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Phylogeography of the subgenus *Transphlebotomus* Artemiev with description of two new species, *Phlebotomus anatolicus* n. sp. and *Phlebotomus killicki* n. sp.

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ABSTRACT

The subgenus *Transphlebotomus* comprises sand fly species with distribution markedly restricted to the Mediterranean basin and suspected of *Leishmania* transmission. Only three species, *Phlebotomus mascittii*, *Phlebotomus canaaniticus* and *Phlebotomus economidesi*, have been described up to the present. Due to their similar morphology, proper identification remains difficult and relies mainly on molecular markers. We studied sand fly species of this subgenus from Crete and south-western coast of Anatolia. Based on the sequencing analysis of mitochondrial genes (cytochrome b, NADH dehydrogenase subunit 4, cytochrome oxidase I), two new *Transphlebotomus* species were identified and subsequently distinguished also by morphological characters: *Phlebotomus anatolicus* n. sp. and *Phlebotomus killicki* n. sp. Moreover, *Ph. economidesi*, previously only recorded from Cyprus, was found in Turkey sympatrically with these two new species. Based on the divergence time estimates, the first split has occurred in the subgenus *Transphlebotomus* ~10 million years ago and the paleogeographic events took place around the Aegean and Mediterranean regions were suggested as the main drivers of the diversification of the subgenus. Our findings indicate that for *Transphlebotomus* species, morphological identification should be confirmed by molecular approaches, especially for investigations concerning their possible vectorial role in *Leishmania* transmission.

1. Introduction

The subgenus *Transphlebotomus* Artemiev, 1984 comprises three currently recognized species with distribution markedly restricted to the Mediterranean basin (Léger et al., 2000). The type species, *Phlebotomus mascittii* Grassi, 1908, occurs in the Mediterranean countries (Turkey, Greece, Croatia, Italy, Spain, and France) and sporadically in North Africa (Algeria) but it is also known to expand its distribution up to Central Europe including Switzerland, Belgium, Germany, Austria, and Hungary (Depaquit et al., 2005; Bosnic et al., 2006; Ivovic et al., 2007; Rossi et al., 2008; Berdjane-Brouk et al., 2011; Farkas et al., 2011; Naucke et al., 2011; Ozbel et al., 2011). With a recent record in the German state of Hesse it represents the northernmost sand fly species in the Palearctic region (Melaun et al., 2014). On the other hand, the known distribution of two remaining members of this subgenus is limited to a very small area; *Phlebotomus canaaniticus* Adler and Theodor, 1931 has been recorded only from the Middle East (Israel, Jordan, Lebanon, and Syria) and *Phlebotomus economidesi* Léger, Depaquit and Ferté, 2000 occurs exclusively in Cyprus (Saliba et al., 1997; Léger et al., 2000; Haddad et al., 2003; Swalha et al., 2003; Maroli et al., 2009).

All three species of the subgenus *Transphlebotomus* are typically characterized by their low density in catches. This peculiar attribute, along with their low biting rates of humans and presumed autogeny, have caused this subgenus to be neglected for a long time. As a result, the taxonomic status of its species, their distribution and population biology have remained understudied. However, occurence of recent sporadical autochtonous cases of canine leishmaniasis in Central Europe, where *Ph. mascittii* is the only recorded sand fly species, led to speculations about its possible involvement in the transmission cycle of *Leishmania infantum* (Naucke et al., 2008) and new interest in *Transphlebotomus* biology.

In the last two decades, molecular methods became routinely applied to the systematics and species identification of sand flies (reviewed by Depaquit, 2014). A comparative molecular study

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using the sequencing analysis of NADH dehydrogenase subunit 4 (ND4) gene confirmed the validity of the three taxa inferred from molecular variation and emphasized the importance of samples from eastern Mediterranean localities, especially from Greece and Turkey, to understand the population structures, distribution patterns and the evolutionary histories of the members of this subgenus (Depaquit et al., 2005).

By applying similar molecular tools, we aimed to define the taxonomic positions of the *Transphlebotomus* specimens originating from south-western Anatolia and Crete, a region characterized by a complex palaeogeographic history. By DNA barcoding approach using cytochrome *b* gene (Cyt*b*), we demonstrated presence of two new species and studied their relations with the previously described species within the subgenus. The use of molecular markers (mtDNA) allowed us to estimate the divergence time of different species and discuss the potential effects of paleogeographical events which took place around the Aegean region on the diversification and speciation of this subgenus. Morphological analysis confirmed the status of newly described species and revealed their species-specific morphological characters useful for species identification.

2. Material and methods

2.1. Sampling

For all analyses we used samples of *Transphlebotomus* spp. from two localities in Crete, the largest island of the Aegean Archipelago, and from two villages on south-western coast of Anatolia, located approximately 500 km away from Crete (Fig. 1 and Table 1). Adult sand flies were collected using CDC miniature light traps. In studied localities in Turkey, traps were placed mostly near animal shelters (September 2007 and August 2011). In Crete, specimens were obtained from both domestic animals enclosures and cavernous habitats with free access of goats and sheep (September 2011). Specimens were stored in 96% ethanol until morphological and molecular examination conducted. Head and genitalia were dissected and slide-mounted in Swan solution or CMCP-10 medium (Polysciences, Germany). The specimens were observed using a BX50 microscope with a camera system and measured with the Perfect Image software (Aries Company, Chatillon, France). Drawings were made using a *camera lucida* installed on the microscope. The thorax of each specimen was kept in ethanol for DNA extraction.

2.2. DNA extraction, amplification and sequencing

DNA was extracted from single specimen's thorax using the Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) or the High Pure PCR Template Preparation Kit (Roche, France) following the manufacturer's instructions and kept at −20 °C until use.

PCR amplifications of cytochrome *b* (Cyt*b*), cytochrome oxidase I (COI) and NADH dehydrogenase subunit 4 (ND4) gene regions were performed in 50 μl reaction volume, using the LCOI490/HCO2198; CB1-SE/CB-R06 and ND4C/ND4AR primer pairs, respectively, as described in previous studies (Folmer et al., 1994; Esseghir et al., 2000; Soto et al., 2001). The amplification products were separated and visualized on 2% agarose gels, purified using the QIAquick PCR Purification Kit (Qiagen) and directly sequenced in both directions using the primers used for DNA amplification (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, USA).

