Mitochondrial phylogenomics and genetic population structure of anchovies (*Engraulis encrasicolus*) along the Moroccan coast using sequence analysis of the mitochondrial DNA cytochrome b

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Abstract A fragment of 680 bp of mitochondrial cytochrome b locus of European anchovies, *Engraulis encrasicolus*, was sequenced for 138 individuals collected from three Moroccan Atlantic areas and from Moroccan Alboran Sea. These samples were surveyed for diversity and differentiation with a range of summary statistics. The results showed that the most dominant clade in Moroccan anchovy is Clade A with a percentage ranging from 89% in Alboran Sea to 91% - 95 % in the Moroccan Atlantic coast. Overall, there was a significant genetic differentiation among the 4 Moroccan anchovy zones ($\Phi st = 0.01283$; $p = 0.03910$). Pairwise Φst among populations and multidimensional scaling revealed a high homogeneity among Atlantic populations but some heterogeneity between Alboran population and Atlantic populations, mainly between the populations from Central Atlantic of Morocco with a significant difference.

Keywords: European anchovy (*Engraulis encrasicolus*), Morocco, mitochondrial cytochrome b, phylogeography, population structure

INTRODUCTION

The European anchovy, *Engraulis encrasicolus* (Linnaeus, 1758) is a small pelagic fish found in a wide range of temperatures (2–30 °C) and salinities (5-41 psu) in the eastern Atlantic, the Mediterranean Sea and the Black Sea (Whitehead, Nelson & Wongratana, 1988). This species

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plays a major socio-economic role in all regions; it is one of the principal target species for commercial fisheries. Indeed, European anchovies with other Engraulidae fisheries representing 14 percent of the world catch of fish (Ababouch & El Marrakchi, 2009). In regard to their economic importance, anchovies like other pelagic fish occupy crucial positions in the oceans ecosystems (Ganias, 2014) particularly in upwelling regions where it occupies fundamental intermediate trophic level (Bakun, 2006). As is known, small pelagic are characterized as 'wasp-waist', being considered as crucial components of pelagic ecosystems (Cury et al., 2000). In addition, anchovy as a pelagic species, it can achieve considerable biomass and undergoes crash–flush cycles (Uriarte, Prouzet & Villamor, 1996; Lavoué et al., 2007). For these reasons, the management of this precious resource is important. In fact, anchovies have been the subject of many genetic studies aimed a better understanding of the stock genetic structure as identifying stocks, discriminating among them, and determining the stock composition of mixed stocks are integral elements of fishery management (Waldman, 1999). Likewise, genetics and fishery management can interact in several ways. When the genetic population structure of a species is known, the distribution of subpopulations in mixed fisheries can be estimated (Utter, 1991).

Previous published data using allozymes, mitochondrial DNA (mtDNA) RFLPs, control region sequence, microsatellites and SNP marker (Bembo et al., 1996a; Bembo et al., 1996b; Bembo et al., 1996c; Magoulas, Tsimenides & Zouros, 1996; Magoulas et al., 2006; Kristoffersen & Magoulas, 2008; Tudela, Garcia-Marin & Pla, 1999; Sanz et al., 2008; Zarraonaindia et al., 2009; Borrell et al., 2012; Vinas et al., 2013; Silva, Horne & Castilho, 2014) suggest the presence of various genetically differentiated groups with spatial and ecological components. In addition to this, Magoulas et al. (1996; 2006) by analyzing mitochondrial DNA restriction fragment length polymorphism, reported a significant phylogeographic structure in both the Atlantic and Mediterranean populations of anchovy with two haplotype clades (A and B) separated by 3.2% sequence divergence. Grant et al. (2005); Vinas et al. (2013); Oueslati et al. (2014) and Silva, Horne & Castilho (2014) confirmed the existence of these clades whose frequency varies within samples across the Mediterranean and Northeast Atlantic.

Along the Moroccan coasts, anchovies fisheries are very important with thousands tons annually cashed. Despite the importance of this species, no widespread genetic studies have focused on the European anchovy population from Moroccan coasts. The objective of this work is to deal with genetic structure of anchovy from this region by using Mitochondrial DNA and to

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examine the hypotheses of existence of tow clades (A and B) in the Moroccan coasts as it was reported in other region.

