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Distribution of two pine processionary moth species in Turkey evidences a contact zone

Kahraman İPEKDAL^{1,4}, Christian BURBAN², Carole KERDELHUÉ³, Selim Süalp ÇAĞLAR⁴

¹Department of Biology, Faculty of Science and Literature, Ahi Evran University, Kırşehir, Turkey

²INRA, UMR 1202 BIOGECO (INRA/Université de Bordeaux), Cestas Cedex, France

³INRA, UMR CBGP (INRA-IRD-CIRAD-Montpellier SupAgro), Montpellier sur Lez Cedex, France

⁴Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey

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Abstract: The pine processionary moths *Thaumetopoea pityocampa* and *T. wilkinsoni* are among the most important Mediterranean pine defoliators. Although recent studies suggest that the two species may coexist in Turkey, the relevant literature lacks precise information on the occurrence and exact distribution of the two species in this country. In particular, whether the two sister taxa form a contact zone is still unknown. In this study, we aimed to reveal the limits of the ranges of the two species in Turkey by using nuclear (ITS-1, photolyase) and mitochondrial markers (COI). Multilocus molecular characterization of the two species showed for the first time that the two species co-occur in some of the sampling sites. In addition, we determined clear signatures of mitochondrial introgression between the two species, and therefore the existence of a possible hybrid zone within Turkey. These results bring up interesting questions relating to the evolution of the identified hybrid zone.

Key words: Pine processionary moth, *Thaumetopoea pityocampa*, *T. wilkinsoni*, mitochondrial introgression, asymmetric hybridization, contact zone, Turkey

1. Introduction

The pine processionary moth (Lepidoptera, Notodontidae), hereafter PPM, is an economically important pine pest in the Mediterranean forests (Masutti and Battisti, 1990), as its larvae feed on needles of pines, and its urticating larval setae cause severe allergy, especially in mammals (Vega et al., 1997; Rodríguez-Mahillo et al., 2012). PPM larval development generally occurs through the winter. The gregarious larvae, if not feeding, rest in silken nests that they build on the tips of pine branches in winter and pupate underground in summer. Adults emerge at the end of summer, live a few days, and die after mating and laying eggs on pine needles (Démolin, 1969).

PPM has been known in Turkey for a long time. The oldest record belongs to Albert Kindermann, a German lepidopterist, who made an expedition in northern Turkey between 1848 and 1850 (Lederer, 1885). We also found records of control efforts in İstanbul in 1904 in the Ottoman archives. Numerous records are available from the Republic period, especially after the 1940s (Acatay, 1953; Sekendiz and Varlı, 2002). Thus, we are confident that PPM has been present for at least 165 years in northern Anatolia.

The PPM species found in Turkey was thought to be *Thaumetopoea pityocampa* (Denis and Schiffermüller, 1775), as in Europe and western North Africa, until Salvato et al. (2002) and Simonato et al. (2007) showed that the species occurring in southern and northern Turkey was actually its sister species, *T. wilkinsoni* Tams, 1924, which is distributed in Anatolia, Cyprus, Crete, and the Middle East (Kerdelhué et al., 2009). Yet, no sample from northwestern Turkey was included in this work.

Kerdelhué et al. (2009) were the first to mention that the ranges of the two species could intersect in Turkey. Due to insufficient sampling in northwestern Turkey, and the use of a single mitochondrial marker, the possibility of a contact zone between *T. pityocampa* and *T. wilkinsoni* was still questionable. Therefore, the objective of the present work is to determine whether a contact zone exists in northwestern Turkey, which is Anatolia's only continental connection to Europe. Furthermore, due to their similar morphologies, habitat requirements, reproductive behaviors, and pheromone chemical structures, Frérot and Démolin (1993) suggested that the two sister species could be crossed to produce hybrids under laboratory conditions. Therefore, we also aimed to search for signs

* Correspondence: kipekdal@gmail.com

of a possible past or recent hybridization between the two species in Turkey. To achieve this goal, we sampled PPM all over Turkey and characterized one mitochondrial and two nuclear markers to: (1) precisely describe the distribution range of both species in Turkey for the first time; and (2) possibly identify signs of introgression between them, which would translate into incongruent molecular identification depending on the marker used for the introgressed individuals.

