Comparison of genetic structure of native and commercial Bombus terrestris populations in the Mediterranean region

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Abstract

B. terrestris dalmatinus is native and the most widespread Bombus terrestris subspecies which occurs at different altitudes and habitats in the Mediterranean region where commercial B. terrestris colonies have been used for pollination in greenhouses crops for more than two decades. In this study, we sampled B. terrestris from greenhouses (commercial populations, CP1-CP7), within the five km areas surrounding the greenhouses (AK, KM, DM and GB) and more distant native populations (TM, BB and FS) in the Mediterranean region to determine the genetic structure of native and commercial B. terrestris populations and to better understand genetic introgression of commercial greenhouse B. terrestris populations into the native B. t. dalmatinus populations using twenty microsatellite markers and two mitochondrial genes (COI and cyt b). Microsatellite analysis showed adequate levels of genetic diversity in both commercial and native populations. However, populations could not been clearly separated from each other (FST = 0.014-0.045; p>0.05). All populations deviated from Hardy-Weinberg equilibrium due to high inbreeding (mean Fis: 0.234). In the mitochondrial analyses, we found two haplotypes for COI and six haplotypes for cyt b gene. Genotypes belonging to the subspecies B. t. dalmatinus were determined in all populations, while genotypes belonging to the subspecies B. terrestris terrestris were determined in some commercial populations and nearby greenhouses populations. Our results thus indicate that native populations are threatened by gene flow from non native B. t. terrestris subspecies. Our data also suggest that aestivated native FS population should be primarily conserved to avoid extinction.

Introduction

Bumblebees are very important pollinators of both crops and native plants and consist of 250 species worldwide (Michener, 2000; Williams, 1998). After the improvements in mass rearing methods, bumblebees are increasingly used for pollination of crops, primarily for tomato pollination in greenhouses (Velthuis & van Doorn, 2006). It is estimated that more than two million commercially reared bumblebee colonies are used for the pollination globally each year (Lecocq, Coppee, Michez, Brasero , Rasplus, Valterova, & Rasmont, 2016). Currently, five species of bumblebees are reared commercially. The main species is the buff-tailed bumblebee, *Bombus terrestris* L. It is naturally distributed mainly in Europe, in coastal North Africa, and in West and Central Asia. Since the late 1980s, commercially reared*B. terrestris* colonies have been transported to many countries, in every continent except Australia and North America (Rasmont, Coppee, Michez, & Meullemeester, 2008; Velthuis & van Doorn, 2006). Shortly after commercial introduction widely beyond its natural area of distribution, it was recognized that *B. terrestris* is invasive and may disturb local ecosystems including; competition for nest sites and food resources with local bee fauna, genetic contamination of local*Bombus spp* ., and spread of parasites and pathogens (Dafni, Kevan, Gross, & Goka, 2010).

B. terrestris comprises nine recognized subspecies which differ in morphology (particularly the pattern of coat colour), genetic, behavior, colony size, etc. and inhabit separate geographic regions, sometimes overlapping. Although many subspecies of *B. terrestris*were used in the early years of commercial rearing, today *Bombus terrestris dalmatinus* has become the main commercialized subspecies due to the suitable characteristics for mass rearing such as the large size of workers and high success rate of colonies. *Bombus terrestris terrestris*

is also currently produced by some companies. Commercial produced colonies probably originate from stocks collected from the Turkey, Greece and Balkan Peninsula (*B. t. dalmatinus*) and the northern Europe (*B. t. terrestris*) (Lecocq, Rasmont, Harpke, & Schweiger, 2016; Rasmont, Coppee, Michez, & Meullemeester, 2008; Velthuis & van Doorn, 2006).