MATERIALS AND METHODS

Fish Sampling

Fish was carried out; using INRH RV "Amir Moulay Abdallah" during species spawning period in 2012, from three Moroccan Atlantic locations, the fourth sample was collected from Moroccan Alboran Sea (Figure 1). (See Field Study Permissions in additional information and declarations)

DNA extraction, amplification and sequencing

DNA was extracted from 25 mg of fin using the "QIAGEN DNeasy Blood & Tissue kit" following the manufacturer's recommendations. A total of 138 individuals were used for mtDNA analyses (Table 1 and Figure 1). For PCR, a fragment of 680 bp was amplified with an initial denaturation at 95 °C for 10 min, followed by 35 cycles (95 °C 60 s; 52 °C 1 min, 72 °C 1 min) with a final extension at 72 °C for 10 min using the primers 5′-AACGACGCAGTAGTAGACC' and 5′-GAGGAAGTATCACTCAGGC- defined in positions 42 and 825 of the cyt-b locus of *E. encrasicolus*, respectively. The amplification products were sequenced at the CNRST/Morocco Platform and 680 bp aligned for individuals between positions 108 and 787 of the gene in BIOEDIT (Hall, 1999) with ClustalW, using the mtDNA cytochrome b sequence of the European anchovy (*Engraulis encrasicolus*) (sequence accession numbers GENBANK KF873783 and KF874179) as a reference with manual adjustments (Oueslati et al., 2014).

Data treatment

DnaSP version 5.10.1 (Librado, & Rozas, 2009) was used to calculate nucleotide diversity per site (π) (Nei, 1987) and haplotype diversity (h) (Nei & Tajima, 1981) from haplotype frequencies and haplotype divergence. The Tajima's D (Tajima, 1989) was also estimated to assess the likelihood that the DNA sequences have evolved in a neutral manner with significance tests (1 000 simulations) (significant, $P<0.05$).

The phylogenetic relationships among mDNA cyto b haplotypes were evaluated by neighbour-joining (NJ) analysis using MEGA 6.06 (Tamura et al., 2013) (Figure 2). Also, the relationship between haplotypes can be understood by Median joining haplotype networks estimated for European anchovy for each clade from four sites in the Moroccan coast (Figure 3).

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These haplotypes networks are made using the Network 4.1 software (available at: www.fluxus-technology.com). The construction of these networks is based on the theory of coalescence (Kingman, 2000) and combines features of Kruskal's algorithm for finding minimum spanning trees by choosing short connections, and Farris's maximum-parsimony (MP) heuristic algorithm (Bandelt , Forster & Röhl, 1999).

Pairwise genetic differentiation between samples (Φst) was estimated in Arlequin 3.5.3.1 (Excoffier & Lischer, 2010) on 10 000 permutations. In addition, the relationships between samples were estimated by multidimensional scaling (MDS) analysis of the pairwise Φst values. To determine homogenous groups of populations, analysis of molecular variance "AMOVA" of haplotypes was carried out in Arlequin 3.5.3.1 with 1 000 permutations to establish significance.

RESULTS

Among all the samples, the Clade A was dominant. Thus, 92% had a haplotype from Clade A and 8% had a haplotype from Clade B (Figure 2). The frequency of Clade A was high (89%) in the Alboran Sea, but its prevalence was lower than in the Moroccan Atlantic coast with a percentage that ranged from 91% in Southern Atlantic to 98% in the Central Atlantic. The median-joining haplotype network revealed two major clades (A and B) separated by 14 mutational steps and showed a star-like genealogy (Figure 3), which means that the European Anchovy population increases. This appears consistent with the values of Tajima's D, significant negative for each population independently, or for all individuals (Table 1).

