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PROTOZOOLOGY - ORIGINAL PAPER

The impact of refugees on leishmaniasis in Turkey: a new Syrian/Turkish *Leishmania tropica* population structure described by multilocus microsatellite typing (MLMT)

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Abstract

Turkey is one of the leishmaniasis endemic countries, and according to the recent reports, more than 45% of the cases were reported from the Southeastern part of Turkey. The disease is endemic in Syria with annually 25,000 cases, and it is emphasized by WHO that the actual number was estimated to be 2–5-fold higher than the reported numbers. Due to the civil war in Syria, more than seven million people were displaced and migrate to neighboring countries. The population structure of *Leishmania tropica* was investigated in the present study using clinical samples, which were obtained from Syrian patients residing in Turkey. Previously reported database was used to compare the results obtained in the present study. According to the multilocus microsatellite typing profiles, three populations (Şanlıurfa, Mediterranean, and Syrian/Turkish) were identified. Syrian/Turkish population, which is a new structure and identified for the first time in the present study, was comprised of clinical samples obtained from Syrian patients. The newly described population structure was homogeneous and solid comparing to previously identified population structures in Turkey. Further analyses revealed two sub-populations under the main Syrian/Turkish population structure. The findings of the present study revealed that the epidemiological status of leishmaniasis is more complicated than it is estimated. We believe that the data presented here will provide valuable information on the leishmaniasis epidemiology.

Keywords *Leishmania tropica* · MLMT · Epidemiology · Syria · Turkey

Introduction

Leishmania is an intracellular obligate parasite, which is transmitted by the bite of female Phlebotominae (Diptera: Psychodidae) sand flies. There are three main clinical manifestations (cutaneous, visceral, and mucocutaneous) of the disease and both visceral and cutaneous forms are seen in the Old World countries. According to the WHO, leishmaniasis is one of the most neglected tropical diseases and it is endemic in 102 countries. More than two million new cutaneous leishmaniasis (CL) and 250,000 visceral leishmaniasis (VL) cases were reported annually in the worldwide. Leishmaniasis is poverty-related, and outbreaks were triggered by wars, famine, and malnutrition. The majority of the infected patients were kids, and malnutrition is the main problem during those epidemics caused by war (Alvar et al. 2012; WHO 2016).

Turkey is classified as one of the endemic countries, and according to the Ministry of Health of Turkey, 45% of the patients were reported from Şanlıurfa province, which is

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located in the Southeastern part of Turkey. Leishmaniasis is one of the notifiable diseases in Turkey and treatment is free of a charge. The number of reported CL cases was peaked between 1990 and 2010 ($n = 40.003$), and it is estimated that the actual number is much higher due to under-report cases (MoH Turkey 2015). Two main complexes (*Leishmania infantum/donovani*, *Leishmania tropica*) are reported to be responsible for CL in Turkey and vectors for both complexes are present in many provinces of Turkey (Özbilgin et al. 2019). In Turkey, most of the CL cases (both autochthonous and imported) were reported to be caused by *L. tropica.* The population structure of cryopreserved Turkish *L. tropica* strains was identified in a previous study, and two main populations and five sub-populations were described. The majority of the identified isolates ($n = 75$, 78%) were found to be Southeastern originated according to the multilocus microsatellite typing (MLMT) analysis (Karakuş et al. 2017).

Cutaneous leishmaniasis has been the major public health risk for Syria since the 1960s, and the disease was restricted to two main provinces, Damascus and Aleppo. In the early 2010s, CL incidence was almost 25,000 and it is emphasized that the actual situation was estimated to be 2–5-fold higher than reported numbers (WHO 2016; Hayani et al. 2015; Hotez 2018). Cutaneous leishmaniasis cases were gradually increased during the civil war in Syria and peaked in 2013, with more than 41,000 CL cases (MoH Syria 2013). Due to the collapsed health system and improper surveillance of leishmaniasis in Syria, it was impossible to determine the exact number of the cases and treated/untreated number of patients. Between 2008 and 2017, almost seven million people were displaced into neighboring countries, which is the major cause of re-emerging of the disease in these countries (Mockenhaupt et al. 2016). Leishmaniasis is one of the most reported diseases among the Syrian refugee community in the World that shows the urgency of proper diagnosis and treatment of the disease.

