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Research Article

The ant *Anochetus ghilianii* (Hymenoptera, Formicidae), not a Tertiary relict, but an Iberian introduction from North Africa: Evidence from mtDNA analyses

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Anochetus ghilianii is the only species of the genus *Anochetus* found in Europe. Its presence in the southernmost region of the Iberian Peninsula (Province of Cadiz) is puzzling. *Anochetus ghilianii*'s poor dispersal capabilities (colony foundation by foot and wingless queens) suggest that its presence in the Iberian Peninsula must be ancient, dating to from at least the last connection period between both Spain and Africa around 5 million years ago. In the Iberian Peninsula, this species is only found at low altitude coastal localities, whereas its mainland counterpart populations of North Morocco show a more widespread distribution, also found at higher altitude inland. In this study, we sequenced a fragment of a rapidly evolving mitochondrial gene (COI) from several individuals throughout the species' Moroccan and Iberian distribution range. The results from the Bayesian Inference (BI) and Median-Joining analyses showed a genetic pattern of decreasing haplotypic diversity from southern to northern localities. Furthermore, our results recovered the same haplotype from all the Iberian localities and from Cap Spartel and Tangier in North Morocco. We attribute this lack of genetic divergence to multiple reoccurring translocations through maritime traffic between ports on either side of the Strait of Gibraltar. Our study raises the concern that two other ants, *Technomyrmex vexatus* and *Stigmatomma emeryi*, also believed to be Tertiary relicts with a similar distribution pattern to *A. ghilianii*, may have also been introduced accidentally in the Iberian Peninsula.

Key words: *Anochetus*, dispersion, Iberian Peninsula, Morocco, Strait of Gibraltar

Introduction

The Strait of Gibraltar, separating the Iberian Peninsula from Morocco, is a well-documented geographic barrier constraining gene flow between land masses since the Late Miocene, around 5 million years ago (mya), although some species are known to have colonized before the separation following the Messinian Salinity Crisis (Albert, Zardoya, & Garcia-Paris, 2007; Kaliontzopoulou, Pinho, Harris, & Carretero, 2011). Despite only being 14 km wide, the Strait of Gibraltar has had a profound implication on biogeographic patterns throughout the area in terrestrial and non-terrestrial taxa after the opening of the Strait, contributing to allopatric speciation events between both land masses (reptiles: Paulo et al., 2008; mammals:

Brändli, Handley, Vogel, & Perrin, 2005; amphibians: Veith, Mayer, Samraoui, Donaire, & Bogaerts, 2004; birds: Guillaumet, Pons, Godelle, & Crochet, 2006; bats: Garcia-Mudarra, Ibañez, & Juste, 2009; arthropods: Gantenbein & Lurgiader, 2003). Nonetheless, several phylogenetic studies have shown that the geography of the area has had little impact on some species with poor dispersal capabilities and which colonized the Iberian Peninsula after this period (Arano, Llorente, Montori, Buckley, & Herrero, 1998; Harris, Carranza, Arnold, Pinho, & Ferrand, 2002; Michaux, Magnanou, Paradis, Nieberding, & Libois, 2003; Planas, Fernandez-Montraveta, & Rivera, 2013; Veith et al., 2004; Mayer, Samraoui, Donaire Barroso, & Bogaerts, 2004).

The genus *Anochetus* is found throughout the world's tropics and subtropics and is represented by more than 100 species. *Anochetus* form small nests (usually < 100

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workers) in soil, in termite nests, under logs, in rotten wood and under rocks (Brown, 1976, 1978; Tinaut, Ruano, Martinez, Bensusan, & Guillem, 2011a). They are known to forage in leaf litter, predated on small invertebrates using their trap-like jaws and sting to capture and subdue prey and are less frequently found foraging in the open. *Anochetus ghilianii* is the only species of *Anochetus* present in Europe, only found in a few localities near the coastal area of the province of Cadiz, in southern Spain, and in Gibraltar (Fig. 1). The occurrence of wingless queens in *A. ghilianii* and field observations (Taheri, pers. observ.) suggest that this species is likely to disperse by foot (i.e. fission), expected to lead to an increase in population structure and promoting an isolation by distance pattern at a local scale (Boomsma, Kronauer, & Pedersen, 2009; Cheron *et al.*, 2011; Doums, Cabrera, & Peeters, 2002; Keller, 1991; Liautard & Keller, 2001; Seppa, Fernandez-Escudero, Gyllenstrand, & Pamilo, 2006), a strong indicator that this species is incapable of crossing the 14 km Strait by flight and that under such dispersal mode, its presence in the Iberian Peninsula must date to at least the Messinian Salinity Crisis. Alternatively, passive dispersal since the Messinian Salinity Crisis could have occurred through marine dispersal (rafting on floating vegetation and logs) and through accidental human introductions. In the Iberian Peninsula *A. ghilianii* is