The polymorphisms investigation defined 118 distinct European anchovy mtDNA haplotypes and 147 segregating sites (S), 59 of which were parsimoniously informative sites. Higher levels of genetic variation were found for the haplotype diversity h = 0.9868 ± 0.0061, but a concatenated mean nucleotide diversity for all samples was 0.008946 ± 0.004740 (Table 1). All locations had a close haplotype diversity, whereas for the nucleotide diversity the Moroccan Atlantic samples were less variable than the Alboran samples, which had the higher nucleotide diversity (π 0.01185 ± 0.00166). Under the infinite mutation model, significant negative values of Tajima's D indicate an excess of low-frequency haplotypes that can arise from selection or rapid population growth (Tajima, 1989).

Overall, there was significant genetic differentiation among the 4 populations (Φst= 0.01283; p = 0.03519). Pairwise Φst revealed a strong homogeneity within Atlantic populations, but less heterogeneity between Alboran population and Atlantic populations,

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especially between Moroccan Central Atlantic which showed a significant difference (Table 2). When using only Clade A haplotypes, the genetic structure stills significant between the populations Fst (Φst 0.01619, p very highly significant). Also the pairwise Φst were significantly different between Alboran population and Moroccan Atlantic populations (Table 2). A multi-dimensional scaling (MDS) ordination, with a stress coefficient 0.001, using all dataset or sequences of Clade A, showed that the Northern Atlantic, Central Atlantic and Southern Atlantic populations of Morocco were structured as a unique unit separated from Moroccan Alboran population (Figure 5).

Using only Clade A sequences, the AMOVA test explained 3.29% of total variation among groups (group 1: Alboran samples and group 2: Atlantic samples) and was significant (p= 0.00587). This percentage decreases when we include all dataset (2.53 %, p= 0.00489) (Table 3).

DISCUSSION AND CONCLUSION

Historical phylogeography

The result of this study showed that the most dominant clade in Moroccan anchovy was Clade A (about 90 %). The origin and expansion of this Clade was previously described by several authors. Magoulas et al. (1996; 2006), by using RFLPs in mtDNA reported two divergent clades (A and B), which differed by 3.2% sequence divergence. The authors postulated that Clade A originated in the Atlantic Sea in the West African coast, in which anchovy was possibly restricted during glacial periods. At a certain time later, approximately 100 000 years ago, under favorable climatic conditions, Clade A anchovy should have migrated to the north east Atlantic up to the west Portuguese coast, while it also entered the Mediterranean and colonized the Black Sea. On the other hand, the different composition of clade in the northwestern Mediterranean and Bay of Biscay populations dominated by Clade B haplotypes, suggest that this clade was restricted to a Mediterranean refuge during the last glacial maximum (LGM), likely the easternmost Mediterranean, from which it expanded to the rest of the Mediterranean during the deglaciation period (Magoulas et al., 2006). Grant (2005) has argued against this hypothesis and suggested that the genetic imprints in Mediterranean populations of European anchovy indicate colonization dynamics on a similar time scale. Extreme climates during glaciations 350 000 and 450 000 years ago likely extirpated populations in the entire Mediterranean Basin, and this was followed by a subsequent colonization of phylogroup ‘B’ fish. A later invasion by phylogroup ‘A’

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fish occurred during one of the pleniglacial cycles (50–25 k years ago) of the Weichselian ice age. The common ancestor of both clades was unknown, but most certainly lay outside the Mediterranean Basin complex from the Atlantic. Because of the salinity and temperature during the Pleistocene, Grant (2005) thinks that the European anchovy was not continuously present in the Black Sea which connects several times with the basins of the Mediterranean during the mid to late Pleistocene (Grant, 2005).

In keeping with distribution of clades found by Magoulas et al. (1996; 2006). In the present study, the clade composition of the Alboran and Moroccan Atlantic coast is almost exclusively from Clade A. This clade composition is similar to the clade composition in the western Portuguese coast, Bay of Cadiz, Canary Islands, the central African coast and the Alboran Spanish Sea and Algerian coast (Magoulas, Tsimenides & Zouros, 1996; Magoulas et al., 2006; Borrell et al., 2012; Viñas et al., 2013; Oueslati et al., 2014; Silva, Horne & Castilho, 2014). Also, in our sequences, we found a very low frequency of mitochondrial Clade B with nucleotide divergence of Clade A around 2.2**%**.