Reported CL cases have shown a rising trend in Turkey and neighboring countries (Yemen, Iraq, Libya, Afghanistan) due to the conflict in Syria (Özkeklikçi et al. 2017). Turkey is one of the most affected countries, and it is estimated that more than three million refugees were accepted into Turkey without prior inspection. Most of the refugees were moved to either camps or homes in the Southeastern part of Turkey, but some moved into the Mediterranean region in order to flee to Europe. Previous studies showed that most of the imported CL cases were reported in neighboring cities (Adana, Gaziantep, Şanlıurfa) of Syria. A dramatic increase in the number of diagnosed Syrian CL cases was reported from several state hospitals in Turkey between 2012 and 2014 (Özkeklikçi et al. 2017; Salman et al. 2014; Korkmaz et al. 2015).

Since the vector sand fly species were present in Turkey, the surveillance of the imported cases is important and the species typing of the infective agent is crucial. MLMT or STR (short tandem repeat) analysis is a powerful tool to understand the molecular epidemiology of the disease. Several primer pairs were used to identify the population genetics of different *Leishmania* isolates, but recently, 12 markers were reported to have high discrimination power among populations/sub-populations of *L. tropica* (Azmi et al. 2017). Population structure of *L. tropica* is quite complex compared to other *Leishmania* species. In Turkey, two main populations and five sub-populations were identified recently (Karakuş et al. 2017). In the present study, we examined the Giemsa-stained smear slides obtained from Syrian refugees residing in Istanbul using highly polymorphic microsatellite markers. Thus, the effects of Syrian refugees on the leishmaniasis epidemiology and the recent population structure of *L. tropica* in Turkey were investigated.

Material and methods

Patients and parasitological examination

Samples were collected from the patients admitted to Bakırköy Dr. Sadi Konuk Training and Research Hospital, Dermatology outpatient clinic. Lesion examinations were made in the dermatology department, and patients have signed the informed consent form. Sampling was done by fine needle aspiration from the outer edge of the lesions. Slide samples were methanol fixed, Giemsa stained, and examined for the presence of amastigote forms. The local ethical committee of the Bakırköy Dr. Sadi Konuk Training and Research Hospital approved this work with protocol number 2018/314.

Molecular identification

After the diagnosis, all samples were included in the molecular species typing studies. Stained smear slides were scraped gently using molecular grade water, and DNA extraction was made using Qiagen DNeasy Extraction kit (Hilden, Germany) following the manufacturer's instructions. In order to obtain high DNA concentration, the last elution step of the DNA extraction procedure was modified to 50 μl. After DNA measurements, a real-time PCR targeting ITS-1 region was performed to identify the causative agents in species level by melting analysis. PCR protocol and reaction mixture was done as published in a recent study (Özkeklikçi et al. 2017).

Multilocus microsatellite typing

Previously reported *L. tropica* specific and fluorescent labeled (HEX, NED, and TED) primers, which were amplifying the STR regions containing the repeat motifs, were used following the procedure reported elsewhere (Karakuş et al. 2017). After the visualizing the specific bands in the gel

electrophoresis, each PCR product was loaded to the Fragment Analyzer device (ABI PRISM 3130XL). Size standard (ROX-500) was used to identify the repeat length in the studied samples. The MLMT data of previously typed 96 Turkish isolates were included in analyses. The peaks obtained as raw data were analyzed using Geneious R8 and Peak Scanner software. Analyzed peaks were exported as an excel file to perform further data analyses (Sup. Table 1).

Data analysis was done using several modeling softwares after the normalization step of the raw fragment data. Microsatellite Analyzer 4.05 (MSA) was used to prepare the appropriate analysis text file for Bayesian-based clustering method (Pritchard et al. 2000). Bayesian statistics method was implemented in the STRUCTURE software to identify the allele frequency and fractions of the genotype for each strain that belongs to each sub/main population. The Markov Chain Monte Carlo iterations were set to 200,000 with 20,000 burn-in period. Delta K values were determined using Structure Harvester with 10 iterations per each K value in order to predict the most appropriate population number among studied samples (Earl and von Holdt 2012). The factorial corresponding analysis (FCA) was done to evaluate the genetic distribution of the studied *L. tropica* samples. Software GDA 1.1 was used to calculate the number of alleles (A), observed (H_o), and expected (H_e) heterozygosity and the inbreeding coefficients (F_{IS}) (Tamura et al. 2011).

