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Comparative molecular and morphological study of *Stenoponia tripectinata tripectinata* (Siphonaptera: Stenoponiidae) from the Canary Islands and Corsica

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Research Paper

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Abstract

Stenoponia tripectinata tripectinata (Tiraboschi, 1902) is the most prevalent subspecies, within the genus *Stenoponia*, in the Mediterranean area. This rodent flea is widely distributed throughout southwestern Europe and the North of Africa including Mediterranean islands and the Canary Islands. Nevertheless, from a taxonomical and systematic point, this flea group has been neglected over the years. Therefore, the aim of this study was to carry out a comparative morphometric, phylogenetic, and molecular study of two populations of *S. t. tripectinata* isolated from rodents collected from different islands from the Canary Archipelago and from Corsica to clarify the taxonomic status of these two isolated populations and to assess the morphological and molecular differentiation between them. For this purpose, we have analyzed several morphological traits and sequenced five molecular markers (*EF1-α*, ITS2, *cox*1, *cox*2, and *cyt*b). We observed slight differences in the overall body size between females of both populations, and two well-defined geographical genetic lineages. This suggests the existence of two cryptic subspecies within *S. t. tripectinata* corresponding to two different island groups. Furthermore, we bring to light the necessity to provide new and updated morphological, molecular, and phylogenetic data to clarify the taxonomic status of *S. tripectinata*.

Introduction

The genus *Stenoponia* Jordan and Rothschild, 1911 is a mainly Palearctic genus with a total of 16 species, two of which occur in the Nearctic region (Lewis, 1993). All the species are found principally on Muridae (rats and mice, voles and gerbils) (Hopkins and Rothschild, 1962). *Stenoponia* is the only genus within Stenoponiinae subfamily; however, the systematic position of this genus within flea systematic has remained controversial over the years. Thus, this genus was first placed within the family Hystrichopsyllidae (Hopkins and Rothschild, 1962); however, Whiting *et al.* (2008) placed the subfamily Stenoponiinae within the family Ctenophthalmidae with Rhadinopsyllinae as sister group. Finally, Zurita *et al.* (2015) used molecular data to assess the monophyly of Stenoponiinae and suggested that this subfamily should be separated from the Ctenophthalmidae at the family level (Stenoponiidae).

Within the genus *Stenoponia*, the most common subspecies in the Mediterranean area is *Stenoponia tripectinata tripectinata* (Tiraboschi, 1902). This rodent flea is widely distributed throughout southwestern area of Europe and the North of Africa including Mediterranean islands and the Canary Islands. It mainly parasitizes Muridae and Arvicolidae hosts, of which *Mus spretus*, *Mus musculus musculus*, and *Mus musculus domesticus* are the most common (Beaucournu and Launay, 1990). From an epidemiological point of view, *S. t. tripectinata* is considered the main vector of plague in Asia Minor and European Russia (Lewis, 1993), furthermore, the proteobacterium *Bartonella elizabethae* has been detected in specimens of this flea subspecies collected from the Canary Islands and Portugal (De Sousa *et al.*, 2006; Zurita *et al.*, 2016; Abreu-Yanes *et al.*, 2018). In this sense, it is known that species belonging to the *B. elizabethae* complex, including *Bartonella tribocorum* and *B. elizabethae* have zoonotic potential when transmitted to humans by a great variety of arthropod vectors, such as fleas, lice, and ticks. *Bartonella* infections in humans can cause different clinical manifestations, such as fatigue, muscle pain, or fever and may develop into serious complications, like neurological signs or endocarditis (Billeter *et al.*, 2008).

Hitherto, only one molecular phylogenetic study of the genus *Stenoponia* has been published (Zurita *et al.*, 2015). Using nuclear (ribosomal internal transcribed spacers 1 and 2 (ITS1, ITS2) and a fragment of 18S rRNA) and mitochondrial gene fragments (*cytochrome c-oxidase* 1 (*cox*1)), these authors found no nucleotide variation within and among *S. t. tripectinata* populations from different islands in the Canary Archipelago.

Yet, Zurita *et al.* (2015) did observe that *S. t. tripectinata* from the Canary Islands and the Iberian Peninsula belong to two different lineages separated by specific restriction endonucleases.

Islands are biologically interesting study systems because: (i) the lower biological complexity of island communities when compared to equivalent mainland ones, (ii) their clearly defined spatial limits and (iii) the availability of a large range of whatever properties are studied (area, latitude, altitude, isolation, etc.) (Pérez-Mellado and Ramon, 2010). These features together with the diverse origin of islands, their geographical settings and locations, their dynamic history and, especially, their persistent isolation through time, have made them outstanding evolutionary tools for research community (Mayr, 1967; Pérez-Mellado and Ramon, 2010). Lastly, archipelagos such as the Canary Islands, have been referred to as speciation machines in the sense that new endemic species, generated from few colonization events, are continuously produced in them resulting in high rates of speciation (Rosenzweig, 1995).

The aim of this study was to carry out a comparative morphometric, phylogenetic, and molecular study of two populations of *S. t. tripectinata* isolated from rodents collected from different islands from the Canary Archipelago and from Corsica to clarify the taxonomic status of these two isolated populations and to assess the morphological and molecular differentiation between them. Furthermore, a possible existence of cryptic subspecies between both populations was evaluated in this work. For this purpose, we have analyzed several morphological traits and sequenced the nuclear *elongation factor 1 alpha* (*EF1*-α), ITS2 ribosomal DNA (rDNA), and the *cytochrome c oxidase* subunit 1 (*cox*1), *cytochrome c oxidase* subunit 2 (*cox*2), and *cytochrome* b (*cyt*b) mtDNA partial genes of several specimens of both populations.