There are many characteristics which may contribute to making *B. terrestris* invasive including; high migration ability, early seasonal emergence, high adaptability under adverse climatic conditions in various habitats, generalist or polylectic foraging strategies, thermoregulatory metabolism that allows it to be active at low temperatures, high reproductive abilities, being able to regulate life cycle in a year with or without hibernation or aestivation (Dafni, Kevan, Gross, & Goka, 2010). Additionally, commercial *B. terrestris* colonies produce more gynes (queens) and are better competitors than the local conspecific populations. (Gosterit & Gurel, 2005; Ings, Ward, & Chittka, 2006). A single commercial *B. terrestris* colony is capable of producing more than a hundred gynes (new queens) and males which potentially could escape from greenhouses. Therefore, commercially produced *B. terrestris* populations are able to establish and spread into the wild of both native and non-native regions. In fact, commercial greenhouse *B. terrestris* populations have become established outside its native range in many countries such as, New Zealand, Tasmania, Japan, Chile and Argentina (Buttermore, 1997; Goka, 2010; Montalva, Dudley, Arroyo, Retamales, & Abrahamovich, 2011; Morales, Arbetman, Cameron, & Aizen, 2013). Within its native range, the large-scale use of *B. terrestris* in greenhouse also raises concerns about the genetic introgression between commercial and native*B. terrestris* populations which include recognized subspecies or distinct populations. For instance, Kraus, Szentgyorgyi, Rozej, Rhode, Moron, Woyciechowski, & Moritz, (2011) in Poland found strong genetic introgression from the sampled greenhouse populations into the adjacent populations also found that more distant populations were much less affected by genetic introgression from the greenhouses. Goulson (2010) suggested that since the beginning of the bumblebee trade in the 1980s,*Bombus terrestris audax* which is endemic to Britain and Ireland may have already merged with commercial produced *B. t. dalmatinus*and *B. t. terrestris* through introgression resulting in a single population. Recently potential hybrids between managed *B. t. terrestris* /dalmatinus and *Bombus terrestris lusitanicus* (endemic in Iberian Peninsula) have been reported by Cejas, Ornosa, Munoz, & De la Rua, (2018) and Seabra et al. (2019).

B. t. dalmatinus is the only native *B. terrestris*subspecies that is found in Turkey. This subspecies of bumble bees are the most widespread and live at different altitudes and habitats in the country. Therefore, there are probably several ecotypes of this subspecies such as aestivated and hibernated populations, each adapted to specific ecological conditions; from the Mediterranean coastal areas to the high mountain conditions of the central Anatolian region. (Gurel, Gosterit, & Eren, 2008; Ozbek, 1997; Yeninar, Duchateau, Kaftanoglu, &Velthuis, 2000). On the other hand, commercially produced *B. terrestris* colonies began to use as a tomatoes pollinator in 1997. The number of commercial colonies used has increased enormously year by year and annually reached to almost 300 000 colonies in 2018 in Turkey. Because of the lack of regulations with respect to introduction of non - native *B. terrestris* subspecies, the movement of commercial *B. terrestris* colonies has largely been without risk assessment and poses a significant disease transmission and hybridization risk. The consequences of commercialization on wild populations, the extent of their spread, the degree of genetic introgression into wild populations are not well understood. Therefore, in this study we sampled *B. terrestris* from greenhouses (belonging to all local and global companies), within the 5 km areas surrounding the greenhouses and more distant native populations in the Mediterranean region to determine the genetic structure of native and commercial *B. terrestris* populations and to better understand genetic introgression of commercial greenhouse *B. terrestris*populations into the native *B. t. dalmatinus* populations using twenty microsatellite markers and two mitochondrial genes (*COI*and *cyt b*).

Materials and Methods

Sampling and DNA extraction

We sampled a total of 408 *B. terrestris* workers from fourteen*populations* in the Mediterranean region in Turkey (Table 1). To assess the genetic structure of native and commercial populations and the potential impacts of introducing non-native subspecies (or populations), we divided the sampled populations into

three groups;1) greenhouses (commercial) populations 2) nearby greenhouses and 3) far from the greenhouses populations. A total of 212 worker samples (one worker bee per commercial hive) from seven different commercial companies (belonging to all local and global companies, CP1-CP7) were collected from greenhouses populations. A total of 100 worker samples from four populations (AK, KU, DE and GB-at least 30 km away from each other) were collected within the 5 km areas surrounding the greenhouses where commercial *B. terrestris* colonies are intensively used for crop pollination. A total of 96 worker samples from three native populations (TM, FS, BB) were collected in the best preserved areas more than 30 km away from greenhouses and each other. In a previous study, Ozbek (1997) observed native *B. terrestris* populations all areas in which we collected field worker samples (seven locations) before the use of commercial *B. terrestris* colonies in Turkey. All sampled workers were stored in 95% ethanol solution at -18 ° C until DNA extraction. Total genomic DNA was extracted from thorax muscles of each worker bee using a 10% Chelex solution (Walsh, Metzger, & Higuchi, 1991).