Results

According to the parasitological examination, 25 patients were diagnosed to be positive for *Leishmania* infection during 4 years (2014–2018) period. Also, previously diagnosed 12 smear samples were included in the present study. According to the species typing, 24 out of 25 clinical samples were diagnosed as *L. tropica* and included in the MLMT study (Sup. Fig. 1).

Multilocus microsatellite typing

Twenty-four *L. tropica* samples (clinical smear samples) were amplified using twelve highly polymorphic markers, and repeat number of each allele was recorded as a text file prior to computer-based experiments. The data obtained from 24 samples were combined with previous MLMT data of Turkish isolates ($n = 96$), and Bayesian statistics analysis was done using STRUCTURE software. Totally 120 Turkish ($n = 79$) and Syrian ($n = 41$) *L. tropica* samples were analyzed.

Two software's (STRUCTURE and Structure Harvester) were used to determine the ΔK and according to those software's, three main populations (Şanlıurfa POP, Mediterranean POP, and Syrian/Turkish POP) were identified (Fig. 1). Most of the analyzed Syrian samples ($n = 39$) were correlated with their population structure, but no clear differentiation was

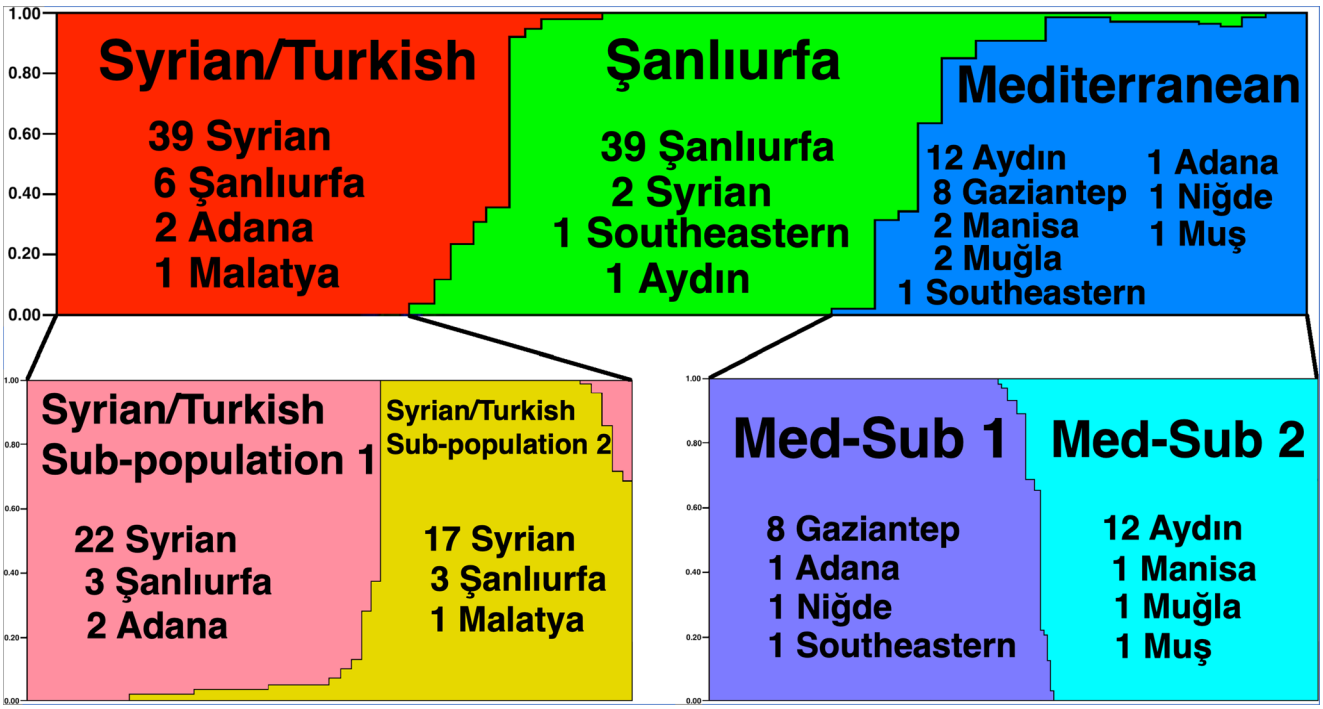

Fig. 1 STRUCTURE analysis of the Turkish *L. tropica* samples (including previous *L. tropica* data). The figure shows fragmented populations according to their repeat numbers obtained from the software STRUCTURE. Each color represents unique population and also sub-population. Three main populations (red: Syrian/Turkish, green: Şanlıurfa, blue: Mediterranean) and four sub-populations (pink: Syrian/Turkish sub-population 1, yellow: Syrian/Turkish sub-population 2, azure: Mediterranean sub-population 1, turquoise: Mediterranean sub-populations 2) are generated by the software