2. Materials and methods

2.1. Sampling and DNA protocols

Eggs and larvae of PPM were collected from 22 locations in Turkey and from 2 locations in Cyprus (Figure 1; Table 1) between 2008 and 2010. Collected egg batches were kept at room temperature until hatching, and then first instar larvae were transferred to 70% ethanol. When larvae were already present in the field, they were directly sampled in the nests and transferred to 70% ethanol. All samples in ethanol were stored at -20°C . DNA was extracted from the whole larval body for the first instars and from the head for later instars by using a standard chloroform-isoamyl alcohol procedure, as described in Gemmell and Akiyama (1996).

From the 24 locations visited, 47 separate nests and egg batches were collected, and one single individual was studied per colony to avoid sampling siblings. Each individual was amplified and sequenced for a 810-bp region of the mitochondrial COI gene using the primers Jerry and Pat (Simon et al., 1994), a 510-bp region of the ITS-1 gene using the primers ITS-1-F and -R (Vogler and DeSalle, 1994), and a 660-bp region of the photolyase gene using the primers described in Simonato et al. (2013). All PCR amplifications were performed in 25 μL reactions. Annealing temperatures for COI, ITS-1, and photolyase were 48, 50, and 50 $^{\circ}\text{C}$, respectively. The concentration and

purity of amplified DNA were estimated using NanoDrop 8000 v. 2.0. Sequencing was performed on an ABI PRISM 3130 genetic analyzer using BigDye Terminator chemistry (Applied Biosystems). In order to compare sequences obtained in this study with reference sequences, and thus identify specimens using molecular taxonomy, we downloaded previously published sequences from GenBank. Concerning the COI gene, we added sequences corresponding to *T. pinivora* (Sweden, GenBank accession number: EF185144.1), *T. wilkinsoni* (Israel, EF210075.1), and *T. pityocampa* (France, EF185138.1). For the ITS-1 marker, we downloaded haplotypes corresponding to *T. wilkinsoni* (Cyprus, EF189687.1) and *T. pityocampa* (France, EF189685.1). Finally, photolyase sequences corresponding to *T. pinivora* (Sweden, JX182492), *T. wilkinsoni* (Turkey, JX182496.1), and *T. pityocampa* (Italy, JX182493) were also added to the data.

2.2. Data analyses

For each gene, consensus sequences were reconstructed from both strands using CodonCode Aligner 1.63 (Ewing et al., 1998) and aligned using the Clustal W 2.0.11 algorithm (Thompson et al., 1994) in BioEdit 7.1.3 (Hall, 1999).

Nucleotides, amino acid composition, and genetic distances were assessed assuming the Jukes-Cantor model sequence evolution (Jukes and Cantor, 1969) with 10,000 bootstraps in MEGA 5 (Tamura et al., 2011). The best-fit model was determined by using the model check option in MEGA 5. A 95% confidence statistical parsimony network was constructed using TCS 1.21 (Clement et al., 2000).