considered to be native to both shores of the Strait of Gibraltar and a relic from the Tertiary (Tinaut, 1989; Tinaut, Ruano, Martinez, Bensusan, & Guillem, 2011a). Furthermore its distribution is puzzling as it is only found near the coast close to urban areas and large ports (Algeciras, Tarifa), which raises the possibility of maritime introduction from North African ports (Guillem & Bensusan, 2009). The Strait of Gibraltar has supported human activity in the area throughout the last 3000 years, having profound consequences in habitat alterations within the Strait's coastal area. Thus the alternative scenario to that of natural dispersal from North Africa, would imply passive or active human introductions or translocations in recent times, as have been reported for other species (Carranza & Arnold, 2003; Carranza, Harris, Arnold, Batitsta, & Gonzalez de la Vega, 2006; Cosson *et al.*, 2005; Dobson, 1998; Pinto-Juma, Quartau, & Bruford, 2008; Pleguezuelos, Fahd, & Carranza, 2008; Recuero, Iraola, Rubio, Machordom, & Garcia-Paris, 2007).

In this study we hypothesize that *Anochetus ghilianii* has been introduced in the Iberian Peninsula from northern Morocco. We base our arguments on the following reasons: (i) *A. ghilianii* has a bizarre distribution, being the only member of a tropical genus present in the south of the Iberian Peninsula, (ii) a close association of Iberian populations to ports with major maritime traffic between

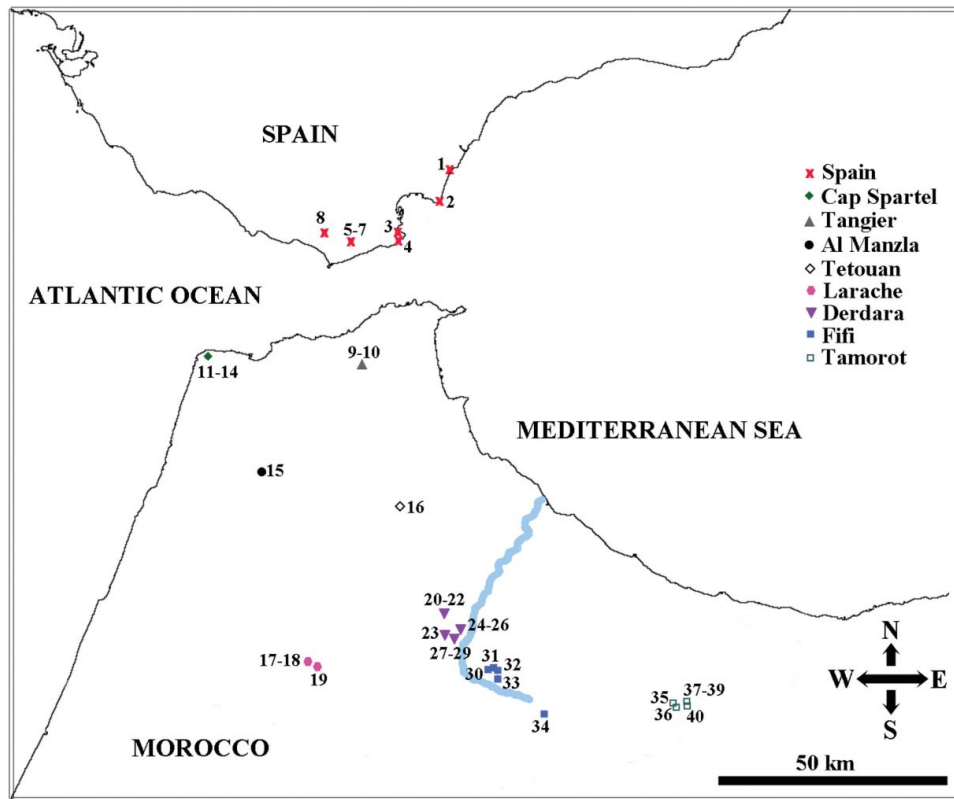


Fig. 1. Map of southern Spain and northern Morocco. *Anochetus* localities are numbered 1 to 40. Blue line follows the Loau River course.

North Morocco and Cadiz and Gibraltar, (iii) wingless queens and restricted dispersal capability by foot suggests an unlikely colonization of the Iberian Peninsula, either through the last connection period or after the opening of the Strait. To test this hypothesis we sequenced a fragment of the mtDNA gene (COI) from several populations throughout the species' Moroccan and Iberian distribution range and employed Bayesian Inference and Median-Joining (MJ) analyses to assess the species' phylogenetic and population structure.