noted in some Syrian samples ($n = 2$) between Şanlıurfa samples, where people movements occur frequently. The FCA and ΔK graphs were also illustrated (Fig. 2).

Totally, 48 samples were clustered in Syrian/Turkish (SYR/TR) population, which originally isolated from four different provinces (Syria, Şanlıurfa, Adana, and Malatya). SYR/TR population was reanalyzed to identify the sub-population structure and as suggested by STRUCTURE software, two closely related sub-populations (SYR/TR1 and SYR/TR2) were identified. SYR/TR1 comprised of 22 samples from Syria, three from Şanlıurfa and two from Adana, while SYR/TR Sub2 consists of 17 samples from Syria, three from Şanlıurfa, and one from Malatya.

According to the ΔK values, Şanlıurfa population was found to be the solid and the most homogeneous population in the present study. This population consists of 39 samples from Şanlıurfa, two samples from Syria, two samples from the Southwestern part of Turkey (exact isolation district is unknown), and one sample from Aydın province. No sub-population structure was identified for Şanlıurfa population due to weak sub-population structure values.

The Mediterranean population was the most heterogeneous population identified in the present study. This population consists of 28 samples from eight provinces (Aydın, Gaziantep, Manisa, Muğla, Adana, Muş, Southeastern Turkey, Niğde) of Turkey. According to the STRUCTURE analyses of the Mediterranean population, two mixed sub-populations were identified. Generated sub-populations were found to be correlated with the origins of the isolates. Those isolated from Southeastern part of Turkey was clustered in Med-Sub1, while western isolates were clustered in Med-Sub2. A phylogenetic tree was constructed using the samples studied in the present study (Fig. 3).

Descriptive analysis of 120 *L. tropica* samples and sub-populations

Descriptive analysis per locus

Descriptive analysis was done per each locus, and the coefficient of relationship value was calculated negative for four markers (GA9n, LIST7027, LIST7040, 27GTGn), while the rest of the markers were noted to have positive value, which suggest a large number of homozygous alleles in the analyzed samples. The inbreeding coefficient (F_{IS}) was found positive for eight out of 12 markers. Overall analyzed loci, observed heterozygosity (H_o) was greater than expected heterozygosity (H_e) for three loci (GA6, LIST7010, and 27GTGn). The mean for H_o, H_e, and F_{IS} of the studied loci was 0.312, 0.234, and 0.455, respectively (Table 1).

Descriptive analysis per main populations

Descriptive analysis was also done for the main populations (ΔK:3) that are identified in the present study. According to the analysis, H_o was found greater than H_e for the SYR/TR

