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High level of genetic variation in mitochondrial 16S rDNA among populations of *Porcellionides pruinosus* (Brandt, 1833) (Crustacea: Isopoda: Oniscidea) in Tunisia

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Abstract

For more than 20 years, the cosmopolitan species *Porcellionides pruinosus* (Brandt, 1833) has been characterised by a controversial taxonomic status. This “species” has a high ecological plasticity, which enabled it to conquer different kinds of habitats. Besides that, this “species” exhibits a geographical variation in its morphological features and its reproduction pattern. In fact, some Tunisian populations had a seasonal reproductive period and while others showed a reproductive activity. A genetic analysis of rDNA 16S sequences has been performed to compare eight populations of *P. pruinosus* from different geographical sites, located in the north, centre, and south of Tunisia. Results reveal that the population of Tunis shows considerable variation in the primary structure of the sequences and has a substantial genetic diversity ($F_{ST} = 0.98$ and $F_{CT} = 0.97$). However, the genetic variation is low for the populations of Bella Regia and Bousalem (0.00041), and null between populations of Elfeija, Chebba, Monastir, Sbiba and Sned. Moreover, the genetic distance of the population of Tunis (≥ 0.35) compared to the other populations raises questions about the taxonomic status of this population. Also, in this latter population transitions are the most dominant variations, whereas for the other seven populations there are only two types of transversion substitutions. The results of the current study emphasise the controversial taxonomic status of *Porcellionides pruinosus* in Tunisia. Consequently, it would appear that the taxonomic status of *P. pruinosus* from Tunisia needs a re-evaluation.

Keywords: Terrestrial isopod, genetic diversity, intraspecific variability, rDNA 16S, Tunisia

Introduction

The cosmopolitan species *Porcellionides pruinosus* (Brandt, 1833), characterised by a controversial taxonomic history and high ecological plasticity, exhibits geographical variations of its morphological features and its reproduction pattern. This species is highly synanthropic and is considered to be the most widely distributed species of terrestrial isopods (Vandel 1962; Garthwaite & Sassaman 1985; Michel-Salzat et al. 2001; Lefebvre & Marcadé 2005; Achouri et al. 2008). Originally native to Asia Minor (Vandel 1962) and carried extensively elsewhere by humans, *P. pruinosus* has colonised the entire world except the polar regions. It presents an important geographical

variation and it is considered to be a polytypic species (Michel-Salzat et al. 2001). Based on morphological criteria about 20 subspecies have been recognised throughout the world (Vandel 1962), although the validity of these subspecies remains questionable (Böhme 1978). More recently several observations have supported the presence of distinct species (Garthwaite & Sassaman 1985; Juchault et al. 1985; Marcadé et al. 1999). Michel-Salzat et al. (2001) and Lefebvre and Marcadé (2005) reported the existence of two sibling species of *P. pruinosus*, one in Western Europe (called the French group) and the other in Greece, Réunion Island and Tunisia.

In Tunisia, the morphology (size and colour) of specimens from populations belonging to

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P. pruinosus varies with geographical locality and also changes with the bioclimatic levels, ranging from albinism to purplish blue (Achouri & Charfi-Cheikhrouha 2002). Furthermore, analyses using scanning electron microscopy and phenetic analysis have presented important phenotypic variations, and showed a polymorphism in some morphological characters such as the length of the antennae and the form of the pleotelson (Achouri & Charfi-Cheikhrouha 2005; Achouri et al. 2008). Moreover, these populations exhibit different reproductive behaviours (Achouri & Charfi-Cheikhrouha 2005). In addition, results from horizontal starch gel electrophoresis, used to assess the levels of their intra and inter-genetic diversity, and to analyse their genetic structure, showed the divergence of one population from North Tunisia (Tabarka; Achouri et al. 2012).

