GWOSP987-11		
			INRGREF add 0046	GWOSP995-11		
			INRGREF add 0039	GWOSP988-11		
			INRGREF add 0044	GWOSP993-11		
			INRGREF add 0043	GWOSP992-11		
			INRGREF add 0036	GWOSP985-11		
			INRGREF add 0037	GWOSP986-11		
			INRGREF add 0047	GWOSP996-11		
	14	12	INRGREF add 0023	GWOSP972-11		*Erica multiflora*
			INRGREF add 0035	GWOSP984-11		
			INRGREF add 0028	GWOSP977-11		
			INRGREF add 0034	GWOSP983-11		
			INRGREF add 0024	GWOSP973-11		
			INRGREF add 0029	GWOSP978-11		
			INRGREF add 0026	GWOSP975-11		
			INRGREF add 0025	GWOSP974-11		
			INRGREF add 0033	GWOSP982-11		
			INRGREF add 0030	GWOSP979-11		
			INRGREF add 0027	GWOSP976-11		
			INRGREF add 0031	GWOSP980-11		
	11	10	INRGREF add 0053	GWOSP1002-11		*Quercus coccifera*
			INRGREF add 0052	GWOSP1001-11		
			INRGREF add 0051	GWOSP1000-11		
			INRGREF add 0054	GWOSP1003-11		
			INRGREF add 0055	GWOSP1004-11		
			INRGREF add 0049	GWOSP998-11		
			INRGREF add 0019	GWOSP968-11	Sejnane	
			INRGREF add 0020	GWOSP969-11		
			INRGREF add 0021	GWOSP970-11		
			INRGREF add 0022	GWOSP971-11		
pupa	2	0				*Eucalyptus camaldulensis*
larval exuvia	1	1	INRGREF add 0017	GWOSP966-11	Nefza	*Quercus suber*
adult	8	8	INRGREF add 0076	GWOSP1025-11	Nabeul (Delhiza)	*Quercus coccifera*
			INRGREF add 0067	GWOSP1016-11		
			INRGREF add 0066	GWOSP1015-11		
			INRGREF add 0063	GWOSP1012-11		
			INRGREF add 0065	GWOSP1014-11		
			INRGREF add 0064	GWOSP1013-11		
			INRGREF add 0069	GWOSP1018-11		
			INRGREF add 0068	GWOSP1017-11	Sejnane	

Figure 2. Sampling data, GenBank accession numbers, sites, host-plant and Barcode sequences of *Orgyia* specimens.

Larval phenotypic variability and host-plant relationships

Each collected individual at the larval stage was assigned to a larval phenotype according to colouration of setae and tubercles. We furthermore noted each host-plant species from where eggs, females and larvae were collected (figure 2).

Correlation between genetic haplotypes and larval phenotypes such as host-plant species were tested using Fisher's exact test contingency tables.

Dissection of genitalia

Six males (5 from Nabeul and 1 from Sejnane) and 4 females (2 from Nabeul and 2 from Sejnane) were used for genitalia dissection as described by Robinson (1976). Dissection was done in the Lepidoptera section of the ZSM with the help of Dr. Andreas H. Segerer.

Results

Molecular diagnosis

A total of 46 barcode sequences belonging to four haplotypes were obtained from 59 specimens of Tunisian *Orgyia* species. All except four which were longer than 600 bp (GenBank accession numbers in figure 2).

The Tunisian *O. trigotephras* specimens were found to be well structured in two homogeneous CO1-clusters,