Evolutionary distances were calculated as the number of base substitutions per site by averaging over all sequence pairs within and between each species. Divergence within species was estimated by using COI, ITS-1, and photolyase haplotypes of *T. pityocampa* and *T. wilkinsoni*; divergence between species was estimated by using COI, ITS-1, and

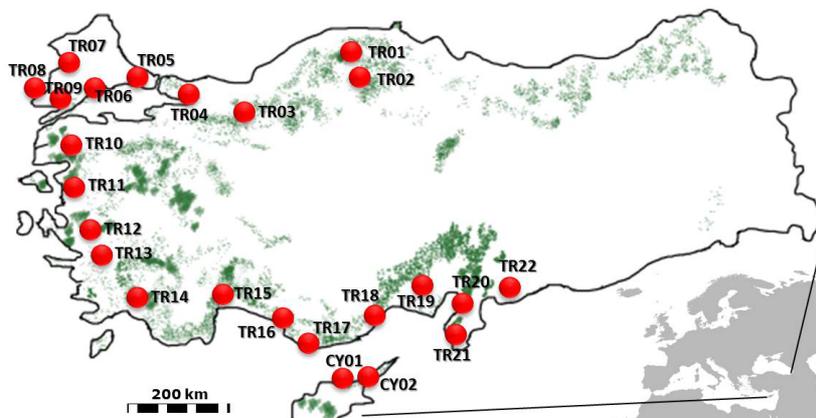


Figure 1. Map of sampling locations and coniferous forests in Turkey and Cyprus (forest data is from EC-JRC Forest Map, 2006).

Table 1. Sampling details.

Locality code	Date	Coordinates		Alt. (m)	Number of sequenced ind.s	Host (<i>Pinus</i>)	Location
		N	E				
TR01	04.2010	41°21'52	33°45'31	850	3	<i>nigra</i>	Kastamonu
TR02	04.2010	41°00'32	34°02'13	838	1	<i>nigra</i>	Tosya
TR03	12.2008	40°45'26	31°34'46	853	2	<i>nigra</i>	Bolu
TR04	03.2009	40°48'04	29°57'04	276	1	<i>nigra</i>	Gündoğdu
TR05	12.2008	41°03'31	28°28'12	91	2	<i>nigra</i>	Kumburgaz
TR06	12.2008	40°40'40	27°04'58	167	2	<i>brutia</i>	Şarköy
TR07	12.2008	41°23'27	26°48'00	106	2	<i>nigra</i>	Kırcasalih
TR08	12.2008	40°44'27	26°43'09	125	2	<i>brutia</i>	Korudağı
TR09	12.2008	40°34'29	26°49'50	35	2	<i>brutia</i>	Körfez
TR10	09.2008	39°28'26	27°21'39	381	1	<i>brutia</i>	Korucu
TR11	09.2008	38°51'01	27°00'59	28	3	<i>nigra</i>	Kurfalı
TR12	09.2008	38°13'51	26°41'25	90	2	<i>brutia</i>	Demircili
TR13	09.2008	37°56'05	27°20'57	38	2	<i>brutia</i>	Selçuk
TR14	09.2008	36°46'32	28°50'45	110	1	<i>brutia</i>	Muğla
TR15	03.2010	36°56'41	30°53'26	12	2	<i>brutia</i>	Antalya
TR16	03.2010	06°20'23	32°12'52	52	2	<i>brutia</i>	Alanya
TR17	03.2010	36°02'10	32°47'15	155	2	<i>brutia</i>	Anamur
TR18	03.2010	36°18'01	33°51'03	22	3	<i>brutia</i>	Silifke
TR19	03.2010	36°56'46	34°49'39	176	3	<i>brutia</i>	Tarsus
TR20	03.2010	36°32'25	36°09'03	151	2	<i>brutia</i>	İskenderun
TR21	03.2010	36°08'48	36°04'18	100	2	<i>brutia</i>	Hatay
TR22	03.2010	36°48'32	36°58'22	444	2	<i>brutia</i>	Kilis
CY01	10.2008	35°19'03	33°35'06	436	2	<i>brutia</i>	Girne
CY02	10.2008	35°18'02	33°34'05	410	1	<i>brutia</i>	Girne

photolyase haplotypes of *T. pityocampa* and *T. wilkinsoni* and COI and photolyase sequences of *T. pinivora* downloaded from GenBank (accession numbers are given above). Analyses were conducted using the Jukes–Cantor model (Jukes and Cantor, 1969) with 10,000 bootstraps. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA 5 (Tamura et al., 2011).