As molecular analyses and DNA polymorphism have become more accessible and methods of measurement of this polymorphism have been multiplied (Ould Ahmed et al. 2010), in the present work we completed intra-specific variability studies by molecular analysis based on the use of a powerful molecular marker, mitochondrial DNA. It has a great value in molecular ecology and allows better estimation of the diversity within populations of *P. pruinosus* and confirmation of the variability observed in morphological, reproductive and biochemical studies. Characterised by their intraspecific polymorphism (Avisé & Lasman 1983; Boursot & Bonhomme 1986), the genes encoding ribosomal RNA are sometimes called “key phylogeny” (Olsen & Woese 1993; Michel-Salzat & Bouchon 2000). Also, from an experimental standpoint, mtDNA is easily amplified (present in the cell in multiple copies; Galtier et al. 2009).

This study presents a useful contribution to the knowledge of the widespread woodlice *P. pruinosus*, which currently exhibits a confused taxonomic status, and could hide a complex of species much like *Oniscus asellus* and *Oritoniscus flavus*.

Materials and methods

Sampling

We sampled 75 specimens (eight populations) of *Porcellionides pruinosus* in the following locations: Elfeija (F1-F10), Monastir (M1-M10), Bousalem (BS1-BS10), Sned (S1-S10), Sbiba (S1-S5), Bella Regia (B1-B10), Tunis (T1-T10) and Chebba (C1-C10) (Figure 1).



Figure 1. Map of the study area. Sampling sites are indicated by numbers from 1 to 8.

DNA extraction, PCR amplification and sequencing

To reduce contamination of the DNA extracted, animals were washed. Tissues taken for DNA extractions were the nervous system and some muscles (Marcadé et al. 2007). Total genomic DNA was obtained with an ABIOPure kit. For Polymerase Chain Reaction (PCR) amplification of a fragment of the gene encoding a large subunit of mitochondrial ribosomal RNA (16S rRNA) in the eight populations of *P. pruinosus*, the following primers were used: LSUF 5' CGCCTGTTTAACAAAGACAT 3' and LSUR 5' TCGGTCTGAACTGA ACTCAGATCACGT 3' (Michel-Salzat & Bouchon 2000). PCR started with initial denaturation, which lasted 4 min at 94°C, before adding *Taq* polymerase, followed by cycles of denaturation (94°C for 50 s), annealing (52°C for 50 s) and

primer elongation (65°C for 50 s) and the final extension (65°C for 5 min) (Michel-Salzat & Bouchon 2000). For the target area, the temperature hybridisation was changed from 47°C to 52°C. For this amplification, we prepared a liquid reaction mixture composed of 0.5 µL primer (50 mM), 2 µL of the DNA template to be amplified, 0.2 µL of Taq polymerase, and 0.5 µL of deoxyribonucleotides (dNTPs – 10 mM): 5 µL buffer 5 X Taq for a final volume of 25 µL (Michel-Salzat & Bouchon 2000). The amplified product was subjected to automatic sequencing after purification using the Big Dye Terminator kit. The obtained sequences were aligned with the MEGA program. DNA polymorphism parameters within and between populations were estimated using the program ARLEQUIN version 3.5 (Excoffier & Lischer 2010). The analysis of molecular variance (AMOVA) was performed on three hierarchical structures: within populations (F_{IS}), among geographic regions (F_{CT}) and among populations of the same geographic locality (F_{sc}). A population-specific F_{ST} index (genetic differentiation among the populations) was determined using both nucleotide divergences and haplotype frequencies (Kimura 1980) for each population pair (Baratti et al. 2005) and used as a genetic distance between populations. The matrix of pairwise F_{ST} values was then used to build a neighbour joining (NJ) dendrogram with MEGA6 software (Tamura et al. 2013).

Results

Composition and nucleotide variations of sequences

The seven populations (Sbiba, Chebba, Monastir, Elfeija, Bella Regia, Bousalem and Sned) have almost the same compositions of cytosine (C), adenine (A), thymine (T) and guanine (G), with percentages that are, respectively, 16.52%, 37.05%, 29.69% and 16.74%. There is a bias towards A-T in all of the populations (Table I). However, in nucleotides, only two kinds of transversion mutations of thymine (T) to guanine (G) were observed in exactly two individuals

of Bella Regia and Bousalem, while 48 substitutions were detected in the population of Tunis. Indeed, the last population presents the majority of the nucleotide variations: 28 transitions, 20 transversions and 11 indels or gaps (insertion/deletion).