Microsatellite analysis

A total of 408 samples belonging to 14 different populations were genotyped at twenty microsatellite markers (BT10, B11, B96, B100, B, B118, B119, B124, B126, BT26, BT28, B132, BT06, BT09, BT20, BTMS0033, BTMS0119, BTMS0131, BTMS0082, BTMS0124 and BTMS0045) previous developed for *B. terrestris* (Estoup, Scholl, Pouvreau, & Solignac, 1995; Estoup, Solignac, Cournet, Goudet, & Scholl, 1996; Reber-Funk, Schmid-Hempel, & Schmid-Hempel, 2006; Stolle, Rohde, Vautrin, Solignac, Schmid-Hempel, Schmid-Hempel,& Moritz, 2009). PCR amplification was performed in 20 µl volumes containing 2-2.5 µl of template DNA, 2 µl, 10X colorless buffer (Geneall), 1.2 µl HQ buffer (Geneall), 2 µl dNTPs (2.5mM), 0.3 µl (10 pmol) of each primer, and 2.5 U of Taq polymerase (Geneall). The PCR conditions consisted of an initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing temperature at 48-60 °C for 1 min, extension at 72 °C for 30 s and a final extension at 72 °C for 10 min. Allele sizing was performed using 96-part automatic capillary electrophoresis system (Fragment Analyzer-Advanced Analytical Technologies-AATI, Ames, Iowa, USA) by comparing alleles with an internal size standard (dsDNA 900 Reagent Kit, 35bp/500 bp).

mtDNA analysis

A total 140 samples (five samples per population) were used for *COI* and *cyt b* analysis. PCR primers CB1 (5'-TATGTACTACCATGAGGACAAATATC-3') and CB2 (5'-ATTACACCTCCTAATTTATTAGGAAT-3') were used to sequence 428 bp region of mitochondrial *cyt b* region (Jermiin & Crozier, 1994) while LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HC02198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primers were used to sequence 625 bp region of mitochondrial *COI* region (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). PCR amplifications were conducted with a 3 min denaturation at 94 °C followed by 35 cycles of

45 s denaturation at 94 °C, 1 min at 48 °C for *cyt b* and 47 °C for *COI* , 1.5 min at 72 °C. The final extension was applied at 72 °C for 10 min. Amplified products were purified and sequenced using forward and reverse primers.

Microsatellite and mtDNA data analysis

Allele size range and private alleles were determined using CONVERT version 1.31 (Glaubitz, 2004). Genetic diversity parameters such as number of alleles per locus (NA), number of private alleles (PA), observed (H_O) and expected (H_E) heterozygosities were calculated by using POPGENE version 1.31 (Yeh, Yang, Boyle, Ye, & Mao, 1997). Polymorphic information content (PIC) and inbreeding coefficient (F_{IS}) were calculated with Microsatellite Tollkit software (Park, 2001) and FSTAT version 2.9.3.2 (Goudet, 1995). Genetic differentiation tests between populations (pairwise F_{ST}) and molecular variance analyses (AMOVA) were conducted using ARLEQUIN (Excoffier, Laval, & Schneider, 2005). The genetic structures among populations and individuals were investigated using STRUCTURE version 2.3.1 (Pritchard, Matthew, & Donnelly, 2000).We estimated the optimal$K$ value using the ΔK statistic as described by Evanno, Regnaut, & Goudet (2005) in STRUCTURE HARVESTER (Earl & vonHoldt, 2012). In addition, GENETIX v 4.05

(Belkhir, Borsa, Chikhi, Raufaste, & Bonhomme, 2004) program was applied for Factorial Correspondence Analysis.

Sequences of mtDNA gene regions were aligned with CLUSTAL W2 (Larkin et al., 2007). mtDNA sequences were checked with reference sequences (Gen Bank accession number for *COI* and *cyt b* , respectively : GU085204.1, JQ820820.1) by using BLAST 2.2.20 (Zhang, Schwartz, Wagner, & Miller, 2000). DNA sequence alignment was performed by uploading all sequence information to the MEGA6 program (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) for both gene regions. Sequences for detection of SNP regions were uploaded to DnaSP v.5 (Librado & Rozas, 2009) software and each bee was then assigned to a mitochondrial haplotype. We compared mitochondrial haplotype frequencies in each population using ARLEQUIN 2.00 (Schneider, Roessli, & Excoffier, 2000). As a result of statistical analyzes intra and inter-group genetic variations (F statistics) and genetic distances of 14 *B. terrestris* populations were calculated. The genetic distance information of the populations was uploaded to SplitsTree 4.0 (Huson & Bryant, 2006) software, to conduct phylogenetic analysis and to construct Neigbour-Joining (NJ) tree. In order to determine the evolutionary ancestral origin of each individual in *B. terrestris* populations, the Maximum Likelihood (ML) method based on the Tamura-Nei model was used (Tamura & Nei, 1993). Trees were created in MEGA X program using 633 bp nucleotide sequences for 66 samples for *COI* gene region and 428 bp nucleotide sequences for 68 samples for *cyt b* region (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). *B. lucorum* , used as an out group (OG) in the present study, were retrieved from Gen- Bank (Accession number: JQ843492.1) to construct both NJ trees.