Material and methods

Collection of samples

A total of 89 fleas were collected from rodents (*M. musculus musculus*) trapped from different islands (Gran Canaria, La Palma, El Hierro, La Gomera, and Tenerife) of the Canary Archipelago (Spain) (28°32′10″N 15°44′56″O), and a total of 41 fleas were collected from rodents (*Rattus* sp., *Apodemus* sp., and *Mus* sp.) trapped from Corsica (France) (42°09′00″N 9°05′00″E). All rodent specimens were captured using live traps. Afterward, each rodent was exhaustively examined for fleas by combing through an inspection of head, neck, body, sides, tail, and ventral regions of each animal. Fleas were collected manually and kept in Eppendorf tubes with 96% ethanol for subsequent identification and DNA extraction.

Morphological identification and biometrical study

For morphological analysis, all whole specimens were examined and photographed under an optical microscope to carry out a first specific classification. Subsequently, 16 flea specimens from different islands of the Canary Archipelago and nine flea specimens from Corsica were put away for DNA analysis. On the other hand, 20 specimens (ten males and ten females) from El Hierro (Canary Islands) and 18 specimens (nine males and nine females) from Corsica were cleared with 10% KOH, prepared, and mounted on glass slides using conventional procedures with EUKITT mounting medium (O. Kindler GmbH & Co., Freiburg, Germany) (Lewis, 1993). No specimens from other islands from the Canary Archipelago were selected for morphometric analysis due to the lack of samples. Once mounted, they were examined and photographed again for a deeper morphological analysis using a CX21 microscope (Olympus, Tokyo, Japan). Diagnostic morphological characters were studied by comparison with figures, keys, and descriptions in Hopkins and Rothschild (1962) and Beaucournu and Launay (1990). After morphological identification, the cleared and mounted specimens were measured using a Zeiss microscope 47 30 11 9901 (Zeiss, Germany) according to ten different parameters for males and 12 different parameters for females (tables 1 and 2). Descriptive univariate statistics (arithmetic mean, standard deviation, and coefficient of variation) for all parameters were determined using Microsoft Excel 5.0. Furthermore, to assess phenotypic differentiation among the samples, morphometric data were explored using multivariate analysis in five measurements (TL, TW, HW, DISTL, and DISTW) in males (see table 1) and eight measurements (TL, TW, HL, HW, BULGAL, BULGAW, APEHILW, and DBMV) in females (see table 2) by Principal Component Analysis (PCA) (Dujardin and Le Pont, 2004). Phenotypic analyses were conducted using BAC v.2 software (Dujardin, 2002; Valero *et al.*, 2009; García-Sánchez *et al.*, 2019).

Molecular and phylogenetic study

The DNA markers sequenced in the present study (*EF1*-α, ITS2 rDNA, *cox*1, *cox*2, and *cyt*b) were amplified by a polymerase chain reaction (PCR) using a thermal cycler (Eppendorf AG; Eppendorf, Hamburg, Germany). PCR mix, PCR conditions, and PCR primers are summarized in the Supporting information (table S1).

The *EF1*-α, ITS2, *cox*1, *cox*2, and *cyt*b sequences were deposited in GenBank (table 3).

The PCR products were checked on SYBR Safe stained 2% Tris–borate–ethylenediaminetetraacetic acid agarose gels. PCR products were purified using the QWizard SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, USA). Once purified, these products were sent to the commercial company Stab Vida (Lisbon, Portugal) for sequencing process. We separately sent purified PCR products and 20 µl of 100 µM of each pair of primers (see table S2) for each molecular marker. Sanger sequencing was carried out using an automatic LI-COR® DNA sequencer. Sequences were aligned with the MUSCLE alignment method (Edgar, 2004) in MEGA, version 5.2 (Tamura *et al.*, 2011). Alignment settings comprised a gap open = −400.00, a gap extend = 0.0, an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) as a cluster method, and a minimum diagonal length = 24. Sequence similarity was expressed as percentage of sequence divergence using uncorrected *p*-distances method as implemented in MEGA, version 5.2 (Tamura *et al.*, 2011). Coding genes sequences were searched for nuclear mitochondrial pseudogenes (Numts) by BLASTN (Altschul *et al.*, 1990). Threshold levels for the inference of Numts from BLASTN hits were taken as expectation values (*E* values) of 10^{-4} or 10^{-14}.

Phylogenetic trees were inferred by: Maximum Likelihood (ML) and Bayesian Inferences (BI). ML trees were generated using the PHYML package from Guindon and Gascuel (2003), whereas BI were generated using MRBAYES, version 3.2.6 (Ronquist and Huelsenbeck, 2003). JMODELTEST (Posada, 2008) was used to determinate the best-fit substitution model for *EF1*-α, *cox*1, *cox*2, and *cyt*b. Models of evolution were chosen for subsequent analyses according to the Akaike information

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Table 1. Biometrical data of males of *S. t. tripectinata* analyzed in this study

	S. t. tripectinata from Corsica/males					*S. t. tripectinata* from El Hierro (Canary Islands)/males				
	MAX	MIN	Mean	SD	VC	MAX	MIN	Mean	SD	VC
TL (mm)*	3.5	3.0	3.2	0.2	6	3.4	2.8	3.0	0.2	7
TW (mm)	1.1	0.9	1.0	0.1	10	1.0	0.9	1.0	0.1	10
HL (μm)	533	498	515	13	3	539	481	505	18	4
HW (μm)	258	205	225	16	7	275	193	220	23	10
PROTW (μm)	264	234	253	10	4	264	240	253	9	4
MESOW (μm)	305	234	269	22	8	293	223	274	20	7
METW (μm)	316	281	298	13	4	334	270	307	18	6
DISTL (μm)	510	440	464	26	6	498	421	462	23	5
DISTW (μm)	103	89	97	5	5	106	89	100	5	5
PROXL (μm)	463	381	425	27	6	433	404	418	10	2

TL, total length; TW, total width; HL, total length of the head; HW, total width of the head; PROTW, total width of the prothorax; MESOW, total width of the mesothorax; METW, total width of the metathorax; DISTL, total length of the distal branch of the IX sternum; DISTW, total width of the distal branch of the IX sternum; PROXL, total length of the proximal branch of the IX sternum; MAX, maximum; MIN, minimum; SD, standard deviation; Mean, arithmetic mean; VC, coefficient of variation (percentage converted).
*Significant differences between both populations of males ($P < 0.005$).