Materials and methods

DNA was extracted from the legs of *Anochetus ghilianii* ($n = 40$) and the outgroup species *A. sp* (Zambia, $n = 1$) and *A. grandidieri* (Madagascar, $n = 1$) (Table 1). Individual DNA was extracted using the Chelex extraction process (Bio-Rad®, Hercules, 12 CA, USA; Walsh, Metzger, & Higuchi, 1991) following slight protocol modifications. Legs were cut thoroughly and incubated for 2 h at 57 °C in 160 µL of 5% Chelex with 40 µL Proteinase K to increase tissue digestion. Following the incubation period it was heated at 100 °C for 15 minutes. After a 4 min centrifugation at 12,500 rpm, all supernatant was transferred into a 1.5 mL tube. We aimed to amplify a fraction of the mitochondrial COI gene. For each PCR, the 20 µL PCR volume contained *c.* 50 ng DNA, 200 µM of each dNTP, 0.15 µM of each primer, 2 µL 10× Buffer, 0.8 µL MgCl₂ and 0.1 unit of taq polymerase (QIAGEN). The thermal cycle profile was as follows: an initial denaturation step of 2 min at 94 °C; 35 cycles of denaturation at 30 s at 94 °C, annealing for 30 s at 52 °C and extension for 45 s at 72 °C; and a final extension for 5 min at 72 °C. Following the PCR, excess primers and dNTPs were removed using enzymatic reaction of *E. coli* Exonuclease I, Antarctic phosphatase and Antarctic phosphatase buffer (all New England Biolabs). Sequencing was carried out in both directions using the BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Labelled fragments were resolved on an automated A3130xl genetic analyser (Applied Biosystems). The primers for the MtDNA COI gene fragment were: LCO: 5'-GGTCAACAAATCATAAAGATATTGG-3', COI HCO 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994) amplified approximately a 700 base pair fraction. Templates were sequenced on both strands, and the complementary reads were used to resolve rare, ambiguous base-calls in Sequencer v.4.9. After removing PCR primers and incomplete terminal sequences, 635 base pairs of the COI gene were available for analyses. Previous nuclear markers used in *Anochetus* (Fisher & Smith, 2008) and other genera (Moreau, Bell, Vila, Archibald, & Pierce, 2006) were tested. Nuclear markers that amplified successfully were sequenced for most individuals (18S rDNA primers; 18H3 and 183WR

(Hillis & Dixon, 1991) and nDNA Wingless primers; Wg/Wg550F (Wild & Maddison, 2008) and Wg/1032R (Abouheif & Wray, 2002)), but these data were not included in the final analyses due to total lack of polymorphisms between localities and populations. Sequences were aligned in Seaview v.4.2.11 (Gouy, Guindon, & Gascuel, 2010) under ClustalW2 (Larkin et al., 2007) default settings. Nucleotide differences and *p*-uncorrected distances (%) analyses were calculated using MEGA v5 (Tamura et al., 2011) and standard errors (SE) were calculated in Excel. The most appropriate substitution model for the Bayesian Inference (BI) analysis was determined by the Bayesian Information Criterion (BIC) in jModeltest v.0.1.1 (Posada, 2008). The tree was constructed using the Bayesian Inference (BI) optimality criteria through the CIPRES gateway (Miller, Pfeiffer, & Schwartz, 2010) under the best fitting model (TPM1uf+G). MrBayes (Huelsenbeck & Ronquist, 2001) was used with default priors and Markov chain settings, and with random starting trees. Each run consisted of four chains of 40,000,000 generations, sampled each 1,000 generations for a total of 40,000 trees. A plateau was reached after few generations with 25% of the trees resulting from the analyses discarded as 'burn in'. Mitochondrial COI haplotype frequencies were estimated in DnaSP version 5.10 (Librado & Rozas, 2009) using a Median-Joining network constructed with NETWORK 4.5 (Bandelt, Forster, & Rohl, 1999). The pattern of isolation by distance was tested by plotting (FST/(1-FST)) coefficients between pairs of colonies against the logarithm (ln) of geographic distances (Rousset, 1997; Slatkin, 1993). The significance of Spearman rank correlation coefficient (two-tailed) between genetic differentiation and geographic distance was assessed using a Mantel test in IBDWS (Isolation by distance web service) v.3.16 (Jensen, Bohonak, & Kelley, 2005). Haplotype frequencies and reduction were estimated using a Median joining (MJ) (Bandelt et al., 1999) with default settings.

Results

The best-fitting model for the BI tree was the TPM1uf+G ($-\ln L = -1492.7297$, $BIC = 3217.7898$). The analyses of all individuals ($n = 40$) recovered 14 haplotypes (excluding indels) and one more haplotype ($N = 15$) including indels (Figs 2, 3). All individuals from Spain, one from Tangier and two from Cap Spartel recovered the same haplotype. Two other individuals from Tangier and Al Manzla recovered this sample haplotype but had an indel within the sequence. Two other haplotypes from Cap Spartel and Tetouan were closely linked to the previously described haplotypes, as shown by their monophyly in the BI analyses. The haplotypes from Dardara were basal to the rest of the populations, suggesting the ancestral state of the remaining populations. Two haplotypes were recovered from Larache and were closely supported

Table 1. Localities, nest codes, map codes, GPS coordinates and altitude.