Results

Population structure based on microsatellite markers

In this study, we divided the populations into three groups; 1) greenhouses (commercial) populations 2) nearby greenhouses and 3) far from the greenhouses populations and assessed genetic structure of populations and groups. *PIC* values were higher than 0.5 in all studied loci (0.88 ± 0.08). All microsatellite loci were highly polymorphic, with 4–27 alleles per locus and an average of 18.30 alleles (Table 2; Table S1). F-statistic and gene flow (*Nm*) values for 20 microsatellite loci in all populations are given Table 2.F_{IT}, F_{IS} andF_{ST} values of the total population consisting of 14 subpopulations were determined as 0.244, 0.203 and 0.053, respectively in *B. terrestris* . These values indicated medium inbreeding and low genetic differentiation among *B. terrestris*populations. Gene flow (*Nm*) for the total population was found to be high (mean 4.9). This suggests that 5 out of every 100 individuals have migrated between populations. Allelic richness (A_R) values ranged from 8.95 to 10.30 among populations (Table 3). The lowest observed heterozygous (*Ho*) value (0.63) was detected in the second group, while the highest*Ho* value (0.75) was observed in the first group (commercial populations). The mean *Ho* and *He* were 0.68 and 0.86 respectively for all populations. The mean inbreeding coefficients were 0.18, 0.17 and 0.22 in group 1, group 2 and group 3, respectively (Table 3).

Pairwise F_{ST} values indicating the genetic differentiation between the sub-populations were given in Table 4**.** The lowest pairwise F_{ST} values were observed between TP5 and TP6 populations (0.014), while the highest pairwise F_{ST} values were determined between TP2 and TP5 (0.045). It was found that there was no significant difference between the populations in accordance with the pairwise F_{ST}values ($p > 0.05$). A large part of molecular variance (AMOVA, Table 5) was found within individuals (65%) rather than between individuals (32%) or between all populations (3%).

Based on the Factorial Correspondence Analysis (FCA), populations were divided into four main clusters. The first cluster consisted of TP1, TP2 and TP3 populations, the second cluster consisted of TP4, TP5 and TP6 populations, the third cluster consisted of TP7, KM, DM and GB populations, and the last cluster consisted of AK, BB and TM populations. The FS population was located between the third and fourth clusters (Fig. 1). The STRUCTURE results were consistent with the FCA. STRUCTURE HARVESTER suggested that $K = 4$ was the most plausible model (Fig. 2). Based on the model populations were divided into four clusters ($K = 4$). Cluster one consisted of TP1, TP2 and TP3 populations (in the purple colored),

cluster two consisted of TP4, TP5 and TP6 populations (in the green colored), cluster three consisted of TP7, KM, DM, GB and FS populations (in the orange colored) and cluster four consisted of AK, TM and BB populations (in the pink colored). Commercial populations were separated from wild populations except TP7.

Population structure based on mitochondrial data

We obtained the DNA sequences of of 633 bp from the *COI* gene and 428 bp from the *cyt b* gene and characterized five individuals randomly selected from each of the 14 population. The analysis of the *COI* gene revealed two different mitochondrial haplotypes in the 66 individuals sampled from 14 populations. The frequency of the haplotype A (GenBank accession no. KP670306.1) was 28.8 % (19 individuals). While haplotype A was common in commercial populations, haplotype B (GenBank accession no. KP670307.1) was common in native and nearby greenhouses populations. The frequency of the haplotype B was 71.2% (47 individuals).

Six different haplotypes (haplotype 1-6) were detected in 68 individuals sampled from 14 populations for the *cyt b* gene. The frequencies of the haplotype 1, 2, 3, 4, 5 and 6 were 7.3 % (5 individuals), 5.9 % (4 individuals), 11.8% (8 individuals), 63.2 % (43 individuals), 5.9 % (4 individuals), 5.9 % (4 individuals) respectively. When the sequences of the haplotypes were checked with the BLAST program in the Gene Bank, it was found that the haplotype 4 and haplotype 5 genotypes clustered within the *B. t. dalmatinus* subspecies. However, haplotype 1, haplotype 2, haplotype 3 and haplotype 6 genotypes clustered within both *B. t. terrestris* and *B. t. dalmatinus* subspecies. Maximum likelihood (ML) phylogeny inferred from *COI* and *cyt* b gene regions sequences of B. terrestris individuals were given in Figure S1 and Figure S2, respectively.