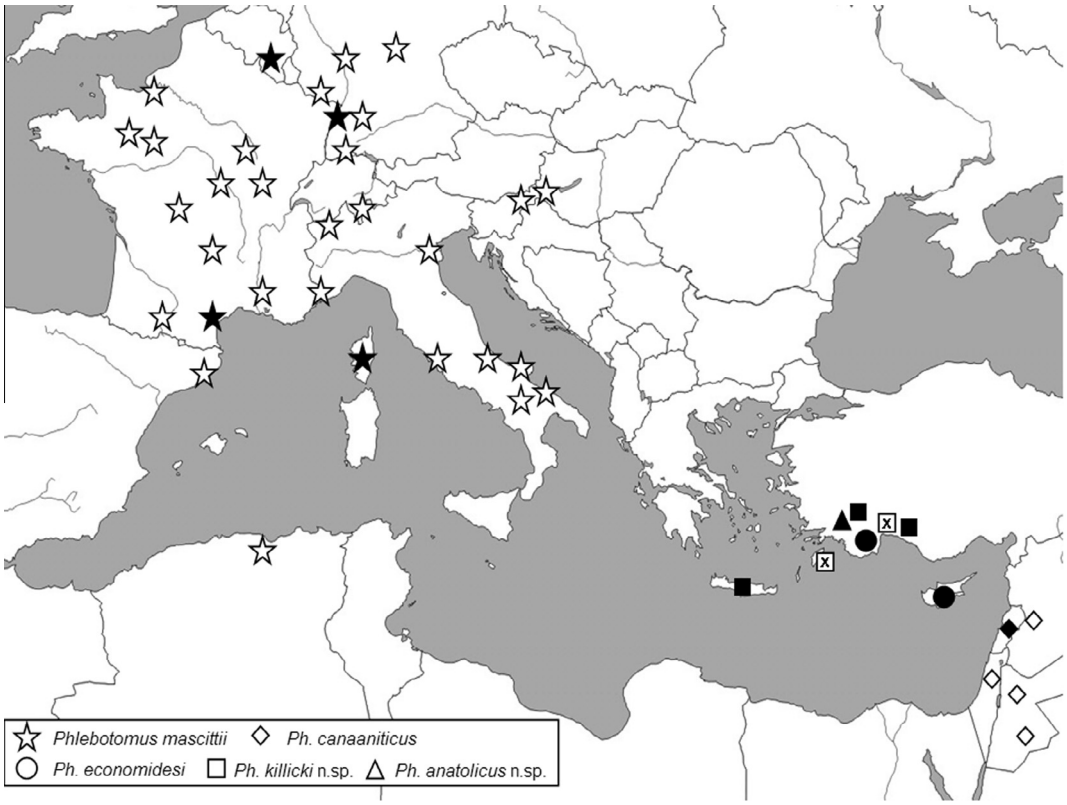

Fig. 1. Map of known distribution of species within the subgenus *Transphlebotomus* (updated from Léger et al., 2000). Collection sites of *Transphlebotomus* sp. specimens used for the morphological and/or molecular analyses in this study indicated by full shapes. Collection sites of specimens previously identified as *Ph. mascittii* and currently reidentified as *Ph. killicki* n. sp. indicated by crossed shapes.

Table 1
Transphlebotomus sp. sampling information and codes for specimens used for morphological and/or molecular analyses.

Locality	Latitude	Longitude	Altitude (m)	Specimen Code	Sex	Species	Cytb Haplotype	CO1 Haplotype	ND4 Haplotype
Turkey, Antalya, Kas, Belendi (12)	36° 12′ 0″	29° 41′ 54″	471	BLD 26	M	*Ph. killicki* n. sp.	CB_TR8	CO_TR9	ND_TR1
				BLD 28	M	*Ph. killicki* n. sp.	CB_TR9	CO_TR7	ND_TR2
				BLD 61	M	*Ph. killicki* n. sp.	CB_TR6	CO_TR7	ND_TR3
				BLD 63	M	*Ph. killicki* n. sp.	CB_TR10	CO_TR7	ND_TR1
				BLD3.73	M	*Ph. killicki* n. sp.	CB_TR1	CO_TR1	ND_TR7
				BLD3.148	M	*Ph. anatolicus* n. sp.	CB_TR2	CO_TR2	
				BLD4.6	M	*Ph. anatolicus* n. sp.	CB_TR3	CO_TR3	
				BLD4.31	F	*Ph. anatolicus* n. sp.	CB_TR4	CO_TR4	
				BLD5.20	M	*Ph. anatolicus* n. sp.	CB_TR3	CO_TR5	
				BLD5.49	F	*Ph. economidesi*	CB_TR5	CO_TR6	ND_TR5
				BLD5.53	F	*Ph. killicki* n. sp.	CB_TR6	CO_TR7	ND_TR6
				BLD5.56	F	*Ph. anatolicus* n. sp.	CB_TR7	CO_TR8	
Turkey, Antalya, Manavgat, Dikmen (1)	36° 49′ 77″	31° 26′ 99″	30	DIK3	M	*Ph. killicki* n. sp.	CB_TR6	CO_TR7	ND_TR4
Greece, Crete, Fournoti Beach (3)	35° 13′ 24″	23° 56′ 07″	3	FB1	M	*Ph. killicki* n. sp.			ND_CR1
				FB2	M	*Ph. killicki* n. sp.			ND_CR1
				FB12	M	*Ph. killicki* n. sp.	CB_CR1	CO_CR1	ND_CR1
Greece, Crete, Agia Roumeli (10)	35° 14′ 34″	23° 57′ 55″	81	AR8	M	*Ph. killicki* n. sp.		CO_CR1	ND_CR1
				AR25	M	*Ph. killicki* n. sp.		CO_CR2	
				AR41	M	*Ph. killicki* n. sp.			ND_CR1
				AR127	M	*Ph. killicki* n. sp.	CB_CR3	CO_CR1	
				AR193	F	*Ph. killicki* n. sp.	CB_CR2		ND_CR1
				AR199	F	*Ph. killicki* n. sp.	CB_CR3		
				AR216	F	*Ph. killicki* n. sp.			
				AR226	F	*Ph. killicki* n. sp.	CB_CR3		
				AR241	F	*Ph. killicki* n. sp.	CB_CR3	CO_CR1	
				AR243	F	*Ph. killicki* n. sp.			
France, Cévennes (1)				P_mas_Fra1	M	*Ph. mascittii*	P_mas_1		P_ mas
France, Corsica, Porto Vecchio (2)				P_mas_Fra2	M	*Ph. mascittii*	P_mas_2		P_ mas
				P_mas_Fra3	M	*Ph. mascittii*	P_mas_3		P_ mas
Germany, Neuenburg (1)				P_mas_Ger	M	*Ph. mascittii*	P_mas_3		P_ mas
Belgium, Sainte Cécile (1)				P_mas_Bel	F	*Ph. mascittii*	P_mas_3		P_ mas
Cyprus, Mandria cave (1)				P_eco_Cyp	M	*Ph. economidesi*	P_eco		P_eco
Lebanon, Drya (1)				P_can_Leb	M	*Ph. canaaniticus*	P_can		P_can

2.3. Data Analysis

Sequences were aligned and edited using BioEdit v.7.0.9.0 (Hall, 1999). To clarify the taxonomic position of the specimens studied, *Ph. mascittii* from Belgium, Germany and France, *Ph. economidesi* from Cyprus and *Ph. canaaniticus* from Lebanon were used as reference sequences (Fig. 1). DnaSP v.5 (Librado and Rozas, 2009) software was used to identify the unique haplotypes. Maximum Likelihood (ML) analyses were conducted using MEGA v.6.06 (Tamura et al., 2013), Bayesian analysis was performed using MrBayes v.3.2 (Ronquist et al., 2011). The Markow Chain Monte Carlo (MCMC) analysis was run for 10 million generations, the trees were sampled every 500th generations and the first 25% samples were discarded as burn-in. MEGA v.6.06 was used to estimate the best fit evolutionary model for both ML and Bayesian analyses. The analyses of the differentiation, gene flow and polymorphism between the different lineages observed after the phylogenetic analyses were also evaluated using Arlequin v.3.1.1 (Excoffier et al., 2005) and DnaSP v.5 software. Parsimony networks were constructed using TCS v.1.21 (Clement et al., 2000) with a 90% connection limit between haplotypes.

Divergence time estimates were conducted using *BEAST implemented in BEAST v.1.8.0 (Drummond et al., 2013) under the multispecies coalescent model. To avoid tricking BEAST into estimating a larger population, we used all the sequence data set we had instead of using the haplotypes. ML test of the molecular clock hypothesis was conducted using Mega v.6.06. BEAST was run for two independent MCMC runs, using a Yule speciation process for 40 million generations, sampling every 4000th generation. We calibrated the molecular clock analysis according to the mtDNA substitution rate suggested by Papadopoulou et al. (2010) and Allegrucci et al. (2011) (3.2% divergence per Myr). Convergence of chains and effective sample sizes were checked using TRACER v.1.5. A Maximum Clade Credibility tree with divergence times and their 95% highest posterior densities (HPDs) was estimated using TREEANNOTATOR and visualized using FIGTREE (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

3.1. Molecular analyses

PCR amplification of the 3′ end of the mtDNA Cyt*b* gene region was successful for all studied specimens. The aligned matrix consisted of 832 characters. The sequences of *Transphlebotomus* spp. produced 18 unique haplotypes with relatively high diversity (Hd: 0.9516) (GenBank accession numbers: KR336642–KR336659). Total number of polymorphic sites was 177, with 153 being parsimony informative.