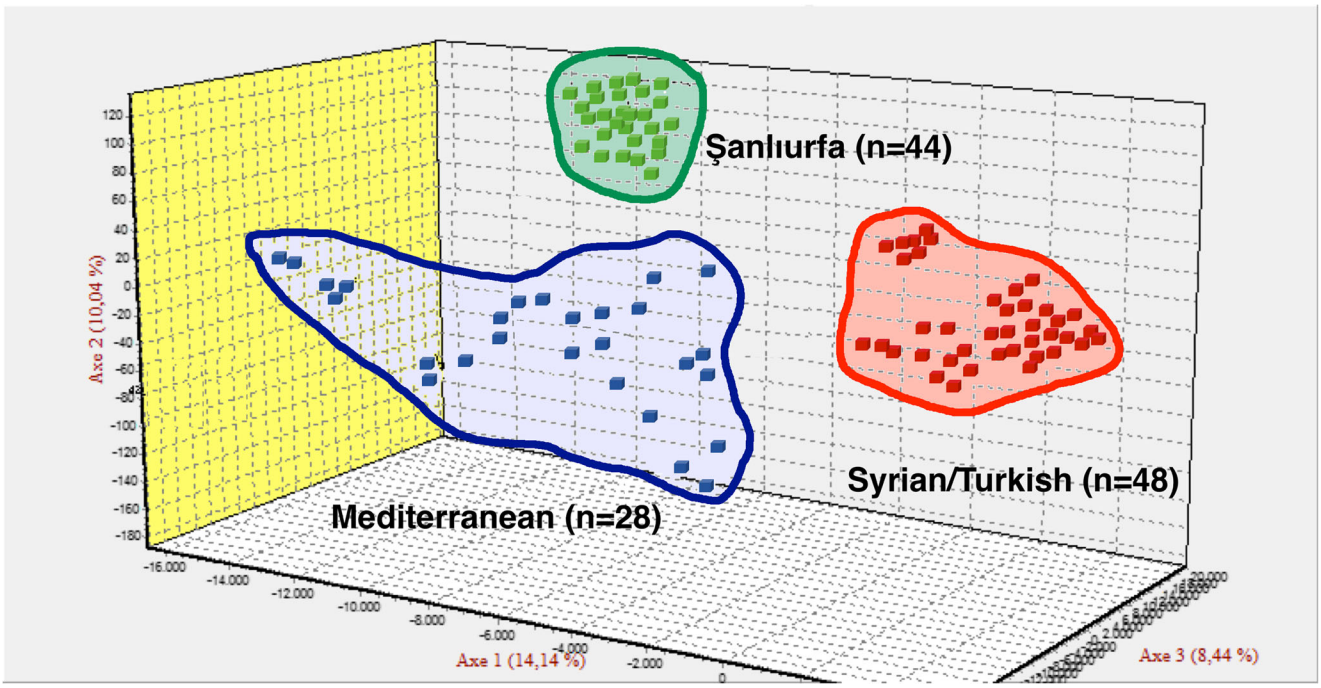

Fig. 2 Factorial correspondence analysis (FCA) of the identified populations. Figure shows factorial correspondence analysis (FCA), which is a plot based analysis of determined populations. This multidimensional figure shows the distance between isolated main populations. Representing colors are congruent with Fig. 1