Table 2. Biometrical data of females of *S. t. tripectinata* analyzed in this study

	S. t. tripectinata from Corsica/females					*S. t. tripectinata* from El Hierro (Canary Islands)/females				
	MAX	MIN	Mean	SD	VC	MAX	MIN	Mean	SD	VC
TL (mm)	4.0	3.4	3.7	0.2	5	4.2	3.3	3.7	0.3	8
TW (mm)	1.4	1.2	1.3	0.1	8	1.5	1.1	1.3	0.1	8
HL (μm)*	586	516	552	26	5	551	510	527	13	2
HW (μm)*	281	229	249	19	8	252	217	236	10	4
PROTW (μm)	322	246	291	27	9	322	293	308	11	4
MESOW (μm)	357	188	310	52	17	322	205	296	34	11
METW (μm)	381	305	344	27	8	369	322	340	14	4
BULGAL (μm)*	115	89	102	8	8	106	89	98	5	5
BULGAW (μm)	103	85	93	7	8	96	80	88	5	6
APEHILL (μm)	136	129	130	2	2	165	106	127	17	13
APEHILW (μm)*	47	28	39	7	18	49	42	46	2	4
DBMV (μm)	416	147	326	87	27	474	240	351	76	22

TL, total length; TW, total width; HL, total length of the head; HW, total width of the head; PROTW, total width of the prothorax; MESOW; total width of the mesothorax; METW, total width of the metathorax; BULGAL, total length of the bulga; BULGAW, total width of the bulga; APEHILL, total length of the apex of the hilla; APEHILLW, total width of the apex of the hilla; DBMV, distance from bulga to ventral margin of the body; MAX, maximum; MIN, minimum; SD, standard deviation; Mean, arithmetic mean; VC, coefficient of variation (percentage converted).
*Significant differences between both populations of females ($P < 0.005$).

criterion (Huelsenbeck and Rannala, 1997; Posada and Buckley, 2004). The concatenated alignment of *EF1*-α, *cox*1, *cox*2, and *cyt*b was analyzed by BI after partitioning and model selection with JMODELTEST. For ML inference, best-fit nucleotide substitution models included TIM2 + I + G (*cox*2), TIM1 + I + G (*cox*1), and GTR + I + G (*EF1*-α and *cyt*b). Support for the topology was examined using bootstrapping (heuristic option) (Felsenstein, 1985) over 1000 replications. The commands used in MRBAYES, version 3.2.6 for BI were *nst* = 6 with invgamma rates (*EF1*-α, *cox*1, *cox*2, and *cyt*b). For BI, the standard deviation of split frequencies was used to determine whether the number of generations completed was enough; the chain was sampled every 500 generations and each dataset was run for 10 million generations. Adequacy of sampling and run convergence were assessed using the effective sample size diagnostic in tracer, version 1.6 (Rambaut and Drummond, 2007). Trees from the first million generations were discarded based on an assessment of convergence. Burn-in was determined empirically by examination of the log likelihood values of the chains. Bayesian Posterior Probabilities (BPP) were used to assess the reliability of nodes.

The phylogenetic analyses, of single gene fragments *EF1*-α, *cox*1, *cox*2, and *cyt*b were carried out using our sequences and those obtained from GenBank (table S2). Phylogenetic trees were rooted using *Panorpa meridionalis* (Mecoptera:

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Table 3. GenBank accession numbers of ITS2, *EF1-α*, and partial *cyt*b, *cox*1, and *cox*2 gene sequences of individuals of *S. t. tripectinata* obtained in this study