Tree topologies constructed with ML and Bayesian analyses under the HKY + G model indicated a similar topology. Using *Ph.* (*Adlerius*) *chinensis* as an outgroup, five well supported lineages located in two major clades of *Transphlebotomus* subgenus were identified: Clade 1 was divided into *Ph. anatolicus* n. sp. lineage comprising the haplotypes from Southern Anatolia, Kas (Haplotypes CB_TR2, CB_TR7, CB_TR3, CB_TR4, and CB_TR7), and *Ph. canaaniticus* lineage which was represented with the P_can haplotype. Clade 2 was composed of three lineages: First lineage

represents all of the *Ph. mascittii* haplotypes from Belgium, Germany and France (P_mas). *Ph. economidesi* (P_eco) from Cyprus and one haplotype from Southern Anatolia (CB_TR5) were found to form the second lineage (*Ph. economidesi*). Finally, all haplotypes from Crete (CB_CR1, CB_CR2, and CB_CR3) and five haplotypes from Southern Anatolia (CB_TR1, CB_TR6, CB_TR8, CB_TR9, and CB_TR10) constituted the third lineage (*Phlebotomus killicki* n. sp.) within the Clade 2. Parsimony network analysis sorted 18 haplotypes into five independent networks which correspond to the five lineages recovered in the phylogenetic analysis (Fig. 2).

Comparisons of the Kimura 2 parameter genetic distances were congruent with the phylogenetic analyses with low intra – lineage versus high inter – lineage distances. Analysis of molecular variance (AMOVA) of the 18 Cyt*b* haplotypes also supported the phylogenetic structuring. Among lineages, variation was found to be significantly (88%, $\Phi_{CT} = 0.879$, $P < 0.005$) higher as compared to those yielded for among populations within these lineages (10%, $\Phi_{SC} = 0.826$, $P < 0.01$) or within populations (13%, $\Phi_{ST} = 0.979$, $P < 0.0001$).

Of all studied specimens, COI fragment was sequenced for 16 *Transphlebotomus* specimens. A total of 11 haplotypes were determined with a haplotype diversity (Hd) of 0.908 (GenBank accession numbers: KR336623-KR336633). Total number of variable sites was detected as 111 and 67 of them were parsimony informative. Although we failed to obtain COI sequences for *Ph. mascittii*, *Ph. economidesi* and *Ph. canaaniticus*, ML analyses of the putative new *Transphlebotomus* species with sequences available from the GenBank revealed the same topology obtained with the Cyt*b* gene region. Statistical parsimony analysis identified three independent networks for the Cretan and Anatolian specimens: First network included the haplotypes from South Anatolia which correspond to the '*anatolicus*' lineage of the Cyt*b* phylogeny; the second network comprised the haplotypes from South Anatolia and Crete which are concordant with the '*killicki*' lineage. The last haplotype from Anatolia (CO_TR6), not connected to the any of these two networks, is *Ph. economidesi* specimen (Supplementary Fig. 1). The length of the aligned sequences of ND4 was 521 bp. In total, 11 haplotypes (Hd: 0.861) were identified for the 21 *Transphlebotomus* specimens (GenBank accession numbers: KR336634–KR336641). Number of the variable sites was 95; 52 of were parsimony informative. The sequence divergence between the remaining of the lineages was found to be in concordance with the Cyt*b* and the COI divergence rates (Table 2).

Table 2
Sequence divergence (%) between the members of the subgenus *Transphlebotomus* (Cytb/COI/ND4).

	1	2	3	4
(1) *Ph. economidesi*				
(2) *Ph. canaaniticus*	0.12/nc/0.10			
(3) Ph. mascittii	0.12/nc/0.10	0.12/nc/0.12		
(4) *Ph. killicki* n. sp.	0.10/0.14/0.10	0.12/nc/0.10	0.10/nc/0.12	
(5) *Ph. anatolicus* n. sp.	0.10/0.15/nc*	0.08/nc/nc	0.12/nc/nc	0.12/0.11/nc

* nc: not conducted.

3.2. Divergence time estimates and biogeography

As we were not able to get the sequences of COI and ND4 gene regions for all of the specimens studied, the estimation of the divergence times between lineages analyses were carried out using the Cyt*b* data set.

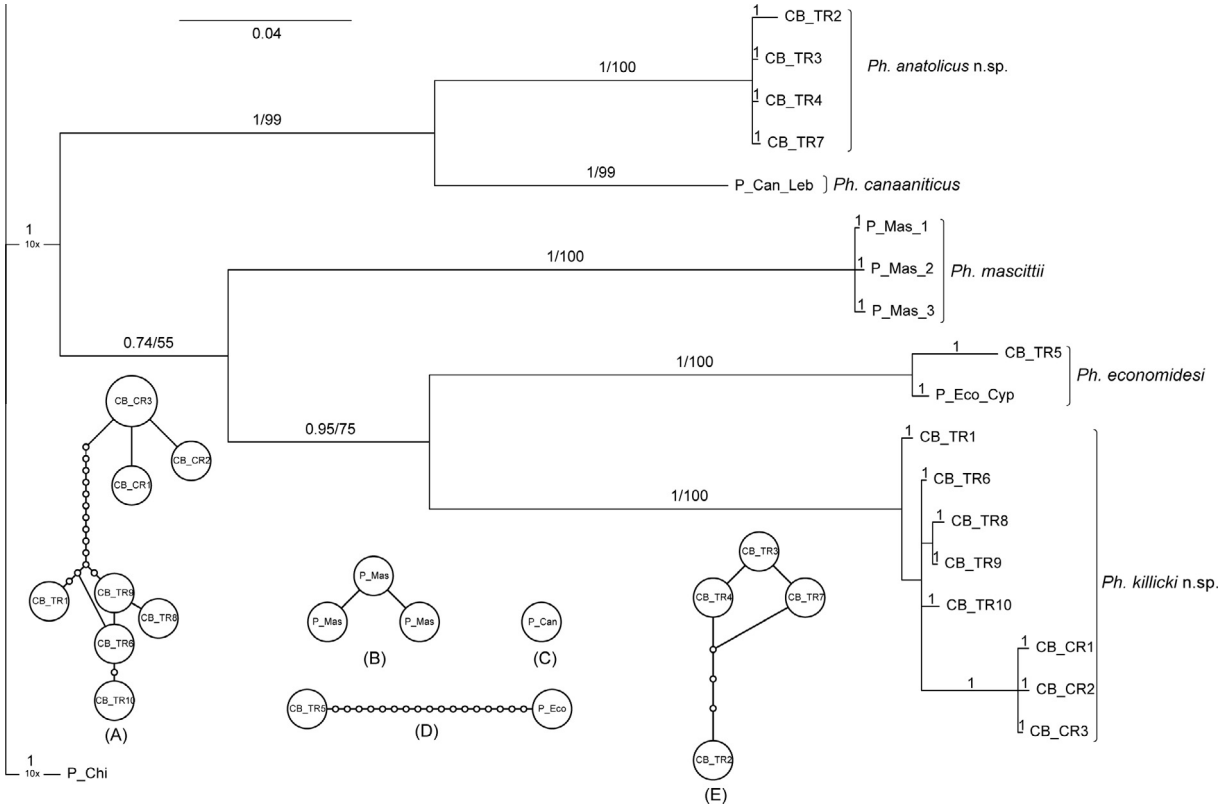

Fig. 2. Bayesian tree (Brunch support: Bayesian inference/maximum likelihood) with corresponding haplotype networks ((A) *Ph. anatolicus* n. sp., (B) *Ph. mascittii*, (C) *Ph. canaaniticus*, (D) *Ph. economidesi*, (E) *Ph. killicki* n. sp.) obtained for each of the *Transphlebotomus* species based on mitochondrial Cyt*b*. Codes for reference sequences are indicated in Table 1.

As the null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level ($P = 0$) in ML analysis, we conducted the *BEAST analysis under an uncorrelated log-normal relaxed clock rate variation model. Divergence time estimates of the five *Transphlebotomus* lineages under the HKY + G model, ranged from 4.5 to 10.5 Mya. Estimated divergence time is 10.5 Mya (95% HPD interval = 6–16) between Clade 1 (*Ph. anatolicus* n. sp. and *Ph. canaaniticus*) and Clade 2 (*Ph. economidesi, Ph. killicki* n. sp. and *Ph. mascittii*). Within the Clade 1, the lineages of *Ph. anatolicus* n. sp. and *Ph. canaaniticus* diverged approximately 4.5 Mya (95% HPD interval = 2.3–7.3). Divergence time estimates within the Clade 2 are as follows: time to the most recent common ancestor (TMRCA) for *Ph. mascittii* and *Ph. economidesi* + *Ph. killicki* n. sp. lineages was estimated as 9 Mya (95% HPD interval = 5.3–14.3) whereas *Ph. economidesi* and *Ph. killicki* n. sp. have split approximately 7 Mya (95% HPD interval = 3.6–11.1) (Fig. 3).