Reference sequences downloaded from GenBank were aligned together with sequences obtained from the present study by using the Clustal W 2.0.11 algorithm (Thompson et al., 1994) in BioEdit 7.1.3 (Hall, 1999). Species identification was conducted by checking the sequences manually and grouping them according to their similarity.

Chromatograms were investigated for traces of hybridization by using BioEdit 7.1.3 (Hall, 1999). In the case of recent or ongoing hybridization, two divergent sequences can be found in a single nucleus for a single marker. This means that a gene sequence belonging to

a hybrid nucleus has two of the parental nucleotides together, and relevant chromatograms have double peaks at polymorphic nucleotide positions (Lefebvre et al., 2008). Finally, mtDNA and nuclear DNA sequences were compared for each individual to discriminate pure individuals that have congruent mitochondrial and nuclear sequences from introgressed individuals, i.e. those exhibiting incongruent species assignment between markers, as suggested by Hansen et al. (2000). Thus, we defined an individual as introgressed if the three markers concerning their specific assignment for that individual are not congruent.

Maximum-likelihood, neighbor-joining, and maximum-parsimony methods were performed separately to reconstruct phylogenetic trees of haplotypes and alleles for each of the three markers (COI, ITS-1, and photolyase) by using MEGA 5 (Tamura et al., 2011). Bootstrap probabilities were obtained with 10,000 replicates. The obtained trees were visualized using TreeGraph 2 (Stöver and Müller, 2010).

3. Results

From the 47 specimens sequenced, 42 COI, 31 ITS-1, and 41 photolyase sequences were clear enough to be reliably used in subsequent analyses.

3.1. Genetic diversity, distance, and taxonomic identification

Summary statistics regarding nucleotide composition and diversity of obtained sequences are given in Table 2. The total number of COI haplotypes was 21 in *T. wilkinsoni* (*w*), while only a single haplotype was found in *T. pityocampa* (*p*). Within *T. wilkinsoni*'s range, haplotype diversity seems

to be higher in the southern regions (18/22) and decreases towards western and northern Turkey (4/22) (Figure 2a). Low diversity was observed for nuclear markers ITS-1 and photolyase, with only 3 (2 *w*, 1 *p*) and 2 (1 *w*, 1 *p*) alleles, respectively (Figures 2b and 2c).

According to the observed genetic distances, which are shown in Table 3, mitochondrial divergence within and between species was larger than divergence observed within and between species for the two studied nuclear sequences. Divergence between *T. pityocampa* and *T. wilkinsoni* (COI 0.0890; photolyase 0.0090) was smaller

Table 2. Nucleotide composition and diversity parameters.

Gene	NC	PI	S	Pi	K
COI	A+T (69.04%)	108	124	0.04931 (±0.00187)	38.071 (±12.858)
ITS-1	G+C (51.13%)	21	21	0.01764 (±0.00107)	8.538 (±2.936)
Photolyase	A+T (61.38%)	6	12	0.00392 (±0.00035)	2.323 (±0.791)

NC: nucleotide composition
 PI: number of parsimony informative sites
 S: number of polymorphic sites