Species	Sample ID/geographical area	Host	Number of fleas	Base pairs (bp)	Accession number
ITS2					
S. t. tripectinata	STT1-4, STT6, STT8-9, STT17-18/Corsica, France	*Mus* sp., *Rattus* sp. and *Apodemus* sp.	9	332	LR983953
*Cox*1					
S. t. tripectinata	STT6/Corsica, France	*Rattus* sp.	1	658	LR989038
S. t. tripectinata	STT1/Corsica, France	*Mus* sp.	1	658	LR989039
S. t. tripectinata	STT2, STT3/Corsica, France	*Apodemus* sp.	2	658	LR989040
S. t. tripectinata	STT4, STT8-9, STT17-18/Corsica, France	*Apodemus* sp.	5	658	LR989041
*Cox*2					
S. t. tripectinata	STT6/Corsica, France	*Rattus* sp.	1	730	LR983966
S. t. tripectinata	STT1-4, STT8-9, STT17-18/Corsica, France	*Mus* sp. and *Apodemus* sp.	8	730	LR983967
S. t. tripectinata	9AZ/Gran Canaria (Canary Islands)	*Mus musculus*	1	730	LR989042
S. t. tripectinata	10AZ, 25AZ, 26AZ/Gran Canaria (Canary Islands)	*Mus musculus*	3	730	LR989043
S. t. tripectinata	13AZ, 14AZ, 15AZ/La Palma (Canary Islands)	*Mus musculus*	3	730	LR989044
S. t. tripectinata	16AZ, 18AZ, 33AZ/La Gomera (Canary Islands)	*Mus musculus*	3	730	LR989045
S. t. tripectinata	19AZ, 20AZ, 21AZ/El Hierro (Canary Islands)	*Mus musculus*	3	730	LR989046
S. t. tripectinata	22AZ, 23AZ, 24AZ/Tenerife (Canary Islands)	*Mus musculus*	3	730	LR989047
*Cyt*b					
S. t. tripectinata	STT1-4, STT6, STT8-9, STT17-18/Corsica, France	*Mus* sp., *Rattus* sp. and *Apodemus* sp.	9	374	LR983954
S. t. tripectinata	9AZ/Gran Canaria (Canary Islands)	*Mus musculus*	1	374	LN897472
S. t. tripectinata	25AZ/Gran Canaria (Canary Islands)	*Mus musculus*	1	374	LR983958
S. t. tripectinata	10AZ, 26AZ/Gran Canaria (Canary Islands)	*Mus musculus*	2	374	LR983960
S. t. tripectinata	13AZ, 14AZ, 15AZ/La Palma (Canary Islands)	*Mus musculus*	3	374	LR983962
S. t. tripectinata	16AZ, 18AZ, 33AZ/La Gomera (Canary Islands)	*Mus musculus*	3	374	LR983963
S. t. tripectinata	19AZ, 20AZ, 21AZ/El Hierro (Canary Islands)	*Mus musculus*	3	374	LR983964
S. t. tripectinata	22AZ/Tenerife (Canary Islands)	*Mus musculus*	1	374	LR983961
S. t. tripectinata	23AZ/Tenerife (Canary Islands)	*Mus musculus*	1	374	LN897473
S. t. tripectinata	24AZ/Tenerife (Canary Islands)	*Mus musculus*	1	374	LR983959
EF1-α					
S. t. tripectinata	STT1, STT18/Corsica, France	*Mus* sp.	2	976	LR989033
S. t. tripectinata	STT2, STT9/Corsica, France	*Mus* sp. and *Apodemus* sp.	2	976	LR989034
S. t. tripectinata	STT4, STT17/Corsica, France	*Mus* sp. *and Apodemus* sp.	2	976	LR989035
S. t. tripectinata	STT6/Corsica, France	*Rattus* sp.	1	976	LR989036
S. t. tripectinata	STT3, STT8/Corsica, France	*Apodemus* sp.	2	976	LR989037
S. t. tripectinata	9AZ, 25AZ/Gran Canaria (Canary Islands)	*Mus musculus*	2	976	LR744006-07
S. t. tripectinata	13AZ, 14AZ, 15AZ/La Palma (Canary Islands)	*Mus musculus*	3	976	LR989029
S. t. tripectinata	16AZ, 18AZ, 33AZ/La Gomera (Canary Islands)	*Mus musculus*	3	976	LR989030
S. t. tripectinata	19AZ, 20AZ, 21AZ/El Hierro (Canary Islands)	*Mus musculus*	3	976	LR989032
S. t. tripectinata	22AZ, 23AZ, 24AZ/Tenerife (Canary Islands)	*Mus musculus*	3	976	LR989031

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Panorpidae) as outgroup. This choice was based on the combination of morphological and molecular data obtained in previous studies, which provided compelling evidence for a sister group relationship between Mecoptera and Siphonaptera (Whiting, 2002; Whiting *et al.*, 2008).

ITS2 sequences were exclusively used to characterize *S. t. tripectinata* population isolated from Corsica and in comparison with those from the Canary Islands (Zurita *et al.*, 2015).

Results

Morphological and biometrical results

All the specimens studied in this work showed morphological characteristics expected for the subspecies *S. t. tripectinata*:

- Eyes vestigial and non-pigmented (fig. 1a).
- Distance between oral angle and first spine of genal comb far more (2/3) than half the length of the genal comb (fig. 1a).
- Genal comb with numerous spines out of which none (sometimes one) of the posterior ones appear out of alignment with the rest (fig. 1b).
- Presence of one pronotal comb and one fully developed abdominal comb (composed by spines which are as long as those of the pronotal comb) (fig. 1c).
- Telomere or movable process of males long and rather straight, rarely somewhat curved (fig. 1d).
- Males showing a dilated apical portion of distal arm of sternum IX with the dorsal and ventral margins subparallel. Dorsal margin usually much more convex than the ventral one (fig. 1e).
- Crochet of males' phallosome variable but usually with a long ventral projection and a short dorsal one (fig. 1f).
- Ventral margin of sternum VII of females with an apical lobe of variable size, which subtended a little sinus of variable size (fig. 1g).
- Spermatheca of females subspherical bulga that projects into the cribriform area without thickened rim around the base of the hilla. The hilla is latter variable in size and shape than bulga but usually much longer than it. Bulga with a posterior little protuberance (fig. 1g).

Based on the morphological features described above, we could not separate both flea populations (Canary Islands and Corsica).

In the PCA, male variables were significantly correlated with PC1, contributing 58% to the overall variation. In the factor map, the two male populations strongly overlapped and as such there was no overall size difference between the males from the El Hierro (Canary Islands) and those from Corsica (fig. 2)

Female variables were significantly correlated with PC1, contributing 66% to the overall variation. In this case, the factor map revealed that the female populations of the El Hierro (Canary Islands) and Corsica show only very limited overlap and hence are well-separated, such that females in Corsica are larger than in the El Hierro island (fig. 3).

DNA sequences results

ITS2 and EF1-α

The length of ITS2 was 332 bp and of *EF1*-α 976 bp (table 3). All ITS2 sequences were identical, whereas the *EF1*-α sequence divergence ranged from 0.6 to 1.4% between both populations. Within the Canary Islands all *EF1*-α sequences were identical; yet, in Corsica there were five haplotypes showing 0.2–1.5% of sequence divergence. In contrast, *EF1*-α sequence divergence between *S. t. tripectinata* and *Stenoponia tripectinata medialis* was slightly higher while the *EF1*-α sequence divergence between *S. t. tripectinata* and congeneric species, such as *Stenoponia americana* and *Stenoponia sidimi*, always exceeded 7.0% (table 4). Lastly, we did not find any Numts in any *EF1*-α sequence.