Population structure

This work showed a heterogeneity among the 4 populations analyzed ($\Phi st = 0.01283$; $p = 0.03519$). In fact, there is a small signal, which may assume the presence of a population in Alboran Sea genetically different from Atlantic populations, particularly when we use dominant clade (Clade A) data only. This observation was indicated in a recent study of Viñas et al. (2013) by surveying sequence variability in the mitochondrial (mtDNA) control region. Viñas et al., (2013) found that the Alboran population was genetically distinct from other Mediterranean and Northeast Atlantic populations, including neighboring populations; Cadiz. Viñas et al., (2013) explained the genetic distinctness of this population might result from the endogenous recovery of the population after the fishery collapse in the 1990s (Irazola et al., 1996; Pertierra & Lleonart, 1996 in Viñas et al., 2013) that led to the accumulation of rare alleles, such as those detected in allozymes (Sanz et al., 2008). Alternatively, Viñas et al. (2013) think that the genetic distinctness might reflect the influx of both Clade A haplotypes from the Atlantic and Clade B haplotypes from the northwestern Mediterranean populations, resulting in a mixed population that is distinct from both sources. However, when we use only the clade A sequences, genetic distinctness increases and becomes more significant, which allows us to reject this hypothesis.

Finally, the observed genetic structure might be the reason of the presence of cryptic species.

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Indeed, the geographical isolation, as shelters of the European anchovy during episodes of glaciation, followed by a secondary contact may be at the origin of the formation of cryptic species, that it is often difficult to distinguish from simple populations genetically structured. Bembo et al. (1996a) by analyzing allozymic markers and morphological differences, observed a genetic structuration between coastal anchovy (> 50 m) and open waters anchovy in the Adriatic Sea differences that Borsa, Collet & Durand (2004) confirmed by studying the polymorphism of the nuclear EPIC marker CK6-2. The existence of coastal forms of anchovies in the Moroccan Alboran Sea had already been reported in the literature on the basis of morpho-anatomical and behavioural differences. Thus, Kada et al. (2009) showed that an anchovy of Nador lagoon in Morocco displays some clear morphological and biological differences from marine *Engraulis encrasicolus*. Lastly, Oueslati et al. (2014) by analyzing mtDNA and six nuclear microsatellite loci, found two strongly differentiated loci and nucleo-cytoplasmic disequilibrium supported the existence of a coastal genetic entity in Nador lagoon in Morocco that is distinct from the more offshore marine populations.

The lack of genetic structure at the Atlantic populations, seems to be related to the fact that this pelagic fish presents relatively high migratory capacities. Indeed, this species is likely characterized by important gene flow, accelerated by a strong dispersion, a large effective population size countering genetic drift (Waples, 1989; Gonzalez & Zardoya, 2007), and the homogenizing forces related to the hydrodynamic environment, such as in the case of the north western African coasts with the Canaries current and the upwelling phenomenon (Le Floch, 1974; Belvèze & Erzini, 1983). However, more suitable sampling methods and more sensitive genetic markers is needed to confirm our results. We believe that additional genetic data can provide insights that will improve the sustainable management of European anchovy fisheries in the Moroccan fishery stocks.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Field Study Permissions

Sampling and analysis techniques used in this work are consistent with the Moroccan legislation Article 14, Section 3, Bill 122.22 and the *Cartagena Protocol on Biosafety to the Convention on Biological Diversity* promulgated by the royal decree "1.09.123".

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Table 1 List of sample and genetic statistics within each of the sample sites for cytochrome b mitochondrial gene in *E. encrasicolus*.

Region sampling locality	Sample Code	FAO fishery area	Date	N	h ± s.d.	π ± s.d.	Tajima's D	D p-value
Moroccan Alboran Sea	Albo	37.1.1	oct-12	37	0.998 ± 0.007	0.01185 ± 0.00166	-1.88700 ± 0.91417	0.01000
Moroccan North Atlantic	N_ATL	34.1.11	nov-12	25	0.967 ± 0.029	0.00704 ± 0.00135	-1.97692 ± 0.89397	0.01600
Moroccan Center Atlantic	C_ATL	34.1.12 & 34.1.13	Jan-13	44	0.993 ± 0.008	0.00702 ± 0.00082	-2.43168 ± 0.92737	0.00000
Moroccan South Atlantic	S_ATL	34.1.3	mars-12	32	0.968 ± 0.024	0,00934 ± 0,00183	-2.20225 ± 0.91166	0.00100
				138	0.9868 ± 0.0061	0.008946 ± 0.004740	-2.12446 ± 0.24399	0.00675

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386 **Table 2** Pairwise Φst among the 4 samples. Below the diagonal using the whole dataset samples and above using only Clade A
387 sequences.