Pi: nucleotide diversity
 k: average paired differences among sequences

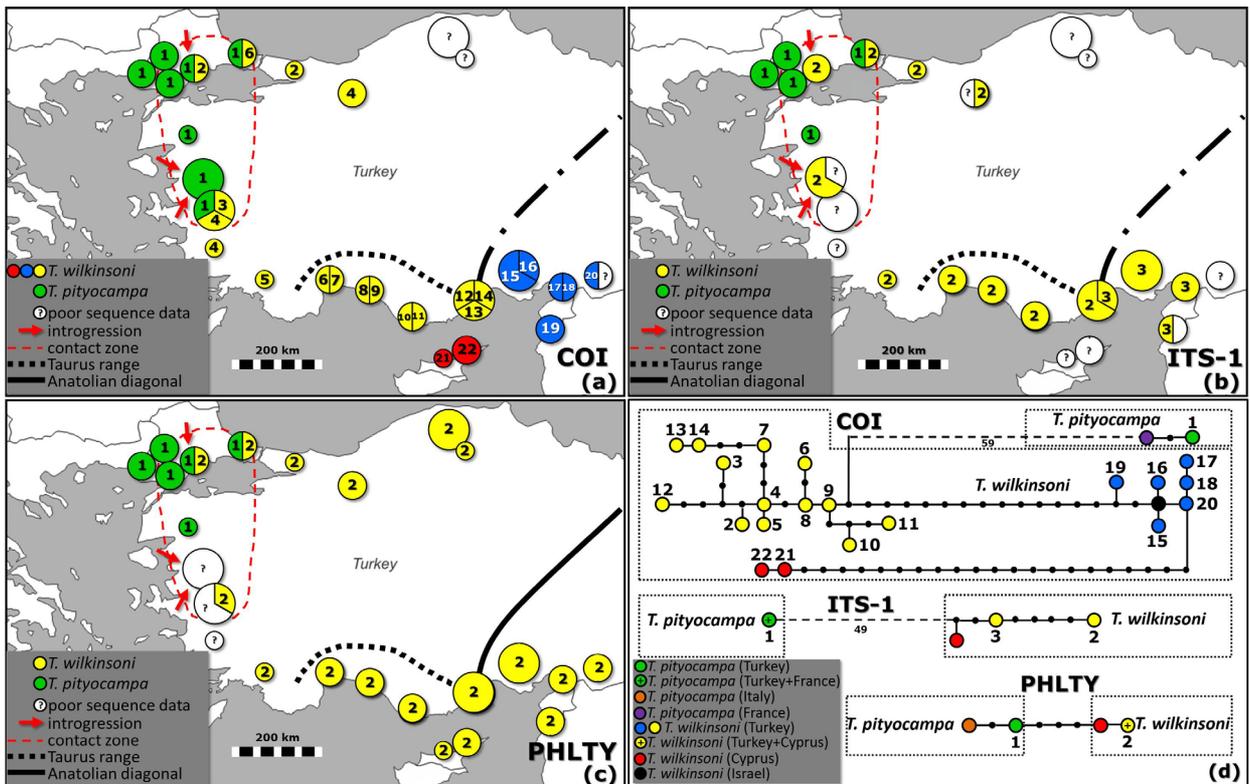


Figure 2. Distribution maps of a) COI, b) ITS-1, and c) photolyase haplotypes. Haplotypes are colored and numbered in accordance with the network. Color and sign codes are given in the legends on the maps. d) Haplotype networks for COI, ITS-1, and photolyase. Numbers on the dashed lines indicate how many mutations separate two relevant haplotypes.

Table 3. Estimates of average molecular distance within and between species regarding the three markers studied.

Distance within species			Distance between species		
	D	SE		D	SE
COI			COI		
<i>T. pityocampa</i>	0	0	<i>T. pityocampa</i> - <i>T. wilkinsoni</i>	0.0890	0.0190
<i>T. wilkinsoni</i>	0.0270	0.0050	<i>T. pityocampa</i> - <i>T. pinivora</i>	0.1670	0.0320
			<i>T. wilkinsoni</i> - <i>T. pinivora</i>	0.1300	0.0240
ITS-1			ITS-1		
<i>T. pityocampa</i>	0	0	<i>T. pityocampa</i> - <i>T. wilkinsoni</i>	0.0370	0.0090
<i>T. wilkinsoni</i>	0.0003	0.0002			
PHTLY			PHTLY		
<i>T. pityocampa</i>	0.0006	0.0004	<i>T. pityocampa</i> - <i>T. wilkinsoni</i>	0.0090	0.0040
<i>T. wilkinsoni</i>	0.0001	0.0001	<i>T. pityocampa</i> - <i>T. pinivora</i>	0.0190	0.0060
			<i>T. wilkinsoni</i> - <i>T. pinivora</i>	0.0240	0.0070

D: evolutionary divergence, SE: standard error.

than that between *T. pityocampa* and *T. pinivora* (COI 0.1670; photolyase 0.0190) and *T. wilkinsoni* and *T. pinivora* (COI 0.1300; photolyase 0.0240).