3.3. Morphological examinations

Evaluation of the discriminative characters suggested previously for the subgenus *Transphlebotomus* confirmed the existence of two new species. Of these, one is described only from southern west Anatolia, whereas the other is described from both Crete and south western of Anatolia. In addition, morphological identification of one female confirmed the presence of *Ph. economidesi* at the same Anatolian region. All measurements are indicated in Table 3.

3.3.1. Description of the male Ph. killicki n. sp. (Fig. 4)

13 specimens examined and measured.
Head:

- AIII long, longer than A IV + A V and longer than the labrum. The antennal formula is 2/III–XV.
- Palpal formula: 1–4–3–2–5. About 15 club-like Newstead's scales.
- Labial furca closed.
- Cibarium with very small lateral denticles which can be visible using phase contrast.
- Pharynx: relatively thin with a well developed pharyngeal armature fully occupying its last third. Anterior teeth pointed and oriented back. Posterior ones dot like, tighter against each other than the anterior ones.

Thorax:

- Wing: length = 2141 μm. Width = 668 μm. α = 503 μm. β = 264 μm. Γ = 382 μm. Theta = 619 μm. Epsilon = 646 μm. Pi = 16 μm. Delta = 121 μm. Pi positive.

Genitalia:

- A group of 10–21 scattered hairs in the middle and the partly on the distal part of the coxite.
- Style shorter than the coxite, carrying 5 spines two terminal, three median.
- Paramere simple.
- Aedeagus rounded at the top, width regularly decreasing.
- Surstyle longer than the coxite.

3.3.2. Description of the female Ph. killicki n. sp. (Fig. 5)

7 specimens examined and measured.
Head:

- Interocular suture not complete.
- One antenna is broken after the 2nd segment and the other one after the 12th segment. A3 long, longer than A IV + A V, shorter than the labrum. Antennal formula: 2/III–XV. Long ascoids not reaching the next articulation.
- Palpal formula: 1–4–2–3–5. From 25 to 30 spoon-like Newstead's scales.
- Labial furca closed.
- Cibarium: Lateral denticles observed using phase contrast.

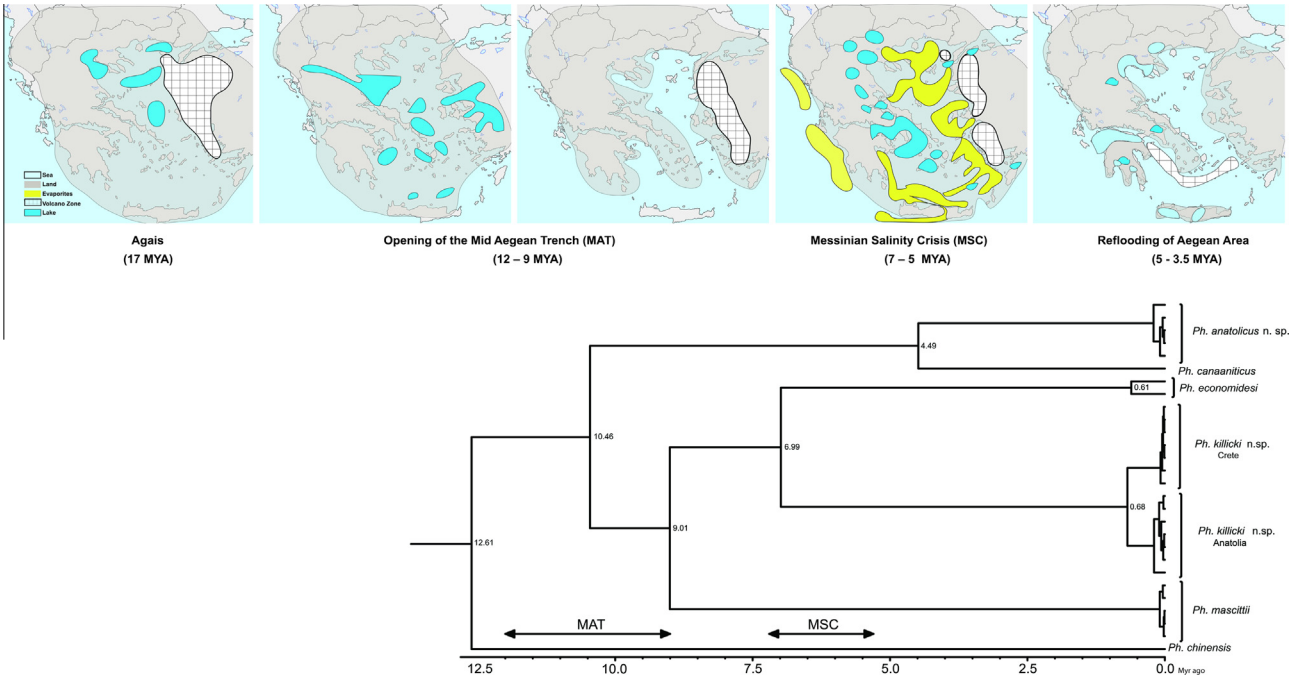

Fig. 3. Divergence times (Myr) indicated by the values on the nodes of the *BEAST chronogram between *Transphlebotomus* species based on mitochondrial Cyt*b*. Horizontal arrows indicate the periods of main paleogeographical events took place around the Aegean area (MAT: Opening of the Mid Aegean Trench, MSC: Messinian Salinity Crisis). The maps showing the main events during the formation of Aegean region redrawn after Meulenkamp, 1985; Dermitzakis, 1990; Poulakakis et al., 2014).

Table 3
Descriptive statistics performed on male specimens of *Transphlebotomus* species. GF: Genital Filament, GP: Genital Pomp.

Species	Specimen	AIII	AIV	AV	AIII/AIV + AV	Labrum	AIII/Labrum	Coxite	Number of coxite hairs	Style	Surstyle	GF	GP	GF/GP	Aedeagus
Ph. anatolicus n. sp.	Mean	286	128	125	1.14	212	1.35	256	30.8	135	334	448	126	3.53	109
	Minimum	270	117	115	1.12	203	1.33	239	26	124	318	411	120	3.43	107
	Maximum	297	135	130	1.16	218	1.36	268	38	144	350	484	133	3.64	111
	Standard error	14.36	9.45	8.39	0.02	7.77	0.02	15.13	4.76	10.15	22.63	51.62	6.66	0.15	2.08
Ph. killicki n. sp.	Mean	420	175	171	1.22	257	1.62	249	14.35	146	344	511	112	4.59	139
	Minimum	380	158	157	1.13	234	1.52	208	10	141	306	421	93	3.79	124
	Maximum	458	188	184	1.30	286	1.74	298	21	166	377	561	124	5.05	157
	Standard error	26.06	9.46	8.82	0.06	14.82	0.07	27.89	2.96	7.77	21.21	42.64	9.21	0.41	10.81
Ph. economidesi	Mean	403	165	166	1.22	297	1.36	427		249	456	522	133	3.93	176
	Minimum	316	149	153	1.15	261	1.26	389	35	227	401	439	117	3	162
	Maximum	495	183	184	1.27	317	1.45	467	40	261	493	603	146	4.46	198
	Standard error	27	10	10	0.04	15	0.06	21		8	26	49	8	0.41	9
Ph. mascittii	Mean	383	173	172	1.11	254	1.5	347		189	383	488	131	3.73	142
	Minimum	316	136	137	1.03	220	1.32	300	35	164	315	407	113	3.33	126
	Maximum	395	222	207	1.29	282	1.76	411	45	223	447	611	153	4.32	164
	Standard error	55	25	24	0.07	19	0.13	33		17	36	57	11	0.26	12
Ph. canaaniticus	Mean	379	160	164	1.17	253	1.5	336		167	368	489	135	3.64	132
	Minimum	329	140	145	1.08	223	1.45	311		151	330	433	113	3.24	116
	Maximum	415	174	181	1.26	283	1.61	358		183	403	567	154	4.23	146
	Standard error	36	16	17	0.07	25	0.07	16		11	27	45	16	0.03	11

– Pharynx: Well developed pharyngeal armature occupying the last third of the pharynx. Anterior teeth relatively long. Posterior ones dot like.