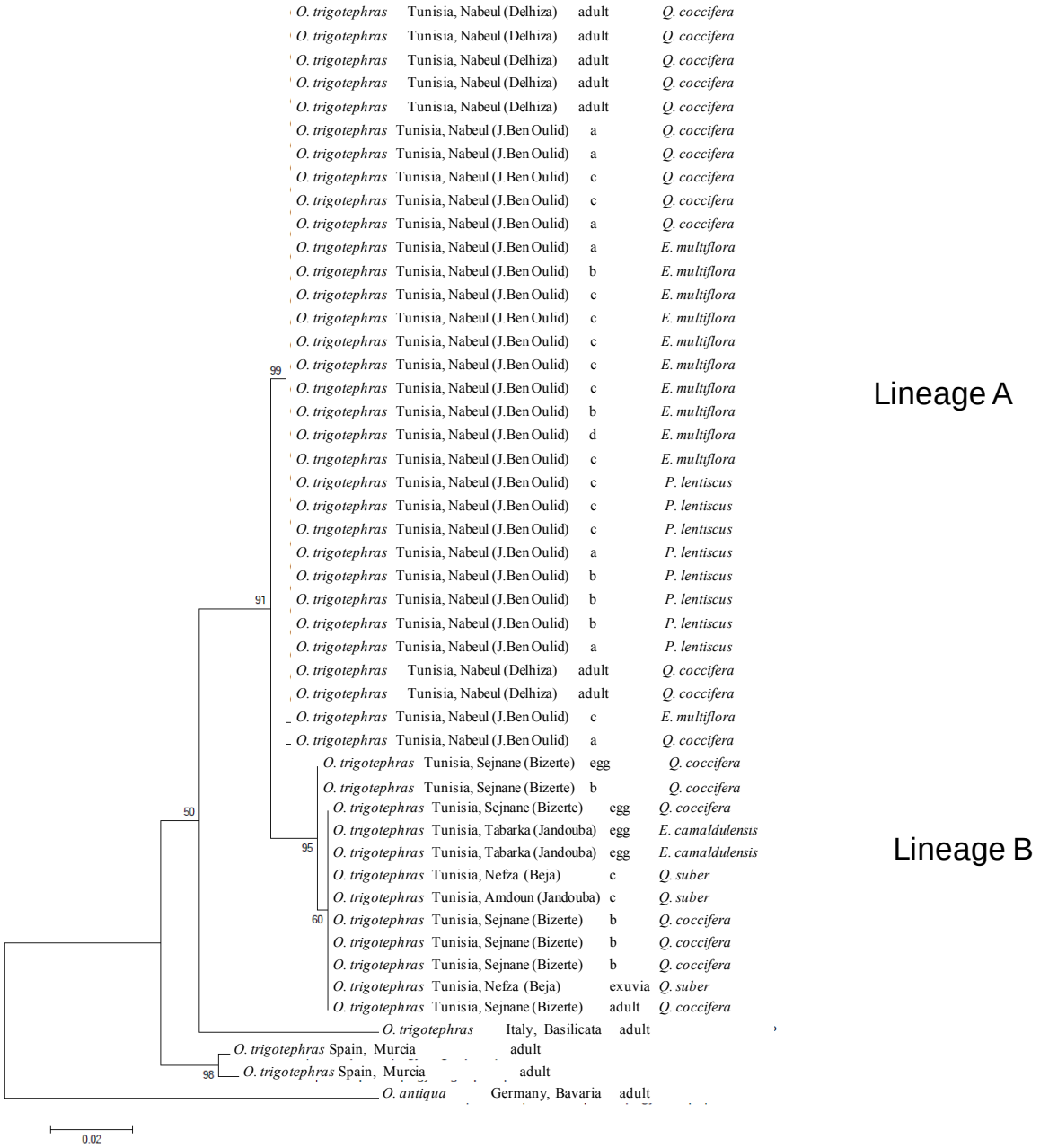

Figure 3. Maximum Likelihood (ML) Tree including 50 *Orgyia* specimens (46 from Tunisia), constructed with MEGA6 (Tamura *et al*., 2013), bootstrap method, 500 replicates, Tamura-Nei model, complete deletion, bootstrap values indicated when >50%.

Figure 4. Morphological aspect of Tunisian *O. trigotephras* larvae in the L5 stage. **a)** The long lateral pencils and the four dorsal tussocks on thorax and first abdominal segments are yellow, the two dorsal spots on segments A6 and A7 are orange; **b)** The long lateral pencils and the four dorsal tussocks on thorax and first abdominal segments are yellow, the two dorsal spots on segments A6 and A7 are yellow; **c)** The long lateral pencils and the four dorsal tussocks on thorax and first abdominal segments are white, the two dorsal spots on segments A6 and A7 are yellow; **d)** The long lateral pencils and the four dorsal tussocks on thorax and first abdominal segments are white, the two dorsal spots on segments A6 and A7 are orange. (Photos Olfa Ezzine).
(In colour at www.bulletinofinsectology.org)

separated by a minimum pairwise distance of 1.0% (figure 1). The two genetic clusters, hereafter named lineages A and B, are geographically separated: Lineage A was found in the province of Cap Bon (Nabeul), in the East, whilst lineage B was collected in the western provinces only, so far. The closest genetic neighbour to the Tunisian specimens were the two barcoded Spanish specimens (minimum pairwise distance 3.6%). The specimen of *O. trigotephras* from southern Italy diverged at a greater genetic distance (5.8%). The genetic distance of *O. antiqua* which was chosen as outgroup was 13.9% (figure 3).

Morphological diagnosis and host use

Within the Tunisian populations four larval phenotypes were differentiated, based on coloration of pencils, lateral 'hairs' (setae) and dorsal spots (figure 4). Larvae in the L5 stage are dark coloured, with orange warts. The long lateral pencils and the four dorsal tussocks on thorax and first abdominal segments are either yellow (figure 4a, 4b) or white (figure 4c, 4d), the two dorsal spots on segments A6 and A7, are either orange (figure 4a, 4d) or yellow (figure 4b, 4c), without being correlated with coloration of lateral and dorsal pencils.

Morphology of male genitalia did not reveal any significant and constant difference between individuals and sites. Since the female bursa copulatrix easily gets damaged during dissection, we were unable to compare female genitalia properly.

No association was found between genetic haplotype, adult morphology (genitalia) and larval appearance. Lineage A contains all four larval phenotypes (figure 3), whereas lineage B is includes only the two phenotypes b and c (figure 3). Phenotypes b and c were the most abundant in both lineages. Only one individual of phenotype d was barcoded, being thus excluded from further analysis. Fisher's exact test contingency table, 2 (lineages) × 3 (phenotypes), showed no significant difference in the distribution of larval phenotypes between the two lineages ($p = 0.208$).