Intra-population diversity

Molecular indices. The mean number of differences between the pairs of haplotypes (π) in a population is the number of nucleotide changes (point mutations) that have occurred since the divergence of each pair of haplotypes in the population (Tajima 1993). Our results showed that the highest value is observed in the population of Tunis, which was equal to 16.82. The two populations of Bella Regia and Bousalem showed the lowest values of 0.2 and 0, respectively. Nucleotide diversity (π_n) was the most used index and most important in intrapopulation analyses. Indeed, the highest nucleotide diversity (π_n) was found in Tunis (0.037), while the lowest was found in Chebba, Sned, Sbiba, Elfeija and Monastir (0).

Standard diversity indices

Table II showed that the 75 individuals analysed carried 10 unique haplotypes. The number of polymorphic sites, among analysed populations, ranged between 0 (Chebba, Sned, Sbiba, Elfeija and Monastir) and 49 (Tunis). The most important haplotype diversity (h) was noticed in the population of Tunis (1.000); populations of Bousalem and Bella Regia showed haplotype diversity that did not exceed 0.20, while the lowest value (0) was observed in the remaining populations.

Inter-population diversity

To determine the genetic diversity between populations, we chose to group them into two groups (AMOVA with regrouping). The first one includes seven populations and the second is represented by the

Table I. Composition and numbers of substitutions (transition, transversion, or insertion/deletion) between the 16S rDNA segments.

Population	Nucleotide composition (%)				Nucleotide variation		
	C	T	A	G	Transition	Transversion	Insertion/deletion
Chebba	16.52	29.69	37.05	16.74	0	0	0
Elfeija	16.52	29.69	37.05	16.74	0	0	0
Bella Regia	16.52	29.67	37.05	16.76	0	1	0
Bousalem	16.52	29.67	37.05	16.76	0	1	0
Monastir	16.52	29.69	37.05	16.74	0	0	0
Sned	16.52	29.69	37.05	16.74	0	0	0
Sbiba	16.52	29.69	37.05	16.74	0	0	0
Tunis	14.62	33.44	36.10	15.84	28	20	11

had a genetic distance of over 0.359 compared with Bella Regia and Bousalem, and more than 0.358 compared with the other populations (Table IV).

Analysis of phylogenetic trees. The topology of the trees found using NJ (Figure 2) allowed us to see two distinct clades. The seven populations (Sbiba, Bella Regia, Sned, Bousalem, Chebba, Elfeija and Monastir) are closely related and formed a monophyletic clade, which diverged first. The second clade contained the population of Tunis (T) with other related taxa (*Porcellio*, *Trachelipus* and *Porcellionides cingendus*). In addition, the trees showed the divergence of specimen T2 compared to others (T1, T3, T4, T5, T6, T7, T8, T9 and T10) in population of Tunis.

Discussion

Although many studies were undertaken on the cosmopolitan species *P. pruinosus*, the systematics of this species remains unclear and controversial. In fact, the conventional approaches for species characterisation are limited and in light of the contribution of the operation of various molecular markers, this work focused on the polymorphism research and estimation of genetic diversity in *P. pruinosus*.

Analysis of the primary structure of rDNA showed a similarity of T-A content of the population's sequences; this result is predictable considering the high degree of sequence conservation except for the sequence from population of Tunis, which showed a high T-A level. This result is in agreement with other studies on other terrestrial isopods, such as *Armadillidium pelagicum* (Charfi-Cheikhrouha 2003). Compared to other population sequences of *P. pruinosus* from France, the 16S fragment length is 348–352 bp, shorter than our sequence which had a length of 463 bp.

The percentage of G-C found in our study was around 30.46% and 33.28%, respectively, almost equal to that determined in populations from France (30.62 and 34.20%; Michel-Salzat & Bouchon 2000). For all analysed sequences, the observed changes were often substitutions. The highest value was detected in the population of Tunis (59 nucleotide variations). In this population, transitions were the most dominant variations, consistent with what has been found in other terrestrial isopods (Charfi-Cheikhrouha 2003). However, for the seven remaining populations, there were only two types of transversion substitutions.