Species	Locality	Collection code	Map code	Coord.x	Coord.y	Altitude	GenBank accession no.
<i>A. ghiliani</i>	Spain (Cadiz)	T4908	1	36.23096	-5.318624	23	KP826660
<i>A. ghiliani</i>	Spain (Cadiz)	A2173	2	36.135063	-5.350393	129	KP826661
<i>A. ghiliani</i>	Spain (Cadiz)	A2556	3	36.085032	-5.442316	46	KP826662
<i>A. ghiliani</i>	Spain (Cadiz)	A0719	4	36.067545	-5.44189	27	KP826663
<i>A. ghiliani</i>	Spain (Cadiz)	A3286	5	36.066378	-5.555151	352	KP826664
<i>A. ghiliani</i>	Spain (Cadiz)	A3290	6	36.066378	-5.555151	352	KP826665
<i>A. ghiliani</i>	Spain (Cadiz)	A3291	7	36.066378	-5.555151	352	KP826666
<i>A. ghiliani</i>	Spain (Cadiz)	T1977	8	36.086966	-5.619787	0	KP826667
<i>A. ghiliani</i>	Morocco (Tangier)	AT790	9	35.762827	-5.531745	183	KP826668
<i>A. ghiliani</i>	Morocco (Tangier)	AT791	10	35.762827	-5.531745	183	KP826669
<i>A. ghiliani</i>	Morocco (Cap Spartel)	A2555	11	35.784535	-5.907078	245	KP826670
<i>A. ghiliani</i>	Morocco (Cap Spartel)	A3299	12	35.785017	-5.907133	280	KP826671
<i>A. ghiliani</i>	Morocco (Cap Spartel)	A2554	13	35.784277	-5.90697	237	KP826672
<i>A. ghiliani</i>	Morocco (Cap Spartel)	A2503	14	35.7842	-5.906933	237	KP826673
<i>A. ghiliani</i>	Morocco (Al Manzla)	AT797	15	35.504417	-5.7697	54	KP826674
<i>A. ghiliani</i>	Morocco (Tétouan)	A1904	16	35.441999	-5.392574	239	KP826675
<i>A. ghiliani</i>	Morocco (Larache)	A2846	17	35.043717	-5.63955	158	KP826676
<i>A. ghiliani</i>	Morocco (Larache)	A2672	18	35.043717	-5.639549	158	KP826677
<i>A. ghiliani</i>	Morocco (Larache)	A2707	19	35.026373	-5.64263	146	KP826678
<i>A. ghiliani</i>	Morocco (Derdara)	A2289	20	35.179388	-5.315114	325	KP826679
<i>A. ghiliani</i>	Morocco (Derdara)	A2288	21	35.179388	-5.315114	325	KP826680
<i>A. ghiliani</i>	Morocco (Derdara)	A2291	22	35.176639	-5.319111	325	KP826681
<i>A. ghiliani</i>	Morocco (Derdara)	A2838	23	35.110783	-5.323883	722	KP826682
<i>A. ghiliani</i>	Morocco (Derdara)	A2492	24	35.115717	-5.289433	388	KP826683
<i>A. ghiliani</i>	Morocco (Derdara)	A2502	25	35.115777	-5.289178	375	KP826684
<i>A. ghiliani</i>	Morocco (Derdara)	A2491	26	35.115717	-5.289433	388	KP826685
<i>A. ghiliani</i>	Morocco (Derdara)	A2174	27	35.099383	-5.302383	485	KP826686
<i>A. ghiliani</i>	Morocco (Derdara)	A2178	28	35.099383	-5.302383	485	KP826687
<i>A. ghiliani</i>	Morocco (Derdara)	A2200	29	35.099383	-5.302383	485	KP826688
<i>A. ghiliani</i>	Morocco (Fifi)	A2007	30	35.026417	-5.225389	1192	KP826689
<i>A. ghiliani</i>	Morocco (Fifi)	A2005	31	35.026417	-5.208889	1194	KP826690
<i>A. ghiliani</i>	Morocco (Fifi)	A2489	32	35.0233	-5.2042	1190	KP826691
<i>A. ghiliani</i>	Morocco (Fifi)	A2290	33	35.007531	-5.201333	1252	KP826692
<i>A. ghiliani</i>	Morocco (Fifi)	S 09-5	34	34.91675	-5.08751	624	KP826693
<i>A. ghiliani</i>	Morocco (Tamorot)	A2558	35	34.935389	-4.787806	973	KP826694
<i>A. ghiliani</i>	Morocco (Tamorot)	A2495	36	34.933717	-4.767367	1081	KP826695
<i>A. ghiliani</i>	Morocco (Tamorot)	A2559	37	34.9395	-4.749222	1103	KP826696
<i>A. ghiliani</i>	Morocco (Tamorot)	A2557	38	34.9395	-4.749222	1103	KP826697
<i>A. ghiliani</i>	Morocco (Tamorot)	A2564	39	34.936	-4.744533	1245	KP826698
<i>A. ghiliani</i>	Morocco (Tamorot)	AT412	40	34.933867	-4.740717	1366	KP826699
<i>A. sp.</i>	Zambia	BLF13580	na	30.29567	-12.55483	1300	KP826700
<i>A. grandidieri</i>	Madagascar	BLF19537	na	49.20074	-17.91801	500	KP826701

by high bootstrap (BPP 1.00) and with high genetic divergence to other closely related populations, thus showing a high level of genetic isolation and reduced gene flow with other localities. Individuals from Fifi and Tamorot are monophyletic (BPP 0.95) and exhibited the highest genetic divergence to all other populations, being terminal to the BI tree and MJ network (Figs 2, 3). This finding corroborates ongoing work that suggests that the populations

from Tamorot and Fifi are probably a separate species (unpublished).