Location	albo	N_ATL	C_ATL	S_ATL
Albo	0.00000	0.02004*	0.02764*	0.03181*
N_ATL	0.01015	0.00000	0.00087	0.00069
C_ATL	0.02990*	0.00409	0.00000	0.00277
S_ATL	0.01291	0.00000	0.00702	0.00000

388 Asterisks indicate significant values after Bonferroni correction (Sample codes as in Table 1).

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413 **Table 3** Analysis of the genetic variability among samples from E. encrasicolus using AMOVA .

	source of variation	Percentage of Variation among groups	Variation Among populations within groups
dataset	Group1 : Albo Group2: N_ATL , C_ATL and S_ATL	2.53178 P-value = 0.00489	0.00% P-value = 0.17204
Clade A	Group1 : Albo Group2: N_ATL , C_ATL and S_ATL	3.29 P-value = 0.00587	0.00% P-value = 0. 24829

414 Sample codes as in Table 1.

415

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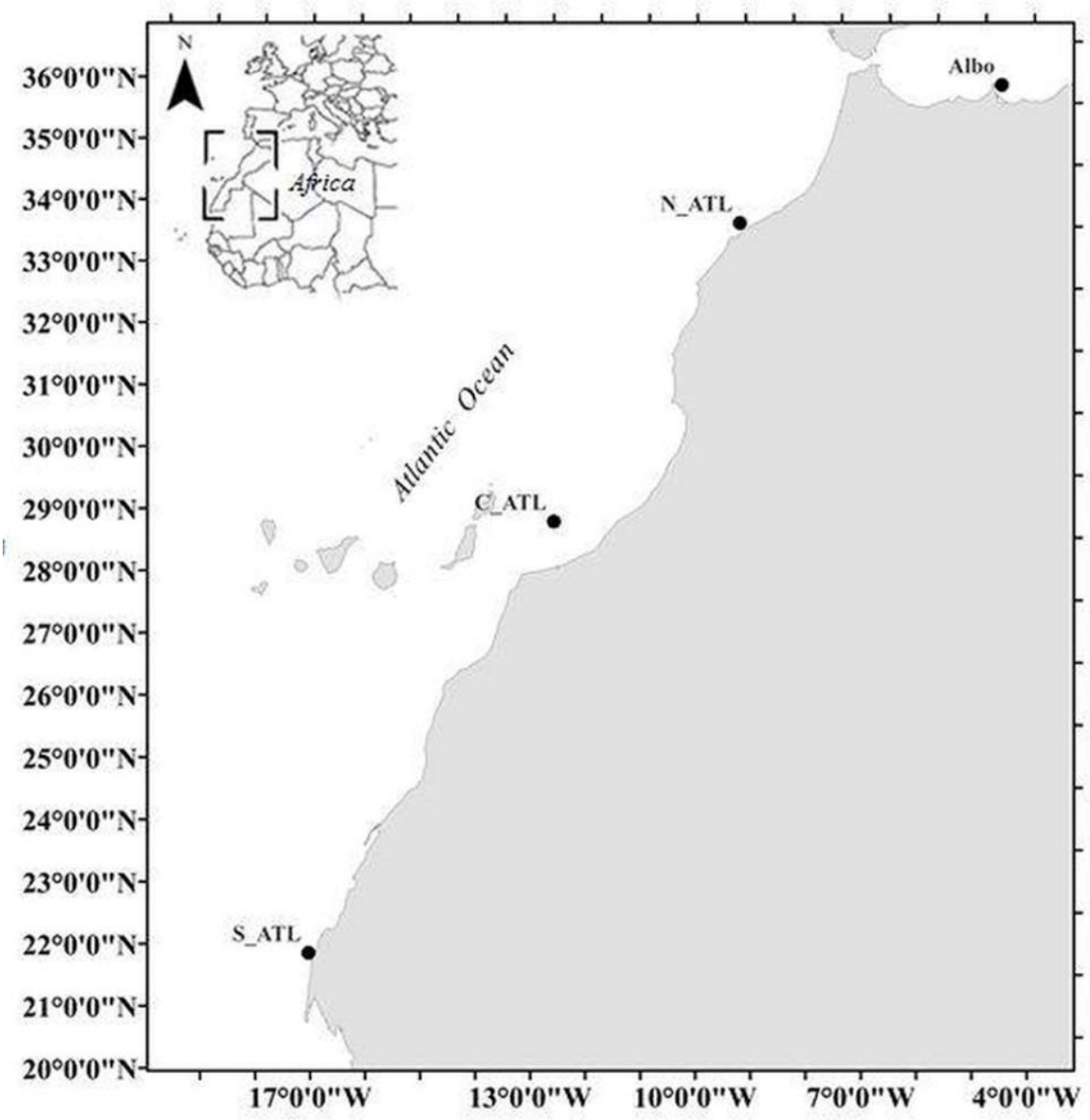