Larvae, pupa and eggs (female oviposition) were mainly found on *Q. coccifera*, *P. lentiscus*, *Erica multiflora* and *Q. suber* (figure 3). Two female individuals were found on the introduced, Australian tree species *Eucalyptus camaldulensis*. Fisher's exact test showed significant differences in the native host-plant choice between the two CO1-clusters ($p = 0.005$). Whereas lineage A was found on *Quercus* sp., *P. lentiscus* and *E. multiflora*, individuals of lineage B were collected from *Quercus* sp.. No association between larval phenotypes and host species was found ($p = 0.171$).

Discussion and conclusions

The CO1 (barcode) marker revealed to be most useful for rapid and objective identification and delineation of subspecies, species and species-groups (Hebert *et al*., 2003). Just a very few young species pairs show genetic distances of less than 2% in the CO1 gene or are even barcode sharing (Hausmann *et al*., 2011a; 2011b; Hausmann and Viidalepp, 2012). Mean intraspecific divergences are usually less than 1%. The mean intraspecific divergence of 1000 North American Lepidoptera species was found to be at 0.43% (Hebert *et al*., 2010). Intraspecific barcode variation of species accepted by traditional taxonomy occasionally exceeds 2%, but often points to cryptic species (Hebert *et al*., 2004) or to geographically isolated (allopatric) populations, reflecting their evolutionary history (Hebert *et al*., 2003; Mutanen *et al*., 2012). DNA Barcoding of Tunisian *Orgyia* specimens clearly yields two distinct clusters at a genetic distance of 1.0% (figure 3), whilst Italian and Spanish specimens diverge from the Tunisian populations by 5.8% and 3.6%, respectively. These data suggest the possibility that the individuals of the three countries may not be conspecific. Further comprehensive, integrative taxonomic study is needed to verify the existence of several cryptic species within the *O. trigotephras* species-complex.

Since sequences of a limited number of specimens from three countries only were available for the present study a substantial increase of the sample size and geographical coverage is required for a comprehensive integrative taxonomic analysis of the *O. trigotephras* species-complex. Further study is required to test the hypothesis that the populations of Morocco and Algeria (*O. trigotephras anceps*) are genetically intermediate between those of Tunisia and those of the Iberian Peninsula. Considering the immobility of adult female sand the maternally inherited mtDNA, human activities (cf. historical routes of commerce through North Africa to the Iberian Peninsula: Constable, 1996), may have reshaped evolutionary genetic patterns more than due to the dispersal ability of the insect itself. Literature data show divergences in the host-plant use of the *O. trigotephras* complex. In north-western Tunisia, where cork oak forests are dense and defoliators are principally feeding on tree oak species (*Q. suber*, *Quercus afares* and *Quercus canariensis*). *O. trigotephras* is rarely found (Mannai *et al*., 2012). In these oak forests other defoliators such as *Lymantria dispar* L. 1758, *Catocala nymphagoga* Esper 1787, *Dryobotodes monochroma* Esper 1790, *Erannis defoliaria* Clerck 1759 and *Operophtera brumata* L. 1758, are much more abundant (Mannai *et al*., 2012). In the North East, dense cork oak forests are replaced by Mediterranean maquis, where *O. trigotephras* is found in higher abundance, mainly feeding on shrub species (*Halimium halimifolium*, *Cistus* sp., *Q. coccifera*, *P. lentiscus*, *Erica arborea* and *E. multiflora*) (Ezzine *et al*., in preparation). In these ecosystems only one defoliator *Anacampsis scintillella* Fischer von Roeslerstamm 1841 was found to compete in numbers with *O. trigotephras* (Ezzine, personal observation). A lack of competitors may have favoured the higher abundance of *O. trigotephras* in these ecosystems, but other ecological or genetic adaptations to these ecosystems and Mediterranean plant species may have also played a role. From present data, the two genetic lineages differed significantly in their host-plants. It cannot be excluded, however, that such an apparent preference is just a result of different availability of host-plant species in certain regions. Further studies are needed to verify if host-plant preferences are really different between the two lineages. This question may be tested in future by feeding experiments.

We conclude that there is considerable cryptic genetic diversity within the *O. trigotephras* complex which may find its origin in the diversity of vegetation structures, habitats and microclimates found in the Mediterranean basin and the immobility of females. Two subspecies of *O. trigotephras* were listed for the fauna of Tunisia, *O. trigotephras transiens* and *O. trigotephras anceps* (Chnéour, 1955). For these two taxa Oberthür (1916) reports different feeding preferences from observations in Tunisia and Morocco. Our study corroborates the existence of two genetically differentiated lineages associated with different plant communities. Further studies, including samples from Algeria and Morocco are needed to confirm the presence of the two taxa on these regions, its distribution and host plants. Integrative taxonomic studies, feeding experiments, hybridization experiments, and studies on predators and parasitoids are further needed for a better understanding of the systematic and the ecology within the *O. trigotephras* complex.

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