Tamorot and Derdara sequences showed the highest within-population divergence, $1.16 \pm 0.16\%$ and $1.1 \pm 0.016\%$, respectively. The highest genetic divergence between localities was between Tamorot/Fifi and all other localities (Spain, Cap Spartel, Tetouan, Al Manzla, Derdara, Larache), with a maximum genetic divergence

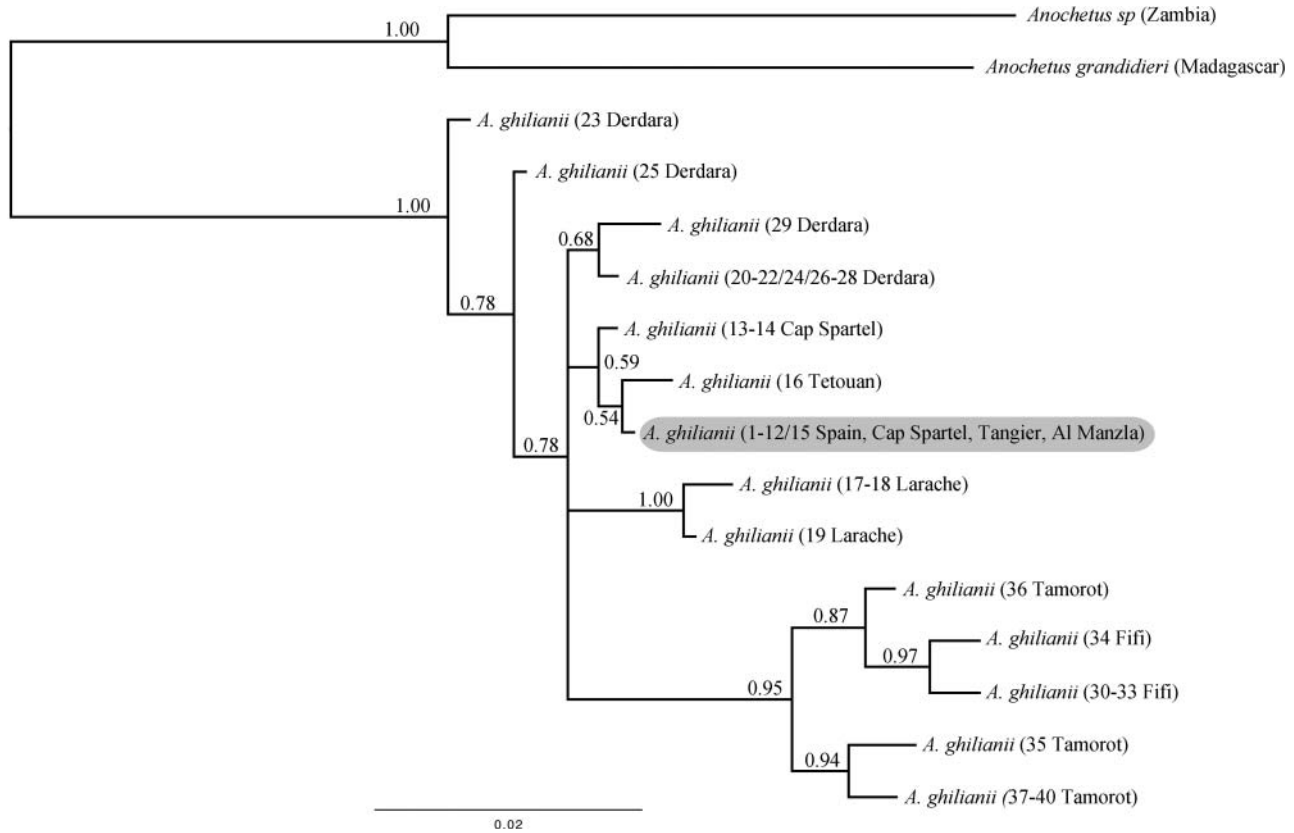


Fig. 2. Bayesian inference phylogram of all *Anochetus* spp. studied (COI, 635 bp). Values on nodes are the posterior probabilities recovered from the BI analyses. Samples from Spain, Cap Spartel, Tangier and Al Manzla that recovered the same haplotype are marked in grey.

of $3.60 \pm 0.07\%$ between Tamorot and Derdara followed by $2.97 \pm 0.10\%$ between Tamorot and Larache. Reduced *p*-uncorrected distances between Tamorot and Fifi ($2.09 \pm 0.29\%$) are in agreement with their close phylogenetic relationship (Table 2, Fig. 2). There was a significant positive correlation between geographic distance and mtDNA genetic differentiation at the regional scale along all sampled populations (14 haplotypes, Mantel test: $r = 0.26$, $P = 0.04$) as expected in DCF mode by fission (queens accompanied by a few workers found a new nest by foot). Similarly, there was a strong correlation between localities and altitude ($r^2 = 0.72$, data not shown), decreasing from north to south (Tamorot: 1145 ± 23 m, Fifi: 1090 ± 52 m, Derdara: 430 ± 12 m, rest of localities: 179 ± 6 m).