Discussion

Impacts of commercially produced *B. terrestris* are more complex in the Mediterranean region where *B. t. dalmatinus* is native subspecies and large numbers of commercial colonies have been used continuously for 25 years. In this study we divided the sampled populations into three groups; 1) greenhouse (commercial) populations 2) nearby greenhouses and 3) far from the greenhouses populations. We examined the genetic structure of different commercial stocks and native populations and assessed the degree of genetic introgression into native populations using twenty microsatellite markers and two mitochondrial genes. Our results demonstrate that the values of F_{ST} between populations were not significant, and the maximum F_{ST} value was only 0.045 (Table 3). Therefore, commercial and native populations were not differentiable based on twenty microsatellite markers. Similarly, structure analyses supported this pattern. According to the best K value (K =4), the populations are separated into four distinct clusters. However, there are transitions between these four main clusters and it has been determined that populations could not been clearly separated from each other. Neighbor-Joining (NJ) trees inferred from the mtDNA *COI* (A) and *cyt b* (B) gene regions could also distinguish groups clearly (Fig. 3). A possible reason for the observed low genetic differentiation between commercial and wild bumblebee populations is that although the origins of the commercial colonies were not well known, commercial and native *B. terrestris* populations probably derived from a common ancestor. A second explanation is that possible selection process in commercial breeding strains may have not significant impacts on the genetic differentiation between the two types of populations. Similarly, a few previous studies of *B. terrestris* showed that the mainland European *B. terrestris* populations had a relatively homogenous genetic structure but had clear differences for some island subspecies (Estoup, Solignac, Cornuet, Goudet, & Scholl, 1996; Lecocq et al., 2013; Widmer, Schmid-Hempel, Estoup, & Scholls, 1998). But a recent study on *B. terrestris* showed that Irish *B. terrestris* populations were highly differentiated from British and continental *B. terrestris* populations, the latter two showing higher levels of admixture (Moreira, Horgan, Murray, & Kakouli-Duarte, 2015). In addition, Cejas, Ornosa, Munoz, & De la Rua (2018) recently found an effective marker (*16S*) to differentiate *B. t. terrestris* and *B. t. lusitanicus* populations (endemic in Iberian Peninsula).

Native *B. t. dalmatinus* populations show considerable differences in the life cycle patterns depending on environmental conditions. Along the some coastal area of the Mediterranean region, native *B. t. dalmatinus*

populations are active from November until July. New queens enter summer aestivation and remain dormant during the dry season until the rainy period starts (September-November) (Gurel, Gosterit, & Eren, 2008). Therefore, life-cycle patterns of aestivated native *B. t.dalmatinus* populations are synchronized with the greenhouses tomatoes production period (September-July) in coastal Mediterranean region where the commercial colonies have been widely used. However, aestivated native *B. t. dalmatinus* populations have become extinct or rare in Mediterranean coastal areas because of both introgressions of commercial stocks and habitat destruction, and heavy urbanization. Some authors have also implied that commercialization processes are capable of changing some colony traits of native *B. terrestris* colonies (Gosterit & Baskar, 2016; Ings, Ward, & Chittka, 2006). The genetic introgression could lead to the loss of specific traits and local adaptations (Evans, 2017). In this study, within the group of the more distant populations FS is the only aestivated population, located more than 30 km away from greenhouses areas and occurs in the one of the best-preserved ancient cities in the Mediterranean region. In our study, although the values ofF_{ST} between populations were not significant, according to PCA results, FS population separated from other populations. Therefore, we suggest that FS population should be initially conserved to avoid extinction.

In the mitochondrial analysis, two haplotypes (haplotype A; 28.8 % samples and haplotype B; 71.2 % samples) were found for the *COI*gene. While haplotype A was common in commercial populations, haplotype B was common in both nearby greenhouses populations and far from the greenhouses populations. In our study, six haplotypes (H1-H6) were found for the *cyt b* gene. Considering haplotypes and their frequencies, we found that the most abundant haplotype was H4, which was detected in 43 individuals (% 63.2). While haplotypes 4 and 5 are known to belong to the *B. t. dalmatinus,* haplotypes 1, 2, 3 and 6 are known to belong to both *B. t. dalmatinus* and *B. t. terrestris.*These haplotypes were found in the commercial populations and nearby greenhouses populations. The detection of haplotypes 1, 2, 3 and 6 in the commercial populations and nearby greenhouses populations potentially suggests that commercial producers in Turkey either have also been producing and selling non -native *B. t. terrestris* in Mediterranean region where *B. t. dalmatinus* is native subspecies or mixing *B. t. terrestris* and *B. t. dalmatinus* in order to set up breeding stocks. We don't know whether *B. t. terrestris*has become established in the Mediterranean region where it is not native. Some environmental factors can prevent its establishment in this region. There is some evidence to support this. Lecocq, Rasmont, Harpke & Schweiger (2016) considered that *B. t. terrestris* would be able to survive as active colonies in south of South America, New Zealand, southeast Australia, and Tasmania based on 19 bioclimatic variables and implied that Mediterranean region is not climatically suitable area for *B. t. terrestris.* On the other hand,because of high demand from consumers for a year-round supply of greenhouse production, highland (at different altitudes and climatic conditions) greenhouse tomato production in Turkey has been increasing in recent years. Therefore, commercial *B. terrestris* colonies are used for pollination all year round. We suppose that *B. t. terrestris* is able to establish in the wild particularly adjacent to the highland greenhouse areas and spread.