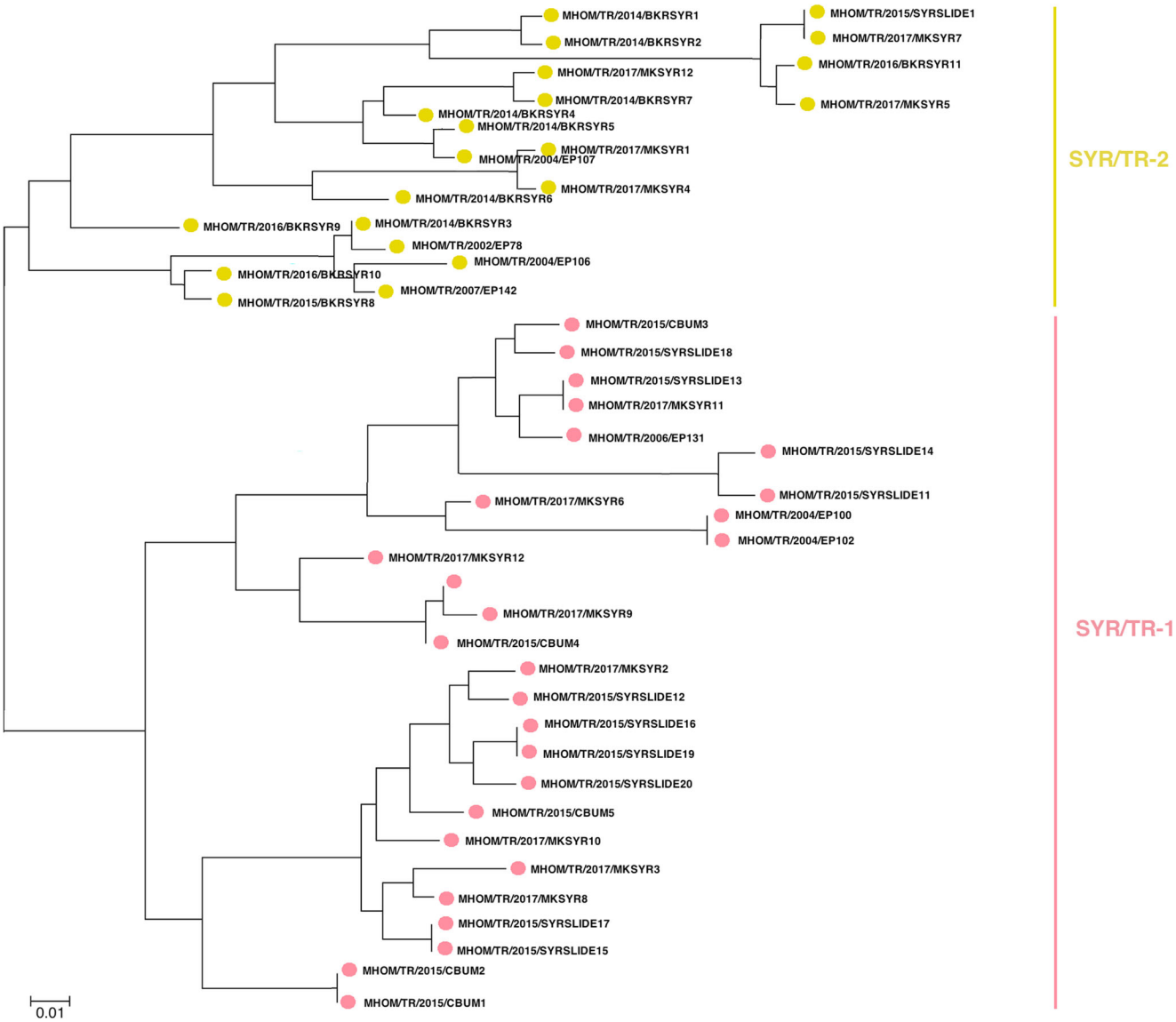

Fig. 3 Phylogenetic tree showing Turkish/Syrian population

and Şanlıurfa populations, while H_o was found lower than H_e for the Mediterranean population. The number of alleles was found lower in SYR/TR population comparing to other populations that suggest homogeneous population structure. According to the F_{IS} values, the Mediterranean population was found to be the most heterogeneous population. The highest F_{IS} value was noted for SYR/TR population, which is described for the first time in the present study.

Descriptive analysis per sub-populations

Sub-populations were further investigated in terms of outbreeding and inbreeding capacity to identify the structure in more detail. For both SYR/TR1 and SYR/TR2 sub-populations, H_e was greater than H_o. The F_{IS} was almost two times higher in SYR/TR1 comparing to SYR/TR2 sub-population.

Mediterranean populations were further analyzed and descriptive values were calculated. Eleven strains were grouped in Med-Sub1 and H_o was found higher than H_e for this sub-population structure. Same results were obtained for Med-Sub2, which has also higher H_e values comparing to H_o values. Inbreeding coefficient was also low in those sub-populations comparing to main populations and SYR/TR sub-populations structure (Table 2).

Discussion

The present study is designed to understand and update the epidemiological dynamics of *L. tropica* caused leishmaniasis in Turkey. The most recent study was performed using autochthonous ($n = 79$), and several Syrian isolates ($n = 17$) and two

Table 1 Descriptive analysis per locus

Marker	Number of strains	Repeat array	Fragment size array (bp)	*A*	H_e	H_o	F_{IS}
GA1	128	GA 11–12	66–68	2	0.117	0.000	1.000
GA2	128	GA 5–8	56–62	3	0.218	0.011	0.262
GA6	128	GA 8	61	1	0.126	0.273	0.953
GA9n	98	GA 7–9	112–116	3	0.231	0.192	− 0.372
LIST7010	98	TA 21–35	176–204	9	0.538	0.607	0.623
LIST7011	96	TA 9–19	174–194	5	0.775	0.627	0.932
LIST7027	89	CA 6–12	169–181	7	0.642	0.204	− 0.105
LIST7033	98	GT 7–8	178–180	2	0.211	0.000	1.000
LIST7039	128	CA 11–28	195–229	9	0.298	0.263	0.62
LIST7040	128	GT 15–24	229–247	3	0.319	0.107	− 0.168
4GTG	128	GTG 4–6	59–65	3	0.172	0.047	1.000
27GTGn	96	GTG 5–9	106–118	4	0.102	0.482	− 0.283
mean	111,92			4.25	0.312	0.234	0.455