Partial cox1, cox2, and cytb analysis

The lengths of *cox*1, *cox*2, and *cytb* were 658, 730, and 374 bp, respectively (table 3). Intrapopulation sequence divergence was always about 0%, whereas interpopulation sequence divergences were 1–2% (tables 5–7). The largest sequence divergences between Corsica and the Canary Islands were observed in *cox*1 and *cyt*b (tables 5 and 7). Furthermore, the *cox*1 sequence divergence between *S. t. tripectinata* from Andalusia (Iberian Peninsula) and Corsica was larger than that between the Canary Islands and Corsica. With respect to *S. t. medialis*, based on *cyt*b sequences, this taxon showed less sequence divergences with population of *S. t. tripectinata* from Corsica than with specimens from the Canary Islands, on the contrary, based on *cox*2 analysis, it was observed less sequence divergences between *S. t. medialis* and *S. t. tripectinata* isolated from the Canary Islands (tables 6 and 7). Based on mitochondrial markers, sequence divergences among *S. t. tripectinata* and other congeneric species (*Stenoponia polyspina*, *S. americana*, and *S. sidimi*) showed quite higher values (always exceeding 10%) than among *S. tripectinata* subspecies (tables 5–7). Finally, we did not find any Numts in any mitochondrial marker assessed.

Phylogenetic analysis

Phylogenetic trees inferred from *EF1*-α, *cox*1, *cox*2, and *cyt*b showed similar topologies (figs S1–S4, respectively). Thus, the genus *Stenoponia* and the family Stenoponiidae each appeared as well-supported clade. Specimens of *S. t. tripectinata* from the Canary Archipelago and Corsica formed two well-supported clades, yet within the Canary Archipelago clade, the specimens from the different islands constituted an unresolved polytomy. In the *EF1*-α phylogenetic tree, two specimens of *S. t. tripectinata* from Corsica formed a separate, but well-supported, clade with *S. t. medialis* within the Stenoponiidae (fig. S1).

The phylogenetic position of *S. t. medialis* was not clear in our study, as this taxon clustered within the Corsica clade (*EF1*-α and *cyt*b), but was placed within the Canary Islands clade on the basis of *cox*2. Nevertheless, *cox*2 phylogenetic tree was unreliable since *S. t. medialis* and Canary Islands clade was only supported by ML with a bootstrap support of 70 (which is really low), while it is not at all supported by BI. Finally, the other *Stenoponia* species (*S. sidimi*, *S. americana*, and *S. polyspina*) always clustered within the *Stenoponia* clade (except *S. sidimi* in *cyt*b analysis) but outside the *S. t. tripectinata* clade.

The concatenated dataset of *EF1*-α, *cyt*b, *cox*1, and *cox*2 comprised 2658 aligned sites and 39 taxa, including *S. t. tripectinata* from Corsica and the Canary Islands and outgroups (*P. meridionalis*). Phylogenetic analysis of this dataset yielded a tree in which *S. t. tripectinata* formed a well-supported clade, as did the genus Stenoponia (fig. 4). Within *S. t. tripectinata*, Corsica and the Canary Archipelago constituted two well-supported clades, though the islands within the Canary Archipelago formed an unresolved polytomy (fig. 4). Furthermore, a third clade corresponding to

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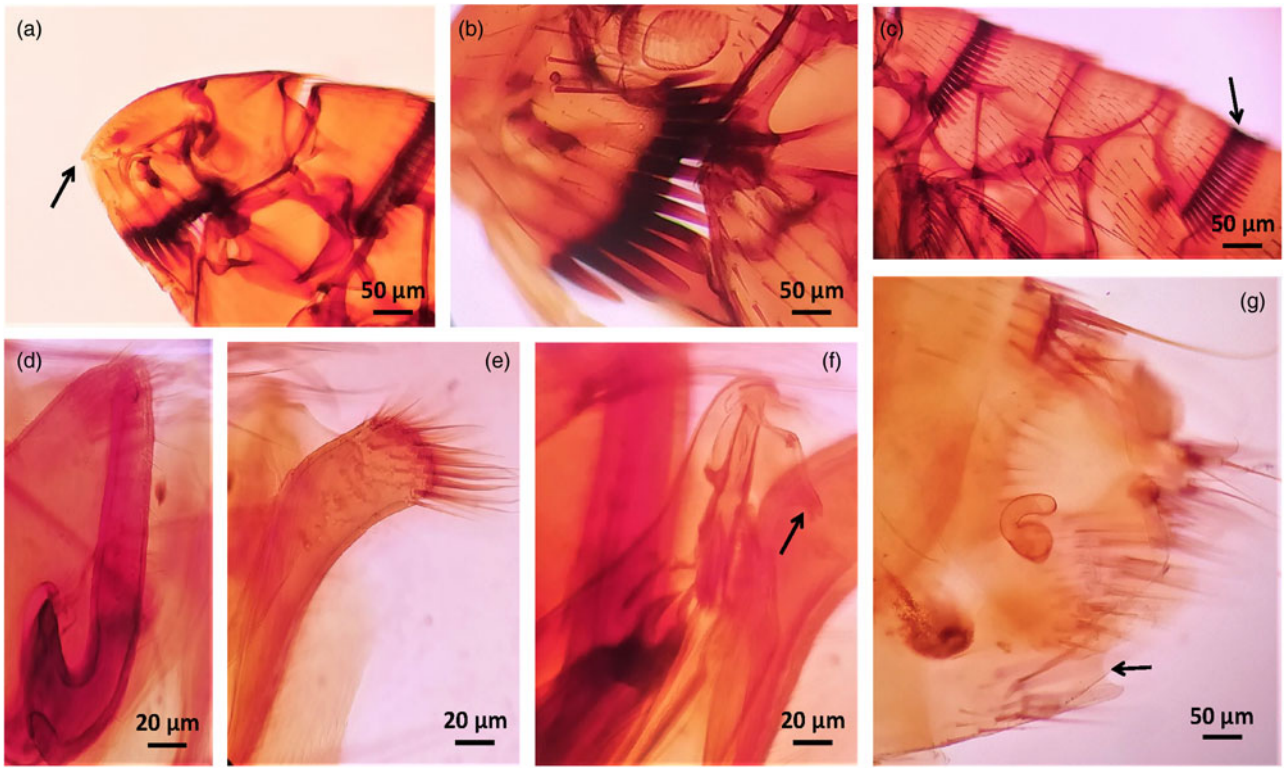

Figure 1. Morphological characteristics of *S. t. tripectinata* specimens assessed in this study. (a) Head and frons with oral angle arrowed; (b) genal comb; (c) pronotal and abdominal (arrowed) combs; (d) telomere or movable process of males; (e) apical portion of distal arm of sternum IX of males; (f) crochet of males phallosome with a long ventral projection arrowed; (g) spermatheca and ventral margin of sternum VII of females showing an apical lobe which subtended a little sinus (arrowed).