It will be useful for future ecological studies to determine locally geographic areas where the establishment success of commercial queens in greenhouses could be higher. In addition, because of morphological similarities and difficulties, new techniques and molecular markers to separate commercial strains, subspecies, and ecotypes and to determine the introgressions are needed. As mentioned in previous studies (Evans, 2017; Goka, 2010; Ings, Ward, & Chittka, 2006; Koide, Yamada, Yabe, & Yamashita, 2008), some special precautions; such as using of mesh to cover glasshouse vents, using of queen excluders to keep gynes in the nest and disposing of old nests to restrict the escape of males and gynes must be taken to prevent both novel subspecies or commercial stocks introductions and the spread of pathogens and pests. Despite some restrictions, regulation of the movement of *B. terrestris*subspecies is problematic and ineffective. We still have little information on the origin of commercial stocks. To reduce the risk of non native subspecies and genetically different populations and the risk of spreading pathogens and parasites, effective control methods would seem to be necessary even without the establishment. Furthermore, there is an urgent need to develop conservation strategies for native bumble bee populations.

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Author Contributions

The study was conceived and the manuscript was written by B.A.K. and F.G. Most laboratory work was performed by B.A.K. Both authors made intellectual contributions to the study design and text.

Data Accessibility Statement

Sequence data will be available in the DRYAD Digital Repository (https://datadryad.org/). Additional tables and figures are available in the Supporting Information.

Supporting Information

Figure S1 Maximum Likelihood (ML) phylogeny inferred from *COI* gene sequences of *B. terrestris* populations

Figure S2 Maximum Likelihood (ML) phylogeny inferred from *cyt b* gene sequences of *B. terrestris* populations

Table S1 Genetic diversity measures of per locus in all*B. terrestris* populations studied

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Table 1 The names, codes and GPS positions of the sampling locations

Code	Location Name	N	Latitude	Longitude	Altitude	Date
CP1-CP7	Commercial Populations 1-7	210	—-	—-	—-	March
AK	Aksu	32	36°57'N	30°51'E	10 m	April
KM	Kumluca	21	36°22'N	30°17'E	15 m	April
DM	Demre	23	36°14'N	29°57'E	12 m	April
GB	Geyikbayırı	24	37°30´N	30°27´E	589 m	May
TM	Termessos	32	36°59'N	30°28'E	1150 m	May
BB	Bayatbademler	32	36°54'N	30°32'E	900 m	May
FS	Phassalis	32	36°31'N	30°33'E	4 m	March

Table 2 F-statistics ($F_{IS,}F_{IT}$ and F_{ST}) and *Nm, PIC* and *Na* values per locus in all populations (*Nm* ; gene flow value, *PIC* ; polymorphism information content, *Na;* number of alleles)

Locus	**N**	F_{IS}	F_{IT}	F_{ST}	Nm	PIC	Na
B100	373	0.07	0.14	0.07	3.29	0.93	19
BTMS0033	370	0.18	0.17	0.06	4.24	0.81	12
BTMS0119	374	0.30	0.35	0.07	3.25	0.92	22
B119	361	0.34	0.38	0.06	4.03	0.64	4
BT28	330	0.63	0.66	0.06	4.18	0.76	8
BTMS0131	358	0.32	0.35	0.05	4.83	0.90	27
BT20	344	0.07	0.11	0.04	5.19	0.91	17
B126	354	0.08	0.11	0.03	7.22	0.93	20
BTMSO45	378	0.17	0.21	0.05	4.74	0.92	25
BT26	355	0.01	0.05	0.04	6.20	0.93	19
B96	349	0.44	0.48	0.07	3.07	0.86	10
BT06	320	0.15	0.20	0.06	4.18	0.90	18
B124	316	0.05	0.08	0.03	6.70	0.94	24
BT09	343	0.11	0.14	0.04	6.70	0.95	27
B118	336	0.12	0.15	0.04	6.35	0.92	22
B132	366	0.05	0.08	0.03	7.40	0.91	23
B11	322	0.26	0.31	0.07	3.54	0.91	18
BTMS0082	312	0.38	0.43	0.08	3.02	0.70	11
BT10	313	0.02	0.05	0.03	6.71	0.90	16
BTMS0124	320	0.36	0.40	0.07	3.16	0.90	18
Mean± SD		0.20±0.16	0.24±0.17	0.05±0.02	4.90±0.60	0.88±0.08	18.3±6.34