Discussion

Anochetus ghilianii (Spinola, 1851) is attributed to be native to both shores of the Strait of Gibraltar, considered a relic from the Tertiary period and the only species within the *Anochetus* genus present in the area of Gibraltar (Tinaut et al., 2011a). Our findings are in contradiction to both of these statements on the reasoning that this

proposition implies an allopatric speciation prior to the formation of the Strait of Gibraltar with subsequent isolation at both sides of the Strait after the formation of the geographic barrier (e.g. Paulo et al., 2008). Another not concordant scenario is the northward expansion colonization route from Morocco to southern Spain through the land bridge formation after the Messinian Salinity Crisis around 5 mya, when the Mediterranean Sea and the Atlantic Ocean along the Betic and Rifan Corridor were progressively restricted until the closure of the Strait (Paulo et al., 2008). According to current genetic divergence estimates based on arthropods, 3.54% per million years (Papadopoulou, Anastasiou, & Vogler, 2010), lack of gene flow resulting in genetic isolation after the opening of the Messinian Salinity Crisis would have resulted in high divergence (over 10% divergence) between both *Anochetus* populations at each side of the Strait of Gibraltar. Hence endemism at both land-masses would imply a genetic divergence for *c.* 5 mya suggesting long genetic isolation between populations, ultimately leading to natural selection acting on reproductive traits between populations at both localities through continuous speciation (Jowers et al., 2014). Hence, the recovery of the same haplotype and thus lack of any genetic divergence, as seen

Table 2. Table of *p*-uncorrected distances (\pm SE) between haplotypes (na: non-applicable due to lack of more than one haplotype recovered at the locality). The Al Manzla and Tangier individuals are included within the Spain population.

	Derdara	Spain	Cap Spartel	Tetouan	Larache	Fifi	Tamorot
Derdara	1.1 \pm 0.016	0.7 \pm 0.14	0.66 \pm 0.09	1.0 \pm 0.14	1.57 \pm 0.07	3.08 \pm 0.08	3.60 \pm 0.07
Spain	0.71 \pm 0.14	na	0.16	0.33	1.26 \pm 0.16	2.96 \pm 0.06	2.88 \pm 0.05
Cap Spartel	0.66 \pm 0.09	0.16	na	0.49	1.34 \pm 0.10	2.82 \pm 0.06	2.80 \pm 0.04
Tetouan	1.0 \pm 0.14	0.33	0.49	na	1.47 \pm 0.16	1.47 \pm 0.16	2.72 \pm 0.05
Larache	1.57 \pm 0.07	1.26 \pm 0.16	1.34 \pm 0.10	1.47 \pm 0.16	0.32	3.06 \pm 0.05	2.97 \pm 0.10
Fifi	3.08 \pm 0.08	2.96 \pm 0.06	2.82 \pm 0.06	3.06 \pm 0.05	3.06 \pm 0.05	0.66	2.09 \pm 0.29
Tamorot	3.60 \pm 0.07	2.88 \pm 0.05	2.80 \pm 0.04	2.72 \pm 0.05	2.97 \pm 0.10	2.09 \pm 0.29	1.16 \pm 0.16