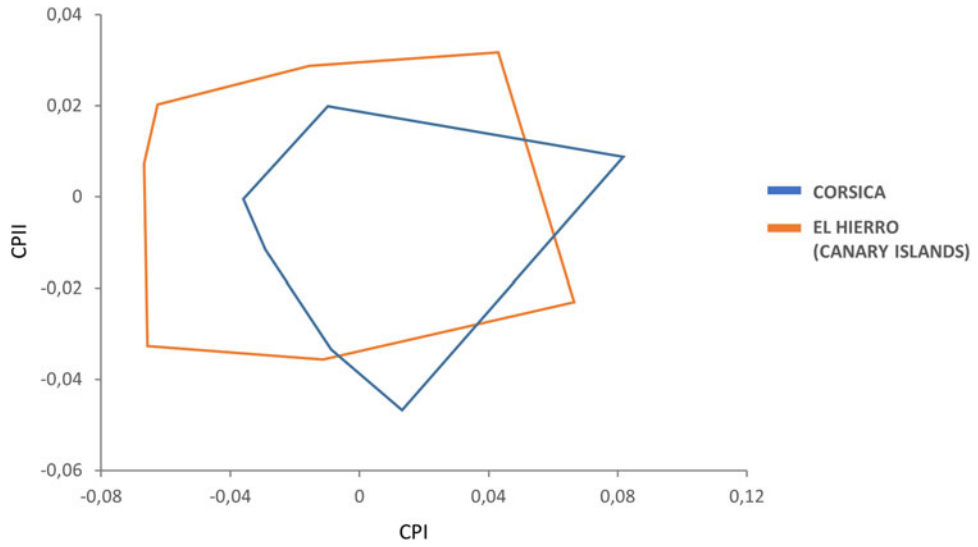

Figure 2. Factor map corresponding to adult of *S. t. tripectinata* males from Corsica and El Hierro (Canary Islands). Samples are projected onto the first (PC1, 58%) and second (PC2, 17%) principal components. Each group is represented by its perimeter.

two specimens from Corsica clustered separately as sister group of the Corsica and Canary Archipelago clades (fig. 4).

Discussion

Island systems have been key to analyze the process of population differentiation, as a result of being discrete, geographically isolated, and small in size relative to continents (Grant and Grant, 2008). We carried out a comparative morphological, biometrical, molecular, and phylogenetic analysis of two islands populations of *S. t. tripectinata*. The presence of this subspecies in rodents from Corsica and the Canary Islands agrees with other authors who revealed the distribution of this taxon throughout the Mediterranean and the North of Africa area, including the

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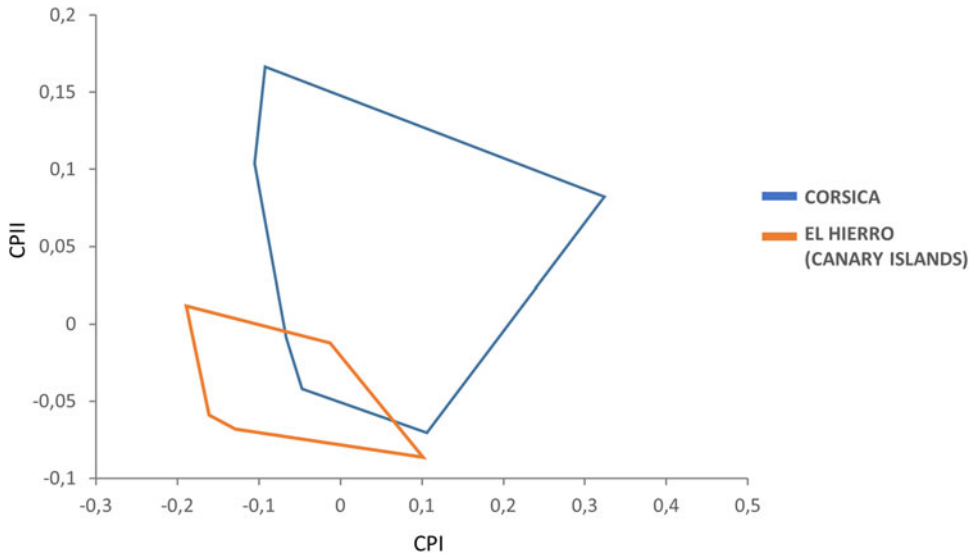

Figure 3. Factor map corresponding to adult *S. t. tripectinata* females from Corsica and El Hierro (Canary Islands). Samples are projected onto the first (PC1, 66%) and second (PC2, 20%) principal components. Each group is represented by its perimeter.

Table 4. Intrapopulation*, interpopulation, and interspecific sequence divergences observed among all the partial *EF1-α* gene sequences of nuclear DNA of different species belonging to *Stenoponia* sp. obtained in this work and retrieved from GenBank database

EF1-α	*S. t. tripectinata* (Canary Islands, Spain) LR744006-07 LR989029-32	*S. t. tripectinata* (Corsica) LR989033-37	*S. t. medialis* EU336263	*Stenoponia sidimi* EU336291	*Stenoponia americana* AF423843 KM890584
S. t. tripectinata (Canary Islands, Spain) LR744006-07 LR989029-32	0.0*				
S. t. tripectinata (Corsica, France) LR989033-37	0.6–1.4	0.2–1.5*			
S. t. medialis EU336263	1.9	0.8–2.0	–		
Stenoponia sidimi EU336291	7.7	7.4–8.0	7.9	–	
Stenoponia americana AF423843 KM890584	7.1	7.0–7.4	7.5	6.4–6.5	0.9*

Values are given in percentages.