Table 3 Genetic diversity measures of *B.terrestris* populations based on microsatellite loci ($A_{R;}$ allelic richness, *PA;* number of private alleles, *Ho* ; observed heterozygosity, *He* ; expected heterozygosity, *PIC* ; polymorphism information content,*Fis* ; inbreeding coefficient)

	Population	A_R	*PA*	*Ho*	*He*	*PIC*	*Fis*
Group 1	CP1	9.88	0	0.68	0.86	0.82	0.20**
	CP2	9.75	2	0.69	0.86	0.83	0.19*
	CP3	10.30	1	0.75	0.87	0.84	0.14*
	CP4	9.34	1	0.66	0.86	0.82	0.23**
	CP5	9.44	2	0.72	0.86	0.82	0.16*
	CP6	9.21	0	0.69	0.85	0.82	0.20*
	CP7	9.11	1	0.73	0.86	0.82	0.15*

Mean ± SD	Mean ± SD	9.58±0.42	1	0.70±0.03	0.86±0.01	0.82±0.01	0.18
Group 2	AK	9.66	2	0.63	0.86	0.82	0.27**
	KM	9.67	1	0.58	0.86	0.81	0.33**
	DM	9.24	0	0.62	0.87	0.82	0.29**
	GB	8.95	0	0.70	0.84	0.80	0.17*
Mean ± SD	Mean ± SD	9.38±0.35	0.75	0.63±0.05	0.86±0.01	0.81±0.01	0.27**
Group 3	TM	9.83	0	0.68	0.87	0.84	0.21**
	BB	9.90	4	0.70	0.85	0.82	0.19*
	FS	9.69	2	0.65	0.87	0.84	0.25**
Mean ± SD	Mean ± SD	9.81±0.11	2.33	0.68±0.03	0.86±0.01	0.83±0.01	0.22**
Overall mean ± SD	Overall mean ± SD	9.57±0.37	1.21	0.68±0.05	0.86±0.01	0.82±0.01	0.21**

(*p<0.05; **p<0.01 significant deviation from Hardy-Weinberg equilibrium)

Table 4 Pairwise F_{ST} (genetic differentiation) values between *B. terrestris* populations

	CP1	CP2	CP3	CP4	CP5	CP6	CP7	AK	KM	DM	GB	TM	BB
CP1	***												
CP2	0.021	***											
CP3	0.018	0.016	***										
CP4	0.035	0.040	0.032	***									
CP5	0.036	0.045	0.035	0.016	***								
CP6	0.039	0.044	0.035	0.015	0.014	***							
CP7	0.030	0.032	0.028	0.027	0.026	0.025	***						
AK	0.031	0.038	0.031	0.030	0.027	0.028	0.029	***					
KM	0.035	0.036	0.030	0.032	0.031	0.032	0.021	0.033	***				
DM	0.037	0.033	0.028	0.029	0.029	0.029	0.022	0.031	0.022	***			
GB	0.038	0.036	0.032	0.032	0.031	0.031	0.021	0.038	0.026	0.023	***		
TM	0.031	0.032	0.026	0.026	0.026	0.026	0.029	0.021	0.032	0.028	0.030	***	
BB	0.030	0.037	0.029	0.033	0.029	0.032	0.032	0.019	0.037	0.030	0.035	0.017	***
FS	0.030	0.035	0.027	0.024	0.024	0.025	0.024	0.027	0.025	0.024	0.027	0.023	0.028

All pairwise F_{ST} values are not statistically significant.