Figure. 1 Map of Moroccan Coast showing sampling sites. (Albo: Moroccan Alboran Sea; N_ATL: Moroccan North Atlantic; C_ATL: Moroccan Center Atlantic; S_ATL: Moroccan South Atlantic).

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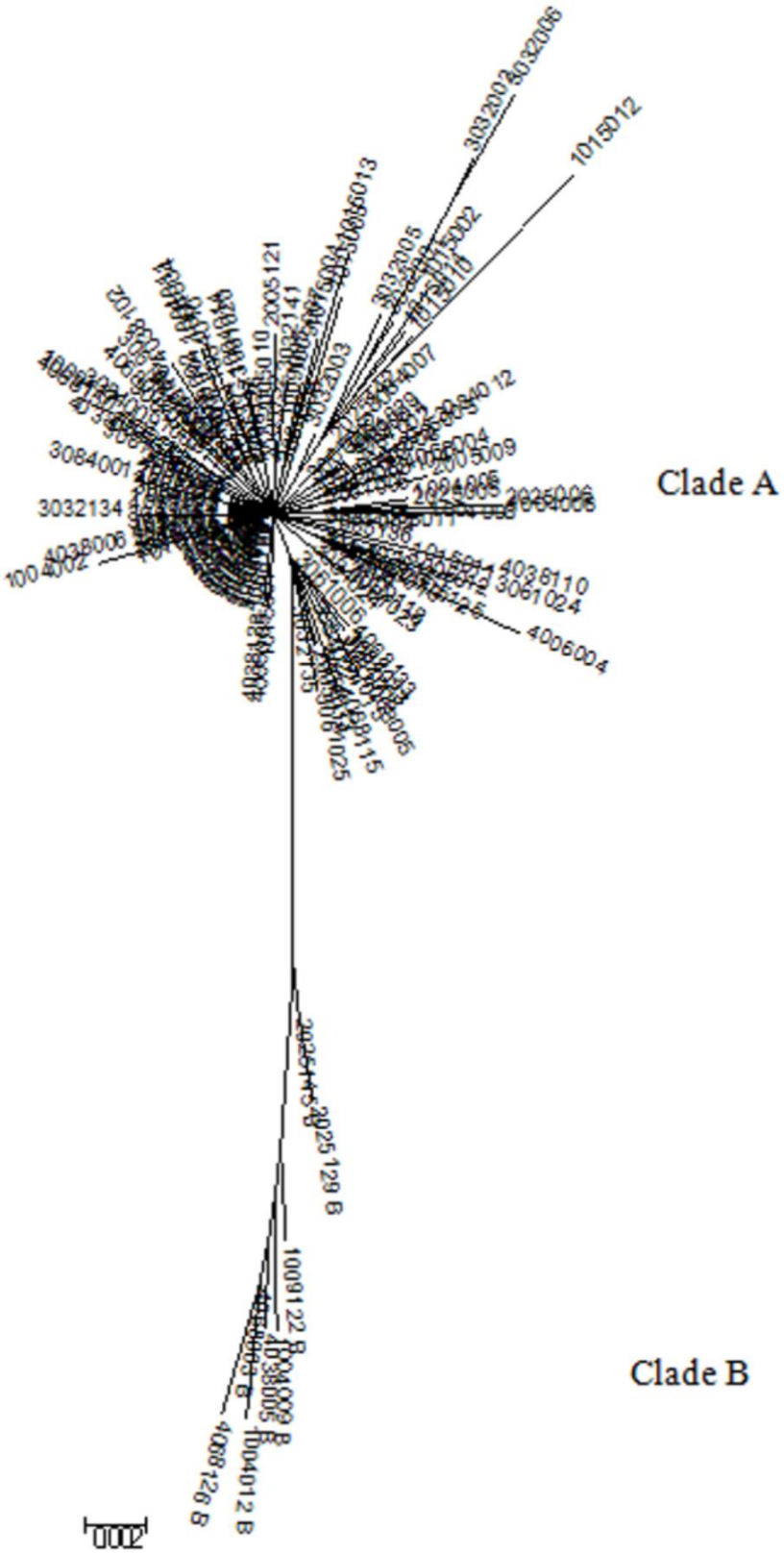