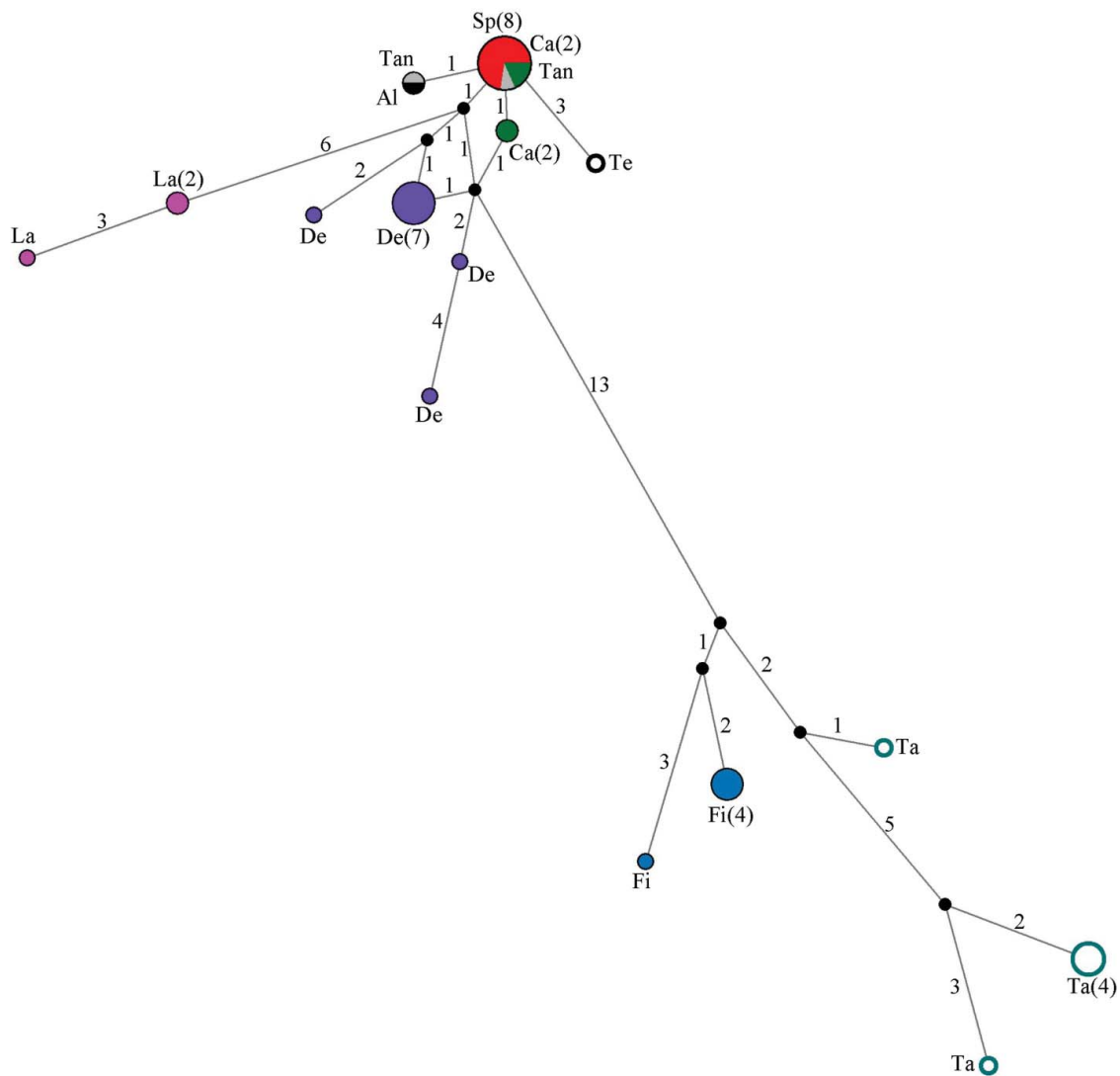


Fig. 3. MJ network depicting all sampled individuals ($n = 40$) and localities are coded by colour. Haplotype frequency higher than 1 is indicated by abbreviated locality names in parentheses. The length of the lines between haplotypes indicates approximate mutational distance. Numbers by lines indicate number of mutations (or indels) between haplotypes. Black dots are median vectors recovered from the analyses. Abbreviation are: Sp (Spain), Ca (Cap Spartel), Tan (Tangier), Al (Al Manzla), Te (Tetouan), La (Larache), De (Derdara), Fi (Fifi), Ta (Tamorot).

from the mtDNA data, is pivotal proof that this evolutionary process never occurred.

The current distribution of *A. ghilianii* is a puzzling one. Distribution range expansions and habitat conditions are quite different on both sides of the Strait of Gibraltar. In the Iberian Peninsula it is restricted to the stretches of land in close proximity to the coast of Cadiz and Gibraltar, and within these localities the populations seem restricted by river systems (Barbate and Guadiaro Rivers at the west and east, respectively) which may act as geographic barriers for further population expansion. Their habitat conditions here are mainly associated with low altitude (up to 390 m) (Tinaut et al., 2011a; Tinaut, Ruano, & Martinez, 2011c) humid conditions in open or shrub habitats and leaf litter, avoiding true woodland. In contrast, in Morocco *A. ghilianii* shows a widespread distribution along the Atlantic coast from Tangier to Ben Slimane and along the Mediterranean coast up to Laou River (collected by AT), tolerating high altitudes (up to 1000 m and oak forest habitat) (Cagniant, 2006).

Clues on this species' dispersal mode derive from morphological examination of the only four reported queens to date (Tinaut, Bensusan, & Guillem, 2011b) suggesting, as in other queens within the genus (Brown, 1978; Tinaut & Ruano, 1992), complete absence of wings indicating their inability to fly and therefore their incapability to colonize or re-colonize from either side of the Strait (Tinaut, Bensusan, & Guillem, 2011b). The four *A. ghilianii* queens described from Spain were accompanied by 20–30 workers, thus indicative of very small nests (Tinaut et al., 2011b). Because *Anochetus* nests are found a few centimetres from the surface, or as reported with *A. ghilianii*, present under rocks and leaf litter, it is possible to assume that whole nests have been transported across the sea in building material such as sand, soil, boulders and woods or most likely in planted flowerpots. Furthermore, a dependent colony-founding queen (through fission) needs part of the colony to survive and thus single or multiple queen colonization events are extremely unlikely.