Thorax: Not observed
Genitalia:

– Spermathecae not visible on the examined specimens.

The authors Dvorak, Votypka, Volf are responsible for satisfying the criteria of availability of the name *Ph. killicki* n. sp. and should be cited as the sole authority of these taxa, according to the Article 50.1 of the International Code of Zoological Nomenclature, 4th edition, 2000.

The holotype (male) and three paratypes (two males and one female) of *Ph. killicki* n. sp. have been deposited in the Museum of Natural History, London. Two paratypes (one male and one female) have also been deposited in the Museum national d'Histoire naturelle, Paris.

The type locality of *Ph. killicki* n. sp. is Agia Roumeli, Chora Sfakion, Greece (35° 14′ 34″, 23° 57′ 55″).

Derivatio nominis: The species is named in honor of Robert Killick-Kendrick, the renowned sand fly expert.

3.3.3. Description of the male Ph. anatolicus n. sp. (Fig. 6)

Three specimens examined and measured.
Head:

– Interocular suture incomplete.
– AIII long, longer than AIV + AV, longer than the labrum. The antennal formula is: 2/III–XI, 1/XII–XIII. A small structure, possibly a relic of ascoid is observable on segments XIV to XV.
– Palpal formula: 1–4–3–2–5. About ten club-like Newstead's scales.
– Labial furca closed.
– Cibarium with lateral denticles visible using phase contrast.
– Pharynx: Well developed pharyngeal armature occupying the quarter third of the pharynx. Anterior teeth are longer and the posterior ones are dot like.

Genitalia:

– From 26 to 38 densely grouped hairs in the middle of coxite.
– Style shorter than the coxite, carrying 5 spines two terminal, three median.
– Paramere: Simple.
– Aedeagus rounded at the top, width regularly decreasing.
– Genital filaments/pump ratio: 3.46.
– Surstyle longer than the coxite.

3.3.4. Description of the female Ph. anatolicus n. sp. (Fig. 7)

Two specimens examined and measured.
Head:

– Interocular suture not complete.
– AIII long, longer than A IV + A V, shorter than the labrum. The antennal formula is: 2/III–XV.
– Palpal formula: 1–4–3–2–5. About 30 Newstead's scales club-like.
– Pharyngeal armature well developed, occupying the last third of the pharynx. It includes two types of teeth.
– Labial furca closed.
– Cibarium: lateral denticles are observed using phase contrast.

Thorax: Not observed.
Genitalia:

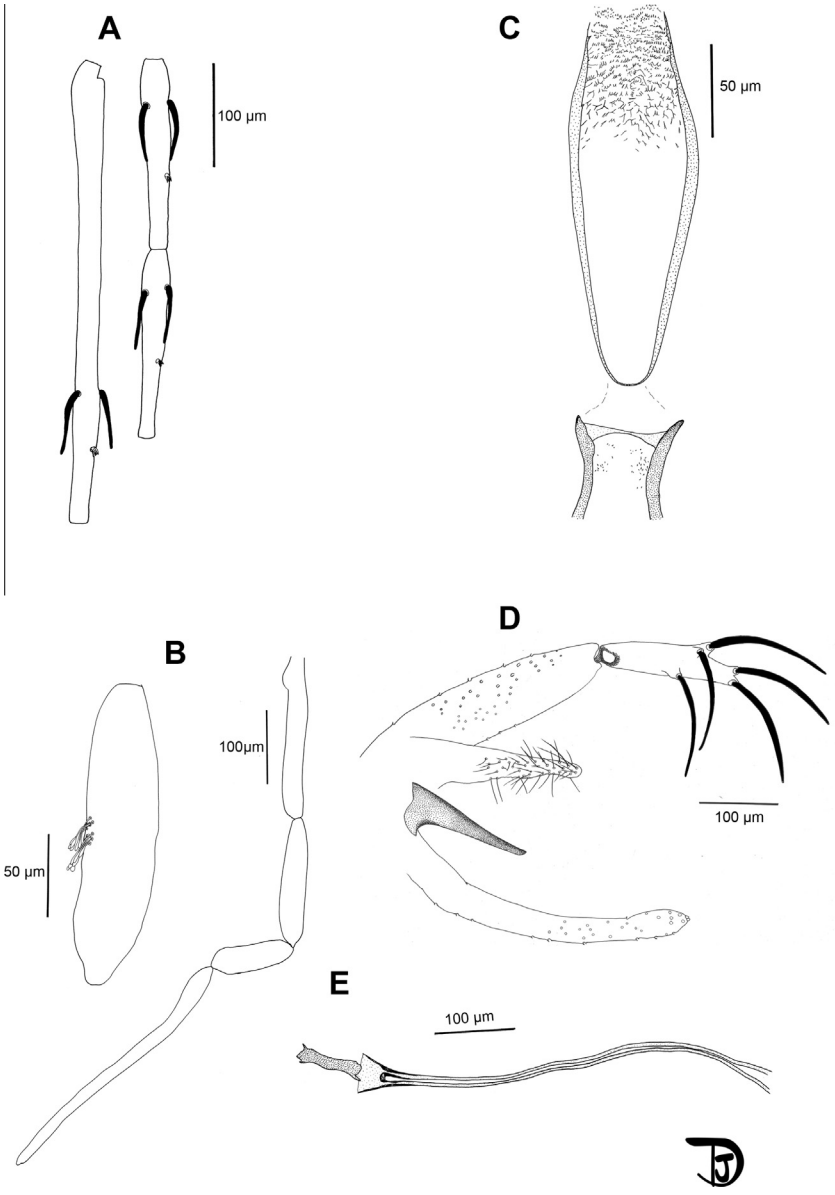

Fig. 4. *Phlebotomus killicki* n. sp. male. (A) Antennal segments III, IV and V, (B) palp (with the third palpal segment bearing the Newstead's scales), (C) pharynx and cibarium, (D) genitalia, (E) genital filament.

– Spermathecae cylindrical, its body is not annealed but striations are still clearly visible. The head is round shaped. The base of the spermathecal ducts was not observable.

The authors Erisoz Kasap, Depaquit, Alten are responsible for satisfying the criteria of availability of the name *Ph. anatolicus* n. sp. and should be cited as the sole authority of these taxa, according to the Article 50.1 of the International Code of Zoological Nomenclature, 4th edition, 2000.

The type locality of *Ph. anatolicus* n. sp. is Belendi, Kas, Antalya, Turkey (36° 12′ 0″, 29° 41′ 54″).

Derivatio nominis: The species name is derived from the name of geographic area (Anatolia) where the type locality is situated.

The holotype (female) and one paratype (male) of *Ph. anatolicus* n. sp. have been deposited in the Museum of Natural History, London. One paratype male has also been deposited in the Museum national d'Histoire naturelle, Paris.

3.4. Identification key for Transphlebotomus males

(1) Third antennal segment shorter than 300 µm	*Ph. anatolicus* n. sp.
Third antennal segment longer than 300 µm	2
(2) Style length more than 200 µm	*Ph. economidesi*
Style length less than 200 µm	3
(3) Coxite length less than 300 µm	*Ph. killicki* n. sp.
Coxite length more than 300 µm	4
(4) A tuft of 35 to 45 coxal setae	*Ph. mascittii*
A tuft of 21 to 27 coxal setae	*Ph. canaaniticus*

4. Discussion

The subgenus *Transphlebotomus* was established by Artemiev in 1984 based on the male genital morphology and female characters for spermathecae and pharyngeal armature; two species, *Ph. mascittii* and *Ph. canaaniticus*, were placed into this newly erected