Table 5. Intrapopulation*, interpopulation, and interspecific sequence divergences observed among all the partial *cox*1 mtDNA gene sequences of different species belonging to *Stenoponia* sp. obtained in this work and retrieved from GenBank database

Cox1	*S. t. tripectinata* (Canary Islands, Spain) (Zurita *et al.*, 2015)	*S. t. tripectinata* (Corsica) LR989038-41	*S. t. tripectinata* (Andalusia, Spain) KF479241-42	*Stenoponia polyspina* MG138242
S. t. tripectinata (Canary Islands, Spain) (Zurita *et al.*, 2015)	0.0–0.5*			
S. t. tripectinata (Corsica, France) LR989038-41	1.2–2.0	0.0–0.6*		
S. t. tripectinata (Andalusia, Spain) KF479241-42	0.3–0.9	1.2–2.0	0.3*	
Stenoponia polyspina MG138242	12.1–12.3	12.0–12.1	12.5	–

Values are given in percentages.

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Table 6. Intrapopulation*, interpopulation, and interspecific sequence divergences observed among all the partial *cox*2 mtDNA gene sequences of different species belonging to *Stenoponia* sp. obtained in this work and retreived from GenBank database

Cox2	*S. t. tripectinata* (Canary Islands, Spain) LR989042-47	*S. t. tripectinata* (Corsica, France) LR983966-67	*S. tripectinata* (La palma, Canary Islands, Spain) KY569104-05-07-10	*S. t. medialis* EU335983	*S. sidimi* EU335996	*S. americana* AF424014
S. t. tripectinata (Canary Islands, Spain) LR989042-47	0.0–0.2*					
S. t. tripectinata (Corsica, France) LR983966-67	0.8–1.0	0.0–0.2*				
S. tripectinata (La palma, Canary Islands, Spain) KY569104-05-07-10	0.0–0.2	0.8–1.0	0.0–0.2*			
S. t. medialis EU335983	1.0	1.8–2.0	1.0–1.1	–		
S. sidimi EU335996	11.9	12.1–12.2	11.9	12.2	–	
S. americana AF424014	14.8–15.0	15.3–15.6	14.7–15.0	15.3–15.5	15.2–15.3	–

Values are given in percentages.

Table 7. Intrapopulation*, interpopulation, and interspecific sequence divergences observed among all the partial *cyt*b mtDNA gene sequences of different species belonging to *Stenoponia* sp. obtained in this work and retreived from GenBank database

Cytb	*S. t. tripectinata* (Canary Islands, Spain) LN897472-73 LR983958-64	*S. t. tripectinata* (Corsica) LR983954	*S. t. medialis* KM890602	*Stenoponia sidimi* KM890611	*Stenoponia americana* KM890757
S. t. tripectinata (Canary Islands, Spain) LN897472-73 LR983958-64	0.0–0.9*				
S. t. tripectinata (Corsica, France) LR983954	1.8–2.1	0.0*			
S. t. medialis KM890602	2.1–2.4	1.2	–		
Stenoponia sidimi KM890611	13.7	13.1	12.8	–	
Stenoponia americana KM890757	15.6	15.0	15.3	12.8	–

Values are given in percentages.

Islands and archipelagos of this Palearctic region (Beaucournu and Launay, 1990; Sánchez and Gómez, 2012).

Jordan (1958) and Hopkins and Rothschild (1962) claimed that the subspecies of *S. tripectinata* constituted an exceptionally difficult problem from a classical taxonomy point of view. They observed two morphological extremes based on dorsal portion of the frons (head) in one of which this part is relatively weakly convex and the genal comb is confined to the genal margin, while in the other, the frons is much more convex and the last three or four spines of the genal comb are out of alignment with the rest and situated on the anterior margin of the antennal fossa. These extremes are so distinct that they have long been considered specifically distinct and some taxonomists would perhaps refer them to different subgenera, additionally, these authors observed that both extremes are connected by a series of morphological intermediates (Hopkins and Rothschild, 1962). Moreover, many populations of *S. tripectinata* analyzed by these authors differ one from another only by the 'mean values' of their diagnostic characteristics, so it becomes necessary either to consider such strikingly different geographical forms to be inseparable or to recognize as subspecies populations which differ from one another only on the average. Both Hopkins and Rothschild (1962) and Beaucournu and Launay (1990) adopted the latter alternative considering eight different subspecies for *S. tripectinata* based on morphological traits; however, they claimed that the number of specimens available was very small and further material might show that certain accepted subspecies could not be maintained.

We did not find clear morphological differences between specimens from the Canary Archipelago and Corsica population; thus, the frons of the head appeared weakly convex and the genal comb was confined to the genal margin for all specimens assessed (fig. 1a, b). Yet, morphometric data showed slight differences between *S. t. tripectinata* specimens from Corsica and El