By comparing the sequences obtained from this study with the reference sequences downloaded from GenBank, 13 COI, 8 ITS-1, and 9 photolyase alleles were grouped together with reference sequences from *T. pityocampa*, whereas 29 COI, 23 ITS-1, and 32 photolyase alleles were grouped together with reference sequences from *T. wilkinsoni*. By further comparing individuals according to the three markers studied, 8 individuals were identified as *T. pityocampa*, 27 as *T. wilkinsoni*, and 3 as introgressed (Table 4). The taxonomy of nine individuals remained unknown owing to inferior sequence quality.

Different phylogeny reconstruction methods yielded similar topologies, summarized in the phylogenetic trees in Figure 3.

3.2. Distribution of PPM species and haplotypes in Turkey

Sequences obtained from the three gene regions were compared to the reference sequences of *T. pityocampa* and *T. wilkinsoni* for taxonomic identification. Our results showed that both *T. pityocampa* and *T. wilkinsoni* were present in Turkey. While most of the pine forests in southern, western, and northern Turkey are dominated by *T. wilkinsoni*, *T. pityocampa* is more prevalent in the Thrace region (European Turkey) (Figures 2a-2c and 4). However, they slightly enter each other's range. In

Table 4. Genetic assignation of the individuals studied and designation of introgressed individuals (Ia, Ib). Haplotype numbers correspond to the numbers on the haplotype distribution maps and haplotype network in Figure 2.

Locality	N	COI haplotype	ITS1 haplotype	Photolyase haplotype	Genetic assignation
Northwestern Turkey	8	1	1	1	P
Gelibolu	1	1	2	1	Ia
İzmir	2	1	2	-	Ib
Southeastern Turkey and Cyprus	27	2-22	2, 3	2	W

p: pure *T. pityocampa*; w: pure *T. wilkinsoni*; Ia, Ib: introgressed individual, N: number of individuals.

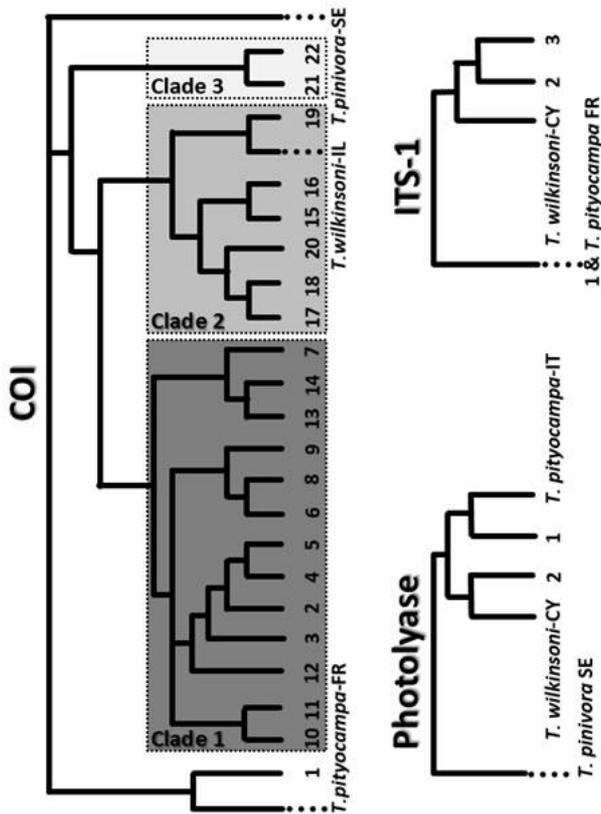


Figure 3. Consensus haplotype trees for COI, ITS-1, and photolyase. Three clades of *wilkinsoni* haplotypes are shown in shaded rectangles. All branches have bootstrap support values > 60%.