419

420 **Figure 2** Unrooted NJ tree showing the relationship of mtDNA cytochrome b

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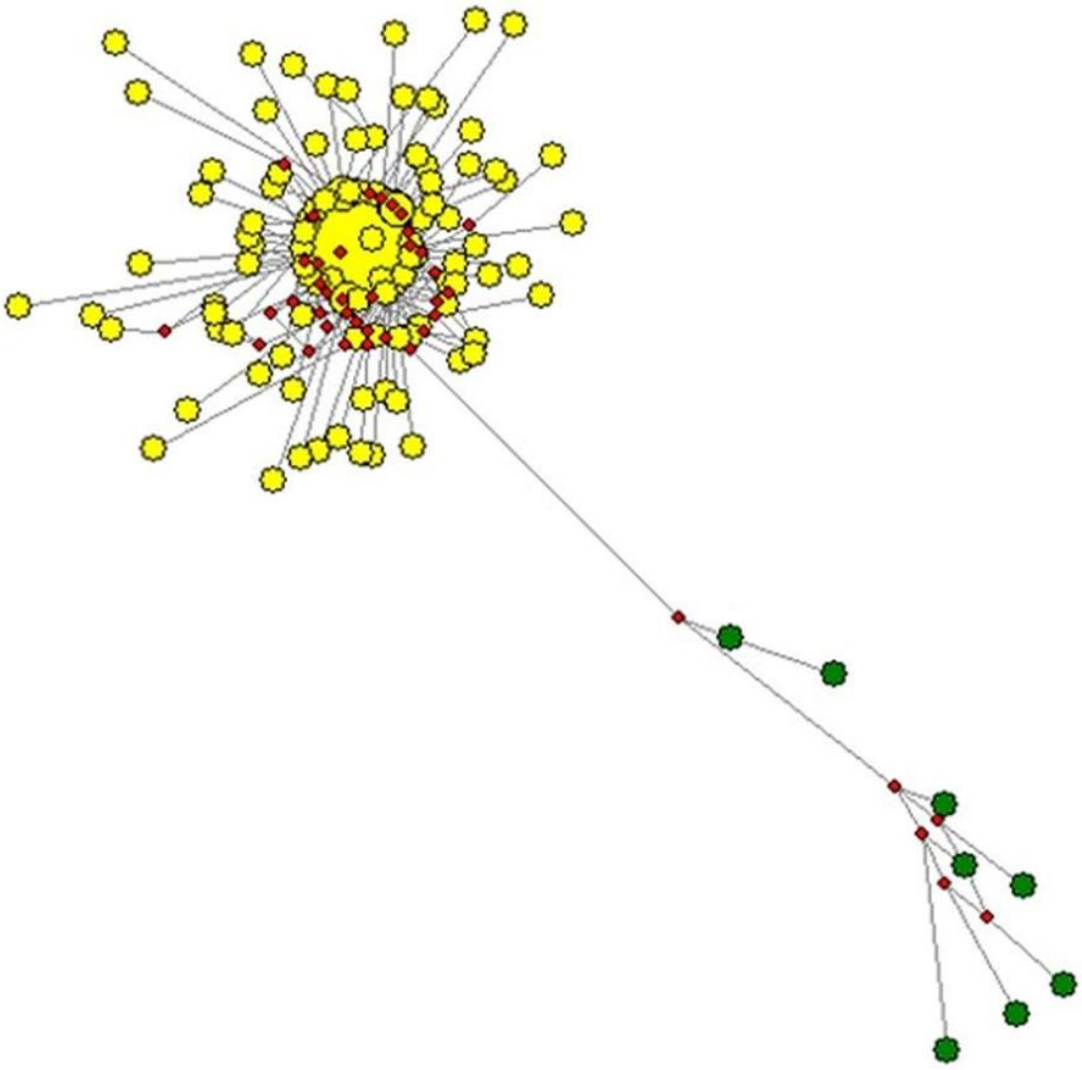