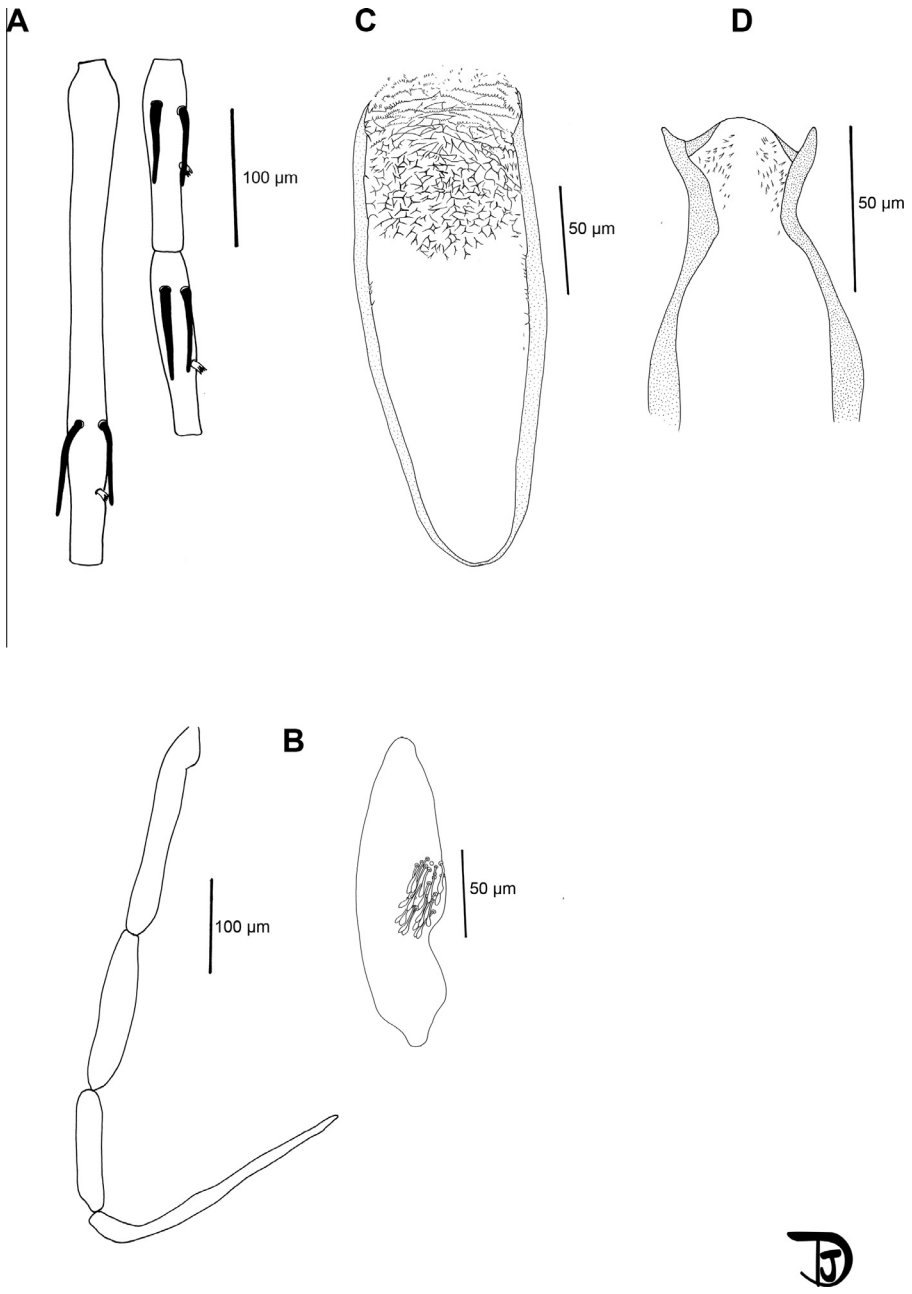

Fig. 5. *Phlebotomus killicki* n. sp. female. (A) Antennal segments III, IV and V, (B) palp (with the third palpal segment bearing the Newstead's scales), (C) pharynx, (D) cibarium.

subgenus (Artemiev and Neronov, 1984). Later, Léger et al. (2000) described *Ph. economidesi* from Cyprus and recorded it as the third member of the subgenus. In the present study, we describe two new species of this subgenus, provide their comprehensive morphological and molecular examination and study their relations with other members of the subgenus *Transphlebotomus*.

Phylogenetic analyses of the mitochondrial cytochrome *b* gene region supported the monophyly of the subgenus *Transphlebotomus* and discriminated the three previously known (*Ph. mascittii*, *Ph. canaaniticus*, and *Ph. economidesi*) as well as two newly described (*Ph. anatolicus* n. sp. and *Ph. killicki* n. sp.) species. Using *Ph. chinensis* as an outgroup, *Ph. canaaniticus* and *Ph. anatolicus* n. sp. lineages form the first major clade of the Cyt*b* phylogeny. Within the second major clade which has a basal position to the first one, *Ph. economidesi* clusters together with *Ph. killicki* n. sp. whereas *Ph. mascittii* appears as their sister species. This is in agreement with the previous study of Depaquit et al. (2005) which also showed the affinity of *Ph. economidesi* to *Ph. mascittii* and their sister relationship with *Ph. canaaniticus*.

From a taxonomic point of view, the inclusion of *Ph. killicki* n. sp. and *P. anatolicus* n. sp. in the subgenus *Transphlebotomus* is justified by following characters: (i) the morphology of the male genitalia, (ii) the morphology of the spermathecae of *Ph. anatolicus* n. sp., and (iii) their phylogenetic position obtained from molecular data. The individualization of these new species is based on the original pharyngeal armature of *P. anatolicus* n. sp. which looks like those observed in the closely related subgenus *Adlerius* Nitzulescu (Supplementary Fig. 2). Regarding the males, the differential diagnosis is mainly based on the number of the setae on the coxite (Fig. 8).

Previously, two males and one female of *Ph. mascittii* were recorded by Houin et al. (1971) in the area where the present study

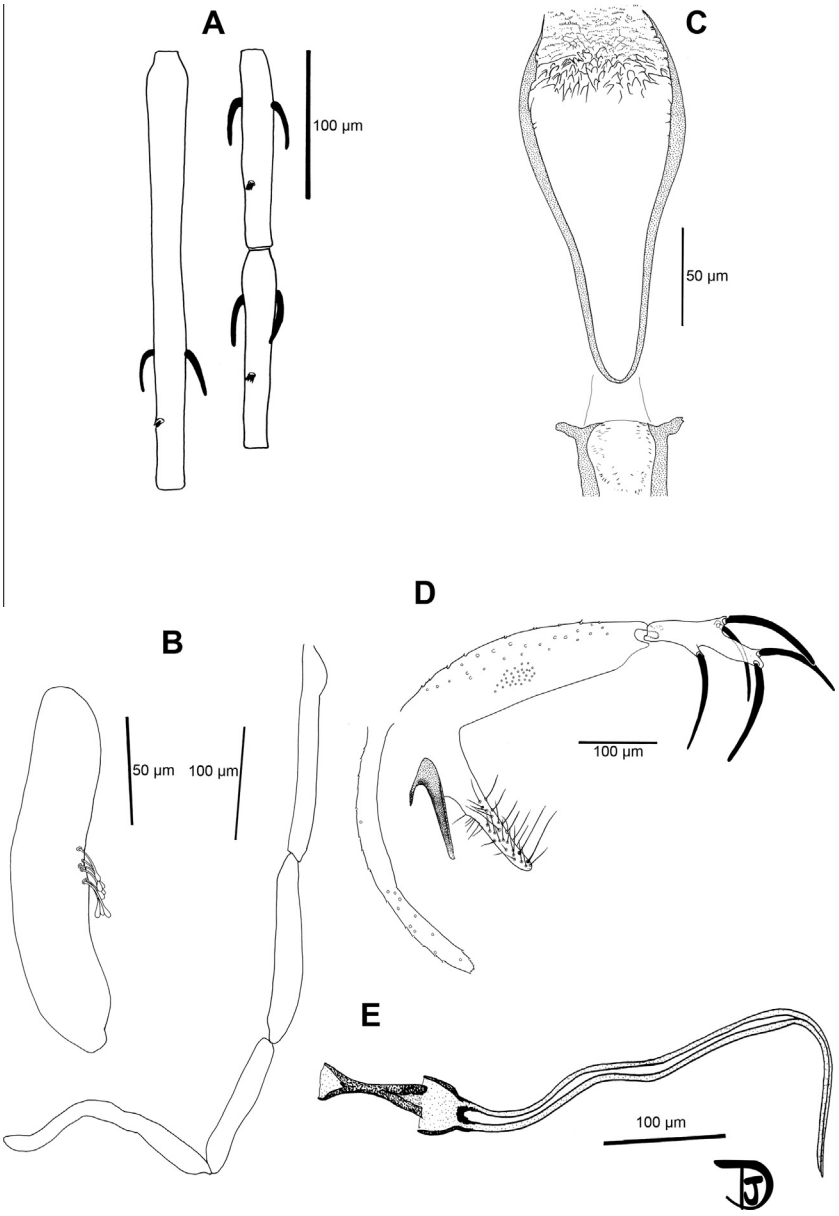

Fig. 6. *Phlebotomus anatolicus* n. sp. male. (A) Antennal segments III, IV and V, (B) palp (with the third palpal segment bearing the Newstead's scales), (C) pharynx and cibarium, (D) Genitalia, (E) Genital filament.