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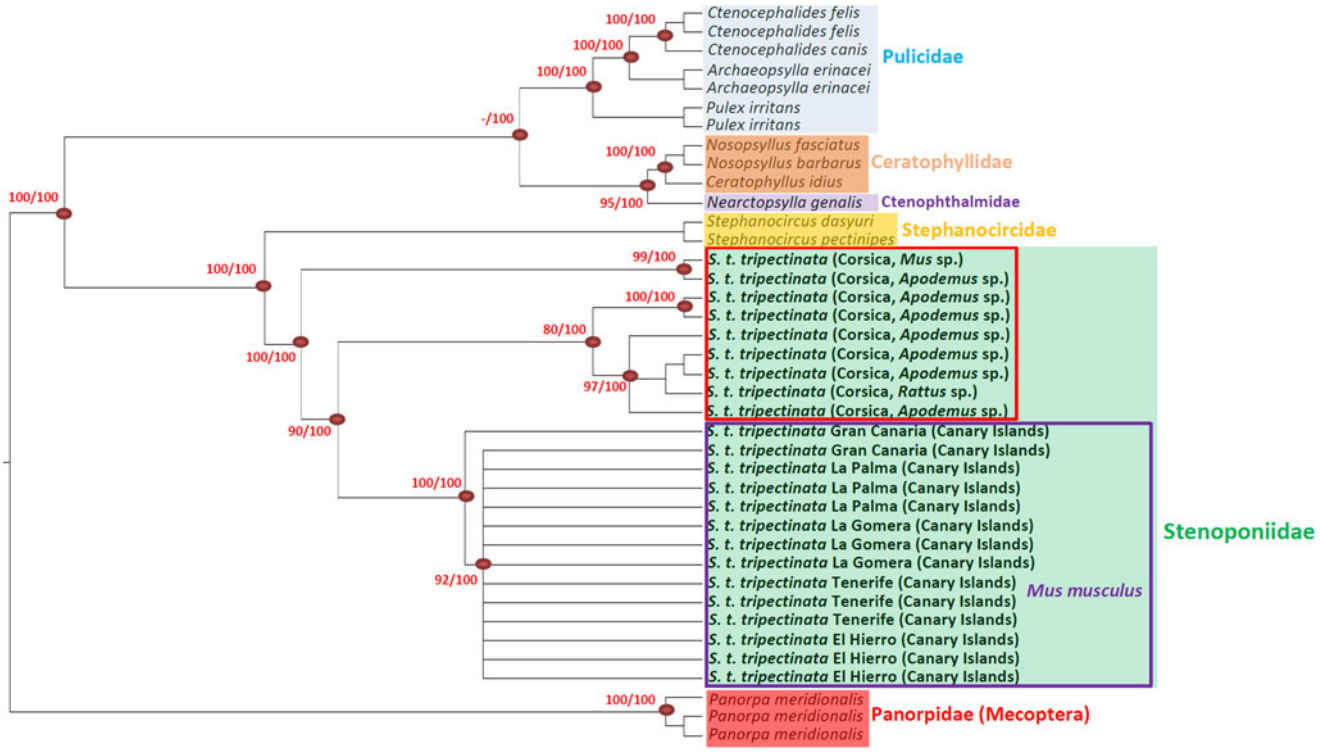

Figure 4. Phylogenetic tree of *S. t. tripectinata* specimens assessed in this study (see table 3). This analysis was based on concatenated sequences of *elongation factor 1 alpha* (*EF1-α*), partial *cytochrome c-oxidase* subunit 1 (*cox1*), *cytochrome c-oxidase* subunit 2 (*cox2*), and *cytochrome* b (*cytb*) gene of mitochondrial DNA inferred using the Bayesian Inference (BI) and Maximum Likelihood (ML) methods and Bayesian topology. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown on the branches (BPP/Bootstrap). The Bayesian Posterior Probabilities (BPP) are percentage converted.

Hierro (Canary Islands). This result was corroborated by PCA, with the Corsica adults showing a slightly bigger global size, especially in females (fig. 2). Although we cannot conclude that we have a diagnostic morphological pattern to separate specimens from both islands based on their global size, we would maybe consider the 'island rule' term coined by Van Valen (1973). It says that island populations show phenotypic changes (e.g. size shifts) in comparison to continental populations. Therefore, it would be interesting to carry out further morphometric studies including samples from the remaining islands of the Canary Archipelago or even continental populations in order to confirm the higher global size trend observed in Corsica specimens.

Our morphological results were not in concordance with the DNA sequence data and phylogenetic trees. In agreement with Zurita *et al.* (2015) we found nearly no nucleotide differences among specimens from different islands from the Canary Archipelago, however these specimens were clearly differentiated from the Corsica clade. The absence of morphological discriminative features together with the sequence and phylogenetic variability observed between Corsica and the Canary Islands populations suggests that *S. t. tripectinata* may involve two cryptic subspecies.

In our study, specimens from Corsica generally showed a higher nucleotide diversity than in the Canary Islands, and this was particularly true for *EF1-α* with its five haplotypes in Corsica. Furthermore, based on *cox*1 phylogeny this lineage appeared more distant to Iberian Peninsula (mainland) population than the Canary Island lineage. The origin theory of *Pulex irritans* provided by Buckland and Sadler (1989) or the coalescent theory supported by Slatkin and Hudson (1991) state that ancestral populations usually exhibit higher genetic diversity values compared with recent populations that have expanded into novel territories. Therefore, our results could mean an earlier colonization of *S. t. tripectinata* or even higher frequencies or on-going introduction events in Corsica than in the Canary Archipelago.

Based on the *EF1-α* and *cyt*b sequence data *S. t. medialis* appeared phylogenetically closer to the Corsica population. Although this subspecies has only been collected from Israel so far (Hopkins and Rothschild, 1962; Krasnov *et al.*, 2002, 2003), the geographical origin of *S. t. medialis* sequences used in this work was not available in GenBank. Thus, basing on *EF1-α* and *cyt*b results, it could mean that *S. t. tripectinata* would be a paraphyletic taxon by the inclusion of *S. t. medialis*. In order to confirm this hypothesis, we strongly encourage the necessity to carry out further taxonomic studies based on morphological and molecular data or even genome-wide SNP markers or whole genome sequencing approaches of *S. t. medialis* and the remaining *S. tripectinata* subspecies to clarify the taxonomical status of these taxa.

In conclusion, the present study provides comparative morphological, biometrical, and molecular data of two different isolated populations of the subspecies *S. t. tripectinata* (Corsica and Canary Archipelago). On the basis of our results, we observed slight differences in size between females from EL Hierro Island (Canary Archipelago) and Corsica and the existence of two well-defined geographical genetic lineages corresponding with the both population assessed. This fact could mean the existence of two cryptic subspecies within *S. t. tripectinata*. Since the lack of knowledge of mitochondrial and ribosomal genomics for this taxon is a major limitation for phylogenetic studies, we bring to

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light the necessity to provide new and updated morphological, molecular, and phylogenetic data in order to clarify the taxonomical status of *S. tripectinata* complex.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485322000098

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Conflict of interest. None.

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