Figure 3 Haplotype analysis for Cytb mitochondrial genes in E. encrasicolus by Median-joining network showing the presence of two main clades (A and B). Yellow nodes represent Clade A, green nodes represent Clade B and red nodes represent undetected haplotypes in the study but necessary to most parsimonious network construction.

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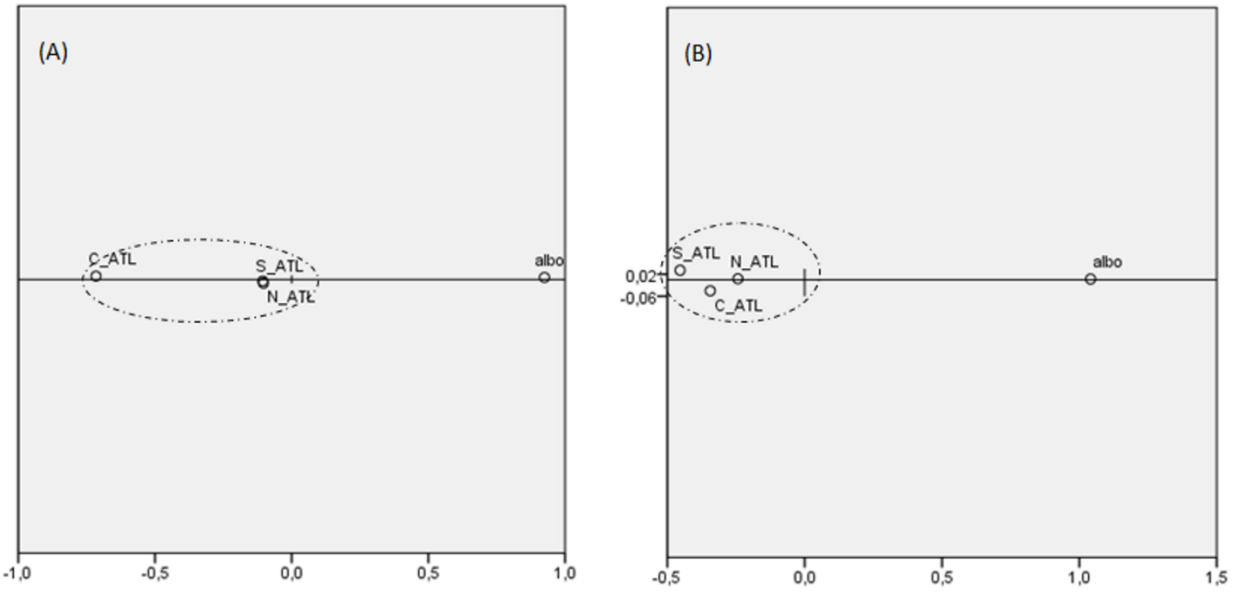

Figure 4 Multidimensional scaling of Φst distance between 4 samples. (A) using the whole dataset samples and (B) using only Clade A sequences (Sample codes as in Table 1).

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