A—number of alleles; H_o—observed heterozygosity; H_e—expected heterozygosity; F_{IS}—inbreeding coefficient. − 1 = outcrossing; 0 = random mating; + 1 = inbreeding

major populations were described (Karakuş et al. 2017). In total, 24 new clinical samples obtained from Syrian patients residing in Turkey were analyzed with previous fragment data of 96 *L. tropica* strains and a new population structure is described. Newly described population, SYR/TR, consists of 39 Syrian, six Şanlıurfa, two Adana, and one Malatya isolates, which have also two sub-populations. According to the results obtained from this study, *L. tropica* population structure is getting more complex in Turkey by introducing new isolates. The previous study performed by our group failed to identify the new population structure due to inadequate Syrian samples (Karakuş et al. 2017). Including new *L. tropica* samples has not only changed the actual population structure but also helped us to identify the new emerging *L. tropica* population consist of Syrian samples in Turkey. Also, new samples included in this study were isolated from Istanbul city, which acts as a bridge between Asia and Europe.

Two Syrian isolates, which clustered in Şanlıurfa POP, showed a distinct profile compared to Syrian isolates that clustered in SYR/TR POP. This finding is also supported by high F_{IS} values, which means high inbreeding is observed in this population structure. Since there is no exact travel history of these patients, some Syrian patients might have infected with Turkish *L. tropica* strains in Turkey. Also, mass people movements between Şanlıurfa and Syria suggest that idea (Özden 2013). A recent study was investigated the refugee

Table 2 Descriptive analysis per populations and sub-populations

Main population	Number of strains	*A*	H_e	H_o	F_{IS}
Syrian/Turkish	48	1.12	0.090	0.107	0.984
Şanlıurfa	44	2	0.521	0.764	0.788
Mediterranean	28	4.32	0.105	− 0.109	− 0.425
Mean		2.36	0.238	0.254	0.449
SYR/TR sub-population					
Syrian/Turkish subpop-1	27	1.63	0.270	0.162	0.837
Syrian/Turkish subpop-2	21	1.78	0.261	0.124	0.482
Mean		1.70	0.265	0.143	0.659
MED sub-population					
Mediterranean subpop-1	11	2.17	0.398	0.871	0.017
Mediterranean subpop-2	17	1.92	0.712	0.982	0.114
Mean		2.04	0.555	0.926	0.065

A—number of alleles; H_o—observed heterozygosity; H_e—expected heterozygosity; F_{IS}—inbreeding coefficient. − 1 = outcrossing; 0 = random mating; + 1 = inbreeding

leishmaniasis using MLMT procedures and showed homogeneity of the studied isolates suggesting that an outbreak probably caused by a specific *L. tropica* strain (Noyes et al. 1998). Of the studied samples, majority of the Syrian samples (37/39) were showed high similarity in marker level. This finding is also supported by low H_o values obtained by the descriptive analysis of the samples. Our findings support the idea suggested by a recent paper (Noyes et al. 1998). Formation of the new distinct population structure is an indication in molecular epidemiological level stating that imported CL cases are forming to an autochthonous population.

Cutaneous leishmaniasis caused by *L. tropica* is a serious public health problem in Turkey and also in the Middle East and North Africa. Leishmaniasis is present in the Southeastern part of Turkey with changing dynamics more than centuries ago (Gürel et al. 2012). There are several *L. major* caused CL cases in the Southeastern part of Turkey but *L. tropica* is the main causative agent for the most of CL cases in Turkey, like it was in Syria. According to the previous studies, the genetical structure of *L. tropica* strains was one of the most heterogeneous complexes in Turkey. So far, six different MON profiles (MON-55, MON-200, MON-303, MON-304, MON-312, and MON315) were identified by multilocus enzyme electrophoresis (MLEE) analyses (Özbilgin et al. 2019). However, this heterogeneity was not supported by MLMT in a previous study and five different MON profiles were clustered in the same population by showing low H_o values (Karakus et al., 2017). Also, a study conducted by researchers reported that no clear association between MON profiles and MLMT profiles (Seridi et al. 2008). By having further discriminatory power comparing to MLEE, MLMT procedure is the best candidate to be the golden standard in the population genetic studies, which is also applicable in stained clinical samples.