particular, *T. pinyocampa* is also seen in the northern Aegean region (western Turkey), and *T. wilkinsoni* is also seen in the Thrace region (European Turkey) around the Marmara Sea. *T. wilkinsoni* mitochondrial haplotypes were structured geographically into three clades (southern, western, and northern Turkey: yellow; southeastern Turkey: blue [closely related to the Israeli haplotype: black]; Cyprus: red, in Figures 2a and 2d) (Figure 3).

3.3. The contact zone, hybridization, and introgression

Although *T. pinyocampa* and *T. wilkinsoni* are mostly distributed in separate areas in Turkey, both species were found on adjacent trees at the same locality in the European part of İstanbul, northern Gelibolu, and İzmir. These localities were taken as the limits of the contact zone, which is depicted by an elliptical circle in Figures 2a–2c and Figure 4.

Evaluation of the chromatograms did not show any hybrid sequences. However, we found taxonomic incongruences between nuclear and mitochondrial markers for three individuals, one of which was found in Gelibolu and the other two in İzmir (Ia and Ib, respectively, Table 4 and Figure 4; I: introgression, a/b: different patterns found) (arrows in Figures 2a–2c). An individual from the Gelibolu sample was classified as *T. pinyocampa* according to mitochondrial and photolyase sequences, while this individual was classified as *T. wilkinsoni* according to ITS-1 sequences (Ia). On the other hand, two individuals from the İzmir sample were classified as *T. pinyocampa*, according to their mitochondrial haplotypes, and were classified as *T. wilkinsoni* based on nuclear genes (ITS-1 and photolyase) (Ib).

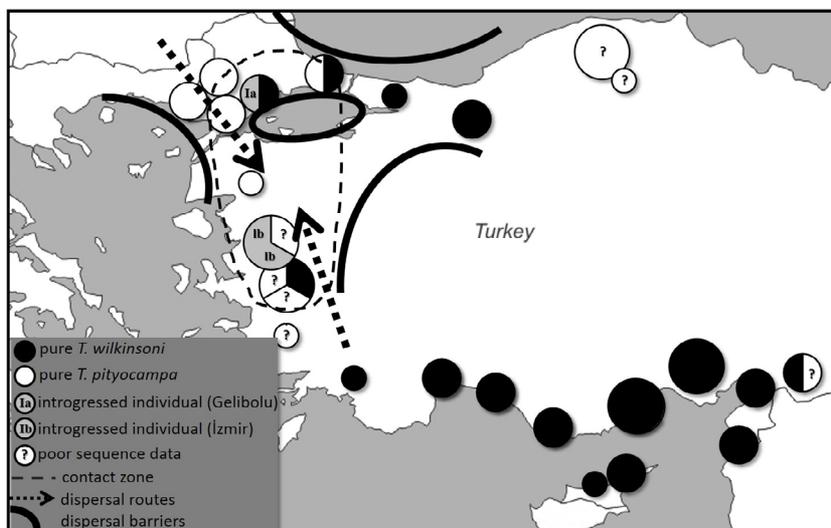


Figure 4. Distribution of pure *T. wilkinsoni* (black) and *T. pinyocampa* (white), introgressed individuals (gray with Ia and Ib), dispersal routes and barriers, and the contact zone. Color and sign codes are given in the map legend.