Directional winds have been suggested to contribute gene flow of the alate males and possibly females of *Cataglyphis emmae* at a local scale in central Moroccan wadis, through which winds are directed (Jowers et al., 2013). However, under the very unlikely scenario of wind-aided dispersal, the winds in the Strait of Gibraltar do not blow in a north to south direction but in a west to east direction, Poniente and Levante winds respectively (Dorman, Beardsley, & Limeburner, 2006). This would not explain the presence of *A. ghilianii* populations in the ports of Tarifa, Algeciras and near Gibraltar. Furthermore, the distance between the narrowest point of Morocco and the Iberian Peninsula (14.3 km) and flightless nature of *A. ghilianii* makes this assumption even less probable. Although *Anochetus* alate males are known to fly in swarms, distances between both sides of the Strait of Gibraltar make

such colonization unlikely for males, and the observed haplotype frequency at both sides of the Strait can only be aided through female and not male dispersal. A few studies have shown low genetic divergence between northern Morocco and southern Iberian lineages, dating to after the closing of the Strait, suggesting rafting as a means of dispersal in the area (Carranza et al., 2006; Harris et al., 2002; Veith et al., 2004). However, marine dispersal would imply multiple colonization events since the closing of the Strait for *A. ghilianii*, resulting in the accumulation of genetic divergence within Iberian and between Iberian and Moroccan populations over time. Furthermore *A. ghilianii*'s current distribution in the Iberian Peninsula would not fit the marine dispersal scenario, dependent on the direction of winds and marine currents. In addition *A. ghilianii*'s niche requirements to non-coastal Moroccan oak tree forests and lack of host (e.g. bark beetle: Horn, Roux-Morabito, Lieutier, & Kerdelhue, 2006) makes the rafting scenario unlikely.

The basal position of the Derdara haplotypes (from Laou valley) recovered from the Bayesian Inference tree (Fig. 2) suggested that the ancestral form may have derived from this locality. Sea level changes through the Pliocene and Pleistocene indicate that the topography of the valley was severely affected throughout the eustatic sea level changes of the Tertiary (El Kadiri, Serrano, Guerra-Merchan, Hlila & Sanz de Galdeano, 2011; Loget & Van den Driessche, 2006) and thus, the sampled sites at Derdara, all close to the Laou River, must be a relatively recent colonization event since the last glacial minima. Furthermore, the recovery of the highest number of haplotypes at Derdara ($n = 4$) implies some sort of past genetic isolation at the local scale, which may be the consequence of habitat change resulting in contractions/expansions and slow movements to newly suitable habitats during interglacial periods (Hewitt, 2001). The Valley and Laou River separates the very proximal Fifi and Derdara populations and coincides with the division of clades observed in the MJ network (southern and northern clades), where Fifi+Tamorot are clearly highly divergent of the remaining haplotypes (Figs 2, 3). Such genetic divergence and ongoing morphometric analyses suggest that the populations from Tamorot and Fifi are likely taxonomically distinct to *A. ghilianii*. Undergoing taxonomic examination should help clarify this matter. The highest number per population haplotype diversity of the southern-most area (Tamorot $H = 3$, Fifi $H = 2$, Derdara $H = 4$, Larache $H = 2$) contrasts with the low genetic diversity of the northern sampled populations, more so considering the large distances between Spanish localities, in agreement with northern expansions and possible founder effects. Comparison of *A. ghilianii*'s inter-population COI uncorrected p -distances (highest divergence $\sim 3\%$) to other wingless *Anochetus* populations (the Madagascan *A. goodmani* and *A. boltoni*, 6.3% and 8% respectively, K2P, Fisher &

Smith, 2008) revealed lower levels of genetic divergence in Moroccan than Madagascan populations.

Interestingly, there are two other attributed Tertiary relict ant species (primarily palaeotropical genera) with a very similar distribution pattern to *A. ghilianii* (Tinaut, 1989). The first ant *Technomyrmex vexatus*, is only found in Ceuta, Tangier and Gibraltar. In Gibraltar it is only found in the Gibraltar Botanic Gardens or surrounding areas where a large number of exotic plants are present. Furthermore, Tinaut's (1989) survey in the vicinity of Gibraltar and other expeditions in the Spanish shore of the Strait have never found it there (Guillem & Bensusan, 2008). The second ant is *Stigmatomma emeryi* (Tinaut, Ruano, & Martinez, 2011c) found near Algeciras and in Ceuta. This species ecology and natural history is regarded to be very similar to *A. ghilianii*'s (Tinaut *et al.*, 2011b; Tinaut *et al.*, 2011c). Another intriguing case is the only record of one worker ant of *Stigmatomma impressifrons* in Jerez (Cadiz) (Garcia, Espadaler, & Gomez, 2009). Thus, molecular work on these species is needed to clarify their colonization time of the Iberian Peninsula, and when possible, assess their population genetic structure.

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Disclosure statement

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