MLMT data of the Syrian samples diagnosed in Istanbul were showed homogeneous population structure than previously collected Syrian samples. Several studies have investigated the relationship between genetic variation and clinical outcomes. The exact contribution of *Leishmania* genotype to clinical outcome is still unclear. In a recent MLMT study, a viscerotropic *L. tropica* was investigated and all markers were found identical to *L. tropica* that causing CL (Karakus et al., 2017). The majority of the newly described SYR/TR subpopulation was not only Syrian samples obtained in the present study, but also there are several autochthonous isolates from the Southeastern part of Turkey. In the present study, we notice that MLMT data of some samples did not overlap with their geographical origin. The presence of nine non-Syrian samples in the Syrian population structure might be the consequence of continuous people movement between Syria and Turkey during the civil war in Syria. Since we do not have any clear data about where the infection has occurred, we can only speculate that those non-Syrian isolates clustered in the Syrian population might be originally Syrian isolates. Same results were obtained for Şanlıurfa population by having two Syrian samples, which might have originally Turkish isolates. Also, the same inconsistencies were reported in previous studies and the possible reason was explained to being infected with parasite while visiting another endemic area (Krayter et al. 2014). Further studies needed to identify those heterogeneous population structures in more detail, and using clinical samples in MLMT studies should be standardized.

Several studies were reported refugee leishmaniasis in Turkey but the exact number of human cases is still unknown (Çulha et al. 2018; Beyhan et al. 2017; Koçarslan et al. 2013). During the civil war in Syria, more than 7 million people were displaced and forced to migrate to neighboring countries. With almost five million refugees, Turkey is one of the top refugee-receiving countries. A large number of people movements have changed the CL dynamics in Turkey. According to the official records, the majority (1459/2002; 72.80%) of the reported CL patients were Syrians during 2013–2015. Southeastern part of Turkey was the most refugee residing area during this period and refugees moved to western parts of Turkey in the following years. This dramatic increase in the number of refugee CL cases was started to decrease gradually in 2015, which might be a result of well-organized CL management in Turkey (Özkeklikçi et al. 2017; Özbilgin et al. 2019). The presence of drug-resistant *Leishmania* isolates was also reported in Turkey following the mass people migration after the civil war in Syria (Özbilgin et al. 2019). In such cases, microsatellite typing is a reliable tool to identify the population origin of the clinical samples, which might help the physician to evaluate the treatment regime.

Previously described two populations were consisting of mostly autochthonous strains that isolated, passaged, and cryopreserved for many years (Karakuş et al. 2017). The effects of passage time on STR regions were shown in previous studies on different *Leishmania* strains (Khan et al. 2017). The oldest Şanlıurfa strain used in MLMT study was isolated in 1999 and compared with most recent Şanlıurfa strain isolated in 2007 from the same area, and no difference was noted. However, it is also reported that some markers or genetic structure might change due to passaging over time (Bussotti et al. 2018; Akopyants et al. 2009; Inbar et al. 2013). In the present study, the high similarity was found between previously cultured Syrian samples and Giemsa-stained Syrian samples in marker level. We believe that using clinical samples in MLMT studies would bring valuable data in terms of the epidemiology of the disease. Including more clinical *L. tropica* data into MLMT studies will help us to understand the epidemiological dynamics of *L. tropica*.

One possible reason for the presence of distinct isolates in the same sub-population structure might be the presence of

different vector/reservoir species and different ecological conditions in Turkey. Turkey has seven different geographical regions and 25 sand fly species reported to be distributed in Turkey (Kasap et al. 2015). *Phlebotomus similis* and *P. sergenti* are the proven vectors of *L. tropica* in several countries as well as in Turkey (Volf et al. 2002; Maroli et al. 2013). The presence of *L. tropica* both in cats and in dogs was reported in a recent study in Turkey (Paşa et al. 2015). This species and reservoir richness might also be another factor for the complex structure of *L. tropica* in Turkey.

In conclusion, a new population structure, SYR/TR, is described in the present study. Also, further analysis suggests that two sub-populations were present in Turkey. The findings of the present study confirm that other factors might play a role in the genetic clustering since not all samples were clustered according to their geographical origins. Also, the impact of large people movement on the leishmaniasis epidemiology in Turkey is described. The data presented here will provide epidemiological knowledge to further studies. More studies should have been performed using clinical samples in MLMT profiling studies in order to eliminate the possible effect of culturing on microsatellite markers.

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Compliance with ethical standards

The local ethical committee of the Bakırköy Dr. Sadi Konuk Training and Research Hospital approved this work with protocol number 2018/314.

Conflict of interest The authors declare that they have no conflict of interest.

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