4. Discussion

4.1. Distribution, diversity, and geographic structure of PPM haplotypes in Turkey

One of our aims in this study was to determine the distribution and geographic limits of the two PPM species by using molecular methods. The haplotype distributions given in Figure 2 show that the ranges of *T. pityocampa* and *T. wilkinsoni* coincide in Turkey, a result that ends former discussions concerning the taxonomy of the PPM species within Turkey.

T. wilkinsoni populations showed significant structuring (similar to what was found in Simonato et al. (2007)) according to mitochondrial haplotypes. High structuring among mitochondrial haplotypes is expected in organisms such as PPM whose females are less mobile than males (Moritz et al., 1987; Salvato et al., 2002). This low mobility along with geographical barriers such as the Mediterranean Sea and the Anatolian Diagonal (the massive mountain range extending from south to northeast in Turkey) explains most of the observed structure among mitochondrial haplotypes.

The dominant pine species in Turkey are *Pinus brutia*, distributed mainly in southern and western Turkey, and *P. nigra*, found mainly in northern Turkey with partial scattered populations in the south. However, no correlation has been found between the distributions of PPM and the host plant species.

Our results suggest a decreasing trend in genetic diversity towards northern latitudes. Although more extensive samplings are necessary to confirm this, such a trend would not be surprising because an increasing number of studies are showing a reduction in genetic diversity from south to north due to postglacial recolonization events (Hewitt, 1996, 2003; Thompson, 2005; Pinho et al., 2007), a phenomenon known as “pioneer and phalanx” (Nichols and Hewitt, 1996).

4.2. The contact zone and possible hybridization between *T. pityocampa* and *T. wilkinsoni*

We found that the ranges of the two species intertwine in northwestern Anatolia. This overlap seems to have resulted in mitochondrial introgression between the two species that reveals itself as discordance between the nuclear and mitochondrial DNA data, a pattern which is not expected in pure individuals. This pattern can also appear as a result of incomplete lineage sorting (Knowles, 2007); however, it is not a convincing explanation in our case, because discordance between mitochondrial and nuclear topologies was found only in the contact zone, not in the entire range as would be expected under an incomplete lineage-sorting scenario (Buckley et al., 2006). Thus, the most plausible hypothesis for the observed pattern is the existence of a hybrid zone.

In this study, a few individuals identified as *T. wilkinsoni* according to nuclear genes were found to carry mitochondrial

genes from the *T. pityocampa* clade. However, the opposite situation was never observed (i.e. *T. pityocampa* individuals carrying mitochondrial haplotypes from *T. wilkinsoni*). Therefore, we conclude the presence of an asymmetric pattern of mitochondrial introgression from the northwestern *pityocampa* clade to the southeastern *wilkinsoni* clade. However this conclusion needs to be clarified by using a larger sample of individuals in order to see if the asymmetric introgression observed in this study is the common pattern or was a chance encounter. We also found an individual (in Gelibolu) which has a discordance between photolyase and ITS-1 haplotypes (i.e. an individual classified as *T. pityocampa* according to mtDNA and photolyase sequences and carrying ITS-1 sequences from *T. wilkinsoni*). This presents further evidence of hybridization between species and hints at the possibility of finding more individuals with mosaic genomes. Further studies using a large panel of markers (i.e. genes) are needed to confirm these findings.

Contact zones often vary in width from a few hundred meters to hundreds of kilometers depending on how they are maintained and the selective forces prevailing in and out of the zone (Barton, 1982; Barton and Hewitt, 1985; Howard, 1986; Moore and Price, 1993). The contact zone that we found in this study is relatively narrow. If our results are not a reflection of sampling bias, the low dispersal capacity of PPM could explain the relatively narrow width of this contact zone and why it has not spread out. However, questions on the selective forces maintaining this hybrid zone and their possible effect on speciation and diversification remain unknown. Extensive sampling from the contact zone and characterization of a wide range of molecular markers, in addition to morphological, demographic, and ecological properties of the two species, are needed in order to better understand and quantify the evolutionary forces acting in this contact zone.

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