Analysis of intra-population diversity shows that populations of Sbiba, Sned, Monastir, Chebba and

Elfeija shared a unique haplotype, which can be explained by the ability of the species to maintain genetic connectedness among populations. However, the population of Tunis has the highest number of haplotypes (10). It should be noted that the population of Tunis exhibits the most important intra-population diversity, followed by the north-western populations (Bella Regia and Bousalem) which exhibit a low intra-population diversity resulting from the two transversions. Otherwise, the other populations show the absence of this diversity (0) and consequently a limited genetic differentiation, which is in agreement with the isozyme studies (Achouri et al. 2012).

Our results showed a very important and significant percentage of variation (98.10%) between the two groups. Nevertheless, the value was very low within populations, which can be attributed to the strong conservation of ribosomal coding regions.

The highest genetic distance between populations of *P. pruinosus* by isoenzyme analysis was equal to 0.18 (Achouri et al. 2012); for comparison, in other studies of operator intraspecific 16S rDNA the genetic distance did not exceed 0.059 between populations of *Armadillidium pelagicum* (Charfi-Cheikhrouha 2003). Moreover, the homology of nucleotide sequences between the population of Tunis and other populations was less than 85%, with a large genetic distance (0.35). In addition, the populations Sned, Sbiba, Elfeija, Chebba and Monastir had the lowest genetic diversity, both intra- and inter-population. This result may be related to the presence of *Wolbachia*, because in this case, populations showed no mitochondrial polymorphism. Indeed, it has been demonstrated that French populations of *P. pruinosus*, almost entirely infected with a unique lineage of *Wolbachia*, showed no mitochondrial polymorphism as compared with populations from Tunisia, Greece and Réunion Island (Marcadé et al. 1999; Grandjean et al. 2005). In connection with this, Delhoumi et al. (2018) detected the bacterium *Wolbachia* in Tunisian populations of *P. pruinosus*.

However, estimates of evolutionary divergence indicated that the population of Tunis was the most distant from other populations, with a molecular divergence value of 0.35 and 0.35 compared to the north-west populations and the other populations, respectively. It should be noted that similar genetic distance values can be observed between two different species, as this was the case between the species *A. pelagicum* and *A. vulgare* (Charfi-Cheikhrouha 2003). Verovnik et al. (2005) reported that an extensive genetic divergence could