has been carried out. A reexamination of these specimens shows that the males belong in fact to *Ph. killicki* n. sp. (number of coxal setae: 18–19; AIII length: 391–455 µm; style length: 277–298 µm). The pharyngeal armature of the female excludes it belongs to *Ph. killicki* n. sp. It could be *Ph. anatolicus* n. sp., but the examination of more specimen is needed to take the intraspecific variability into consideration. Consequently, the record of *Ph. mascittii* in Turkey is not valid anymore. The record of *Ph. mascittii* (Depaquit et al., 1996) in Rhodes Island, a Greek island located in front of the studied area in Turkey was also reexamined. The male and two females collected during this study belong, in fact, to *Ph. killicki* n. sp.: the male exhibits 17 setae on the coxite and the pharyngeal armature of the female depicted by Depaquit et al. (1996) corresponds with current description of *Ph. killicki*.

Lastly, *Ph. mascittii* had also been recorded in Cyprus (Depaquit et al., 2001) from one female specimen only. The authors indicated this female was not that of *Ph. economidesi* due to spermathecal morphology but could be that of *Ph. mascittii* or that of *Ph. canaaniticus*. The slide has not been stored due to the epidemiological initial goal of the study carried out in Cyprus. However, the atypical pharyngeal armature of *Ph. anatolicus* n. sp. excludes this identification. In the light of this present work's results, the record of *Ph. mascittii* is doubtful and further studies need to be carried out in this island in order to investigate a possible sympatry of several *Transphlebotomus* species.

Parsimony network analysis of *Transphlebotomus* Cyt*b* sequences resulted in the discrimination of five independent networks, corresponding to the five lineages revealed in the phylogenetic analyses. First network contains all *Ph. mascittii* haplotypes from Belgium, France and Germany while the second one includes *Ph. economidesi* from Cyprus and south western Anatolia. All the Cretan haplotypes along with five haplotypes from Anatolia formed the third network which well matches with the *Ph. killicki* n. sp. lineage recovered in the Cyt*b* phylogeny. The remaining four Anatolian haplotypes were found to cluster in the fourth network – the representative of the

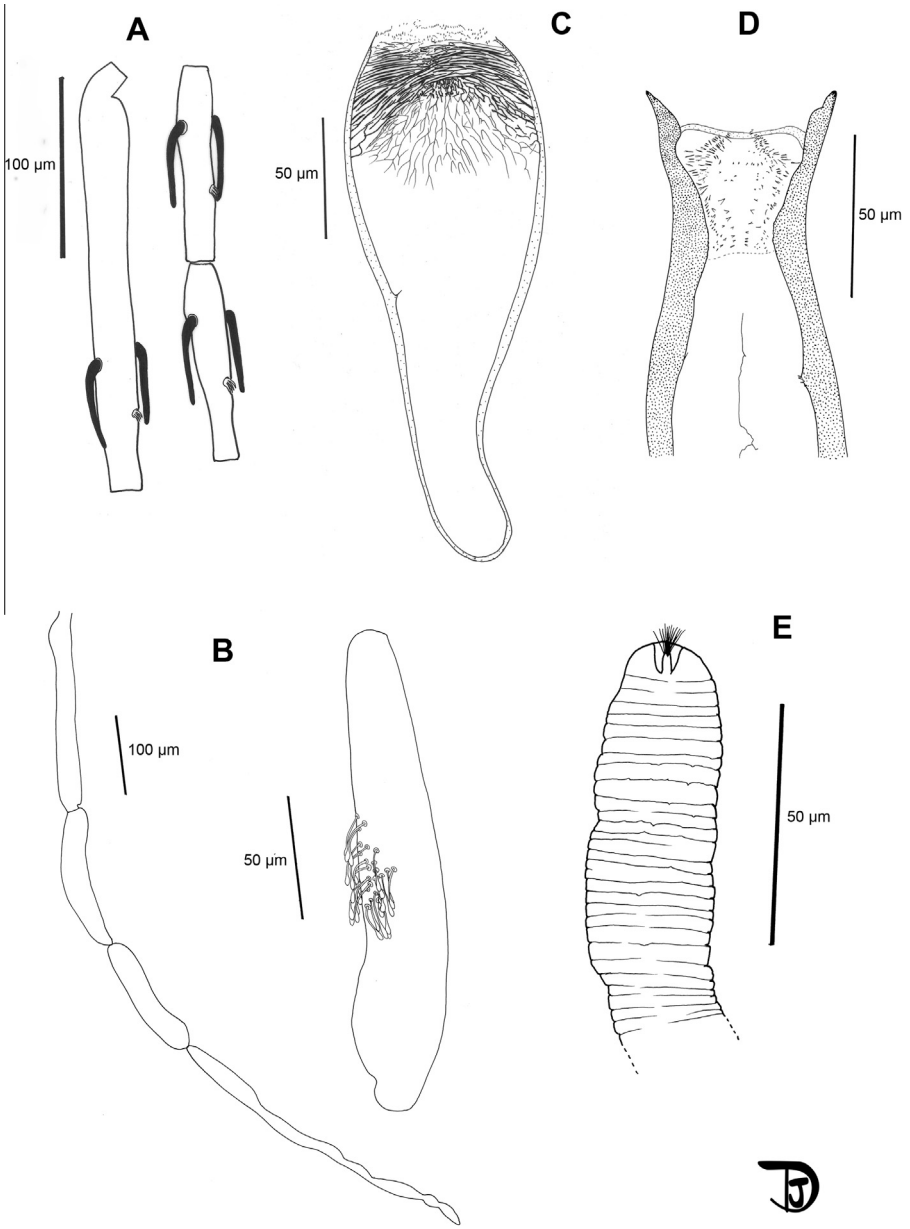

Fig. 7. *Phlebotomus anatolicus* n. sp. female. (A) Antennal segments III, IV and V, (B) palp (with the third palpal segment bearing the Newstead's scales), (C) pharynx, (D) cibarium, (E) spermathecal body.

Ph. anatolicus n. sp. lineage. *Ph. canaaniticus* sequence from Lebanon whose sister species relationship with *Ph. anatolicus* n. sp. was well indicated in the phylogenetic analyses was rejected to be included in any of the four networks. Genetic distances ranging from 9.5% to 12.4% between these five lineages are also comparable with those recorded previously for *Transphlebotomus* species (Depaquit et al., 2005) as well as for the other closely related sand fly species (Testa et al., 2002; Boudabous et al., 2009; Erisoz Kasap et al., 2013). Based on available data on COI and ND4 gene regions, divergence between sequences were found to be congruent with the Cyt*b* data. *Ph. anatolicus* n. sp., *Ph. economidesi* and *Ph. killicki* n. sp. lineages were found to be separated by genetic distances in the range between 12% and 15% for COI gene region and 10% to 12% for the ND4 sequences of *Ph. canaaniticus*, *Ph. economidesi*, *Ph. killicki* n. sp. and *Ph. mascittii*.

The estimated age for the split of the *Transphlebotomus* subgenus closely matches with the biogeography of the Aegean Region. Located at the margins of the Eurasian and African Plates, this area is characterized by a complex palaeogeographic history. The formation of the mid-Aegean Trench (MAT) (12–9 Mya), the occurrence of several land bridges between western Anatolia and Greece during the Messinian Salinity Crisis (MSC) (7–5.5 Mya) and changes in the sea level during Pliocene (5.3–2.5 Mya) and Pleistocene (2.5 Mya to 11.7 Ka) were shown as the most influential geological events that shape the current diversification and distribution of the terrestrial and aquatic taxa occur in this area (reviewed by Poulakakis et al., 2014).