Table 5 Results of the molecular variance (AMOVA) analysis for populations of *B. terrestris*

Source of variation	Degrees of freedom	Sum of squares	**Variance (%)**
Among Populations	13	326.93	3
Among Individuals / Within Populations	389	4.531,05	32
Within Individuals	403	2.354,00	65
Total	805	7.211,98	100

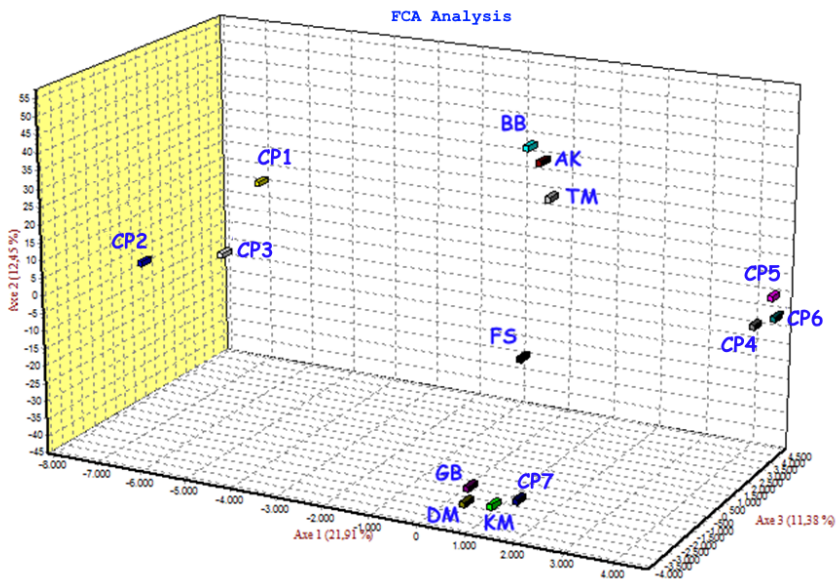

Figure1 Factorial Correspondence Analysis based on 19 microsatellite loci of *B. terrestris* populations

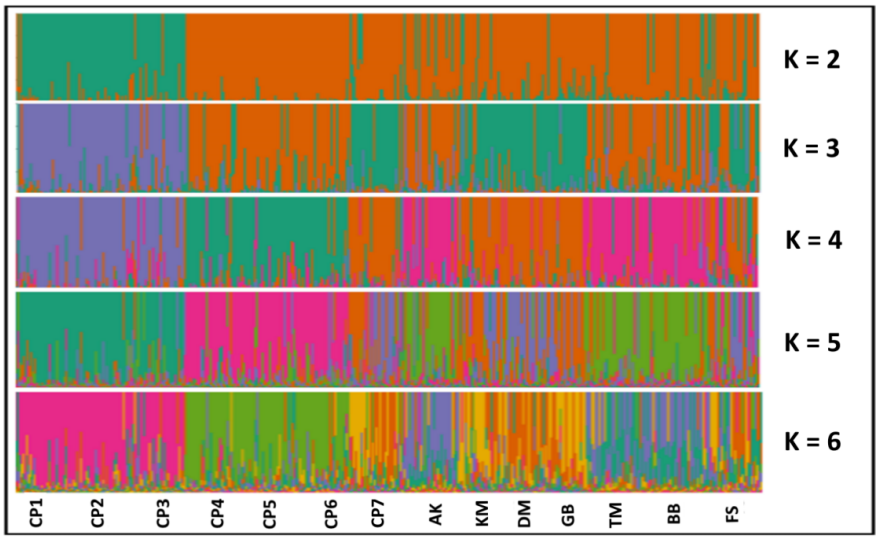

Figure 2 STRUCTURE diagrams for all *B. terrestris*populations studied. Letters on the x-axis correspond to populations. Each color represents the probability of assignment to a specific cluster. The $K = 4$ diagram had the highest support in the population structure analyses.

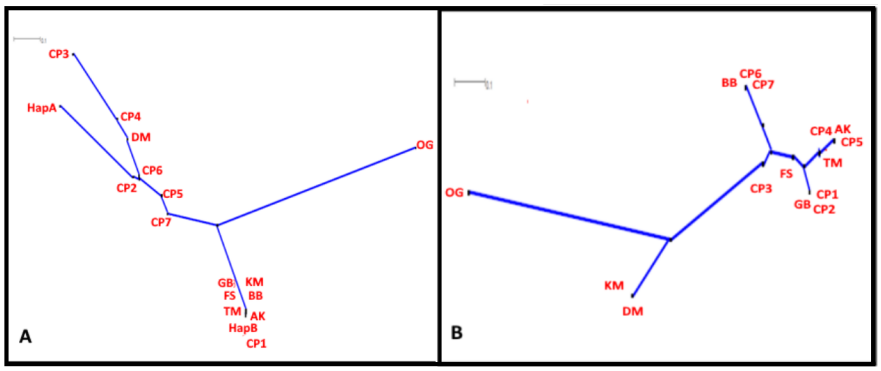

Figure 3 Neighbor-Joining (NJ) trees inferred from the mtDNA *COI* (A) and *cyt b* (B) gene regions