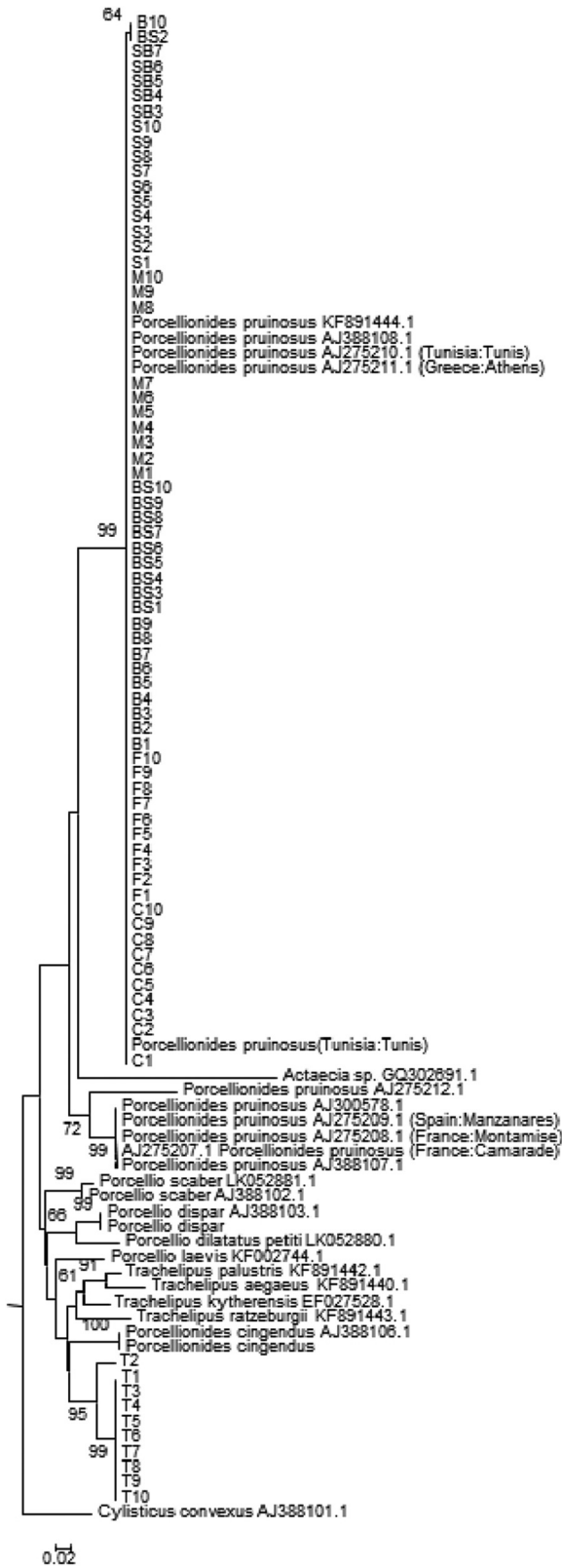


Figure 2. Neighbour joining tree showing the phylogenetic relationships among the analysed populations. Tunis: T1–T10, Sbiba: SB1–SB5, Chebba: C1–C10, Monastir: M1–M10, Elfeija: F1–F10, Bella Regia: B1–B10, Sned: S1–S10, Bousalem: BS1–BS10.

induce a cryptic speciation or fragmentation between allopatrically evolved populations as in the case of the population of Tunis. Indeed, despite the uniform morphology, genetic divergence indicates the presence of overlooked or cryptic species in isopods, as shown by Brix et al. (2014). Hence, the divergence and the genetic distance separating the population of Tunis from the other ones, on one hand, and the specimen T2 as compared with other specimens from Tunis support the hypothesis of cryptic species. Subsequently, it is necessary to re-evaluate the taxonomic status of *P. pruinosus* in Tunisia.

The tree obtained by the NJ method showed two clusters. The first groups the populations of *P. pruinosus* from Sbiba, Chebba, Bousalem, Bella Regia, Sned, Elfeija and Monastir, and the second includes the individuals collected from Tunis with other species (*Porcellio*, *Armadillidium pelagicum* and *Porcellionides cingendus*). In addition, the tree showed the existence of two groups in the population of Tunis, T2 and the other specimens (T1, T3, T4, T5, T6, T7, T8, T9 and T10). In fact, we can conclude the existence of more than two groups in the studied populations. These results showed the existence of a taxonomic problem among Tunisian populations of *P. pruinosus* analysed in this study, whereas it was consistent with previous studies of *P. pruinosus* (Garthwaite & Sassaman 1985; Marcadé et al. 1999; Lefebvre & Marcadé 2005). Indeed, the tree (NJ) revealed that populations of *P. pruinosus* were the most divergent from the population of Tunis and the other species, *Porcellio* and *Trachelipus*, on one hand, and from *Porcellionides cingendus* and the population of Tunis in the other hand. This result is partially in agreement with Vandel (1943), Schmalzfuss (1989) and (Carefoot & Taylor 1995) who considered that the genetic basis of these species is explained by the divergence of the Armadillidiidae family from family Porcellionidae.

Conclusion

As mentioned above, Achouri et al. (2012) studied the genetic differentiation of some Tunisian populations of *P. pruinosus* based on allozymic data and reported a notable divergence of one population (Tabarka) from the others, highlighted by a mean genetic distance equal to 0.18. This genetic differentiation was correlated with a variable reproductive behaviour and an important phenotypic variation. The present study corroborates these results. Indeed, the population of Tunis exhibited a continuous reproductive activity while all the others presented seasonal reproduction which is likely to be a barrier to gene flow.

Further investigations of this “species complex” using other molecular markers including nuclear ones and crossing experiments would contribute to elucidate its taxonomic status.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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