During the upper and middle Miocene (23–12 Mya), Aegean region was a part of a united landmass known as Agäis. Formation of the MAT (12–9 Mya) that was initiated by tectonic movements resulted in the separation of the west Aegean from the east Aegean islands and Asia Minor. These dates fit well with our divergence time estimate for the first split in the *Transphlebotomus* subgenus and let us suggest that the MAT is the main driver for the

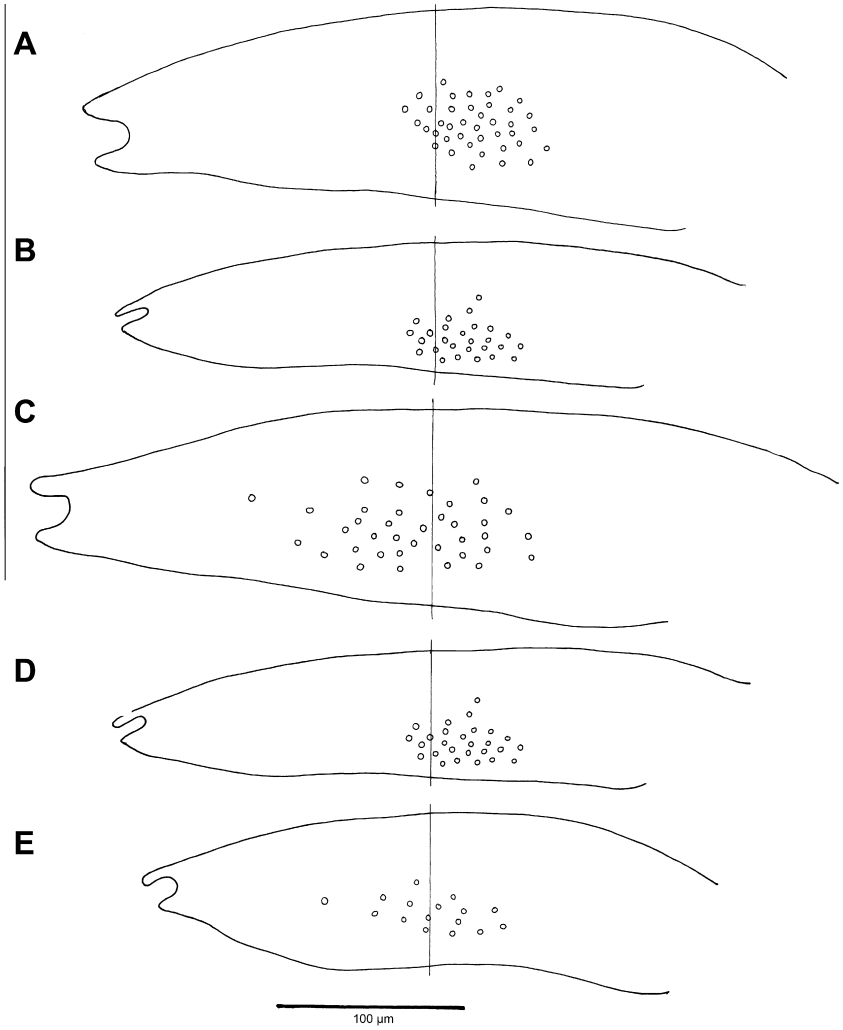

Fig. 8. Comparison of coxite setae of *Transphlebotomus* spp. males. (A) *Ph. mascittii* (Corsica), (B) *Ph. canaaniticus* (Topotype), (C) *Ph. economidesi* (Topotype), (D) *Ph. anatolicus* n. sp., (E) *Ph. killicki* n. sp.

separation of the Clade 1 (*Ph. anatolicus* n. sp. and *Ph. canaaniticus*) and Clade 2 (*Ph. economidesi*, *Ph. killicki* n. sp., and *Ph. mascittii*), approximately 10.5 Mya. Based on these data we also assume that the ancestor of the *Transphlebotomus* was present in the old Aegean region before the formation of MAT which is the case for several animals' phylogeographic history around the region such as *Mesobuthus* scorpions (Parmakelis et al., 2006), legless skinks *Ophiomorus* (Poulakakis et al., 2008) and *Troglophilus* crickets (Kaya et al., 2012).

Second divergence in the subgenus took place in the western Clade 2. Separation of *Ph. mascittii* lineage from *Ph. economidesi* and *Ph. killicki* n. sp. lineages appears to have occurred approximately 9 Mya and thus possibly related with the same event (MAT) which also caused the isolation of Crete from mainland Greece and Cyclades (9.7–8.9 Mya). The formation of MAT is known to last three million years and progressed gradually northwards. Therefore, Crete was the first part to be isolated from the united landmass of Agäis. Separation of several Cretan lineages were suggested to be dated back to this historic event as reported for the gecko *Cyrtopodion kotschyi* (Kasapidis et al., 2005), land snail of the genus *Mastus* (Parmakelis et al., 2003), scorpion *Mesobuthus gibbosus* (Parmakelis et al., 2006) and frog of the genus *Pelophylax* (Akın et al., 2010).

Time to the most recent ancestors of *Ph. economidesi* and *Ph. killicki* n. sp. was estimated as 7 Mya corresponding to Messinian Salinity Crisis. During the end of Miocene, connection between Atlantic Ocean and Mediterranean Sea was lost and Mediterranean Sea dried up. Several land bridges were established which allowed organisms to disperse between west Aegean region and east Aegean islands plus Asia Minor. When the Mediterranean reopened, the basin was filled and western and eastern Aegean regions separated vicariantly. Based on this palaeogeographic event, we assume that the ancestor of *Ph. economidesi* and *Ph. killicki* was represented on the western side of the Aegean region, probably in Crete before the Messinian Crisis. During this latter period, this ancestor probably dispersed to the east by temporary land bridges. Reflooding of the Mediterranean caused the isolation of Cretan lineage *Ph. killicki* n. sp. on the west side and gave rise to the *Ph. economidesi* lineage on the east in southern west Anatolia. Our finding of *Ph. killicki* n. sp. in Anatolia indicates a more recent

dispersal between Crete and Anatolia which is also reflected in the very low level of genetic divergence between the two lineages (1.7%). This long distance dispersal can be facilitated through the satellite islands of Crete and/or Rhodes and Karpathos islands which may have acted as stepping stones.

Being one of the most isolated Mediterranean islands, the biogeography of Cyprus has been controversial. It is known that the island originated from the raised seabed and according to many researchers, it has never had a connection with the neighboring mainland (Hadjisterkotis et al., 2000). Others, however, suggest that there used to be a land bridge which connected the island with the mainland, most probably with southern Anatolia and/or Syria during Messinian Salinity Crisis (Kornilios et al., 2012). However, considering the estimated divergence time between Anatolian and Cypriot *Ph. economidesi* (0.6 Mya), colonization of the island took place more recently, being not a result of geodispersal but rather an oversea (most probably passive) dispersal from Anatolia, as was demonstrated for several other taxa (Poulakakis et al., 2013).

The last split within the subgenus *Transphlebotomus* appears to have occurred approximately 4.5 Mya. Uplifting of Taurus Mountains during late Miocene and Pliocene with subsequent environmental changes (Seyrek et al., 2008) may have caused a split from *Ph. anatolicus* n. sp. lineage and gave birth to eastern *Ph. canaaniticus* lineage.

In conclusion, our analyses showed that the subgenus *Transphlebotomus* has been diversifying in the Aegean and Mediterranean regions since ~10 Myr mainly driven by the old paleogeographic events that took place around these regions. Yet, these estimates of diversification should be evaluated with caution when considering the limited data we have especially for *Ph. economidesi* and *Ph. canaaniticus.* Inclusion of more specimens from eastern Aegean islands and Balkans into an analysis would improve these conclusions on the distribution and diversification of Anatolian and Cretan members of the subgenus. In the light of our results that we based on both morphological and molecular data we also suggest to reinvestigate the records of *Ph. mascittii* from south eastern and western Turkey and from Palestinian West Bank (Volf et al., 2002; Sawalha et al., 2003; Ozbel et al., 2011).

Conflicts of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2015.05.025.

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