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A molecular study of the tardigrade *Echiniscus testudo* (Echiniscidae) reveals low DNA sequence diversity over a large geographical area

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ABSTRACT

In the present study we investigate the genetic diversity within the asexually reproducing tardigrade Echiniscus testudo. *The present study is the first to sample a tardigrade species for comparison of DNA sequence diversity between widely separated samples.* Echiniscus testudo *was sampled at 13 localities spanning three continents. DNA sequences of the mitochondrial COI gene and the nuclear ITS2 sequence were used to investigate the genetic diversity and phylogeographic structure of the various asexual lineages. Terrestrial tardigrades with the capability of entering a cryptobiotic state are assumed to have a high passive dispersal potential through airborne transport. Our results show moderate (ITS2) to high (COI) haplotype diversity and low sequence diversity that indicate evolution of haplotypes within distinct asexual lineages and a high dispersal potential. No isolation by distance was detected by Mantel tests. Different phylogeny inference methods (neighbor-joining, maximum parsimony, maximum likelihood and Bayesian inference) revealed little topological resolution, but minimum spanning networks showed some phylogeographic patterns. The COI and ITS2 minimum spanning networks show patterns that indicate dispersal of several haplotypes from founding populations. In conclusion our data show a low genetic diversity and a relatively high haplotype diversity indicating that* E. testudo *is a young species with a high dispersal potential.*

Key words: terrestrial Tardigrada, asexual lineages, sequence diversity, COI, ITS2

1. INTRODUCTION

Tardigrades, or water bears, are minute metazoans closely related to arthropods and onychophorans (Garey *et al.* 1996; Giribet *et al*. 1996; Mallatt *et al*. 2004). The terrestrial tardigrades constitute a major component of the cryptic fauna in mosses and lichens. This environment is subject to continued drying and soaking, and in cooler areas freezing. The ability of many tardigrades to enter a cryptobiotic metabolic state is shared with e.g. nematodes and rotifers and is important for the survival and dispersal of terrestrial species. It is assumed that terrestrial tardigrades in their cryptobiotic state have a high passive dispersal potential through airborne transport (Pilato 1979).

The cosmopolitan *Echiniscus* C.A.S. Schultze, 1840 is the most species rich (currently 146 described species) of the 105 tardigrade genera (Guidetti & Bertolani 2005). The distribution of *Echiniscus testudo* (Doyère, 1840) covers most of the palaearctic biogeographic region. Until recently the species in the heterotardigrade genus *Echiniscus* were thought to reproduce parthenogenetically, only producing females (thelytoky) (Kristensen 1987). Males have recently been found in some *Echiniscus* species from Himalaya, Australia and Antarctica, perhaps showing the remains of an ancient Gondwanaland distribution (reviewed in Miller *et al*. 1999). In these populations males are not rare spanandric males but rather appear in equal numbers to females (Miller *et al*. 1999). The specific type of parthenogenesis remains unknown for *Echiniscus*.

When continuous parthenogenetic (or asexual) reproduction occurs in a species it results in the absence of gene flow between populations, reduced genetic variability and if parthenogenesis is prolonged it might eventually lead to speciation (Pannebakker *et al*. 2004). However, recent investigations indicate that at least the parthenogenetic bdelloid rotifers have undergone substantial speciation (Birky *et al*. 2005). Parthenogenesis occurs in tardigrades (Bertolani 1994) and many other invertebrate groups, i.e. aphids, crustaceans, nematodes, rotifers, etc. (reviewed in Lushai *et al*. 2003; Normark *et al*. 2003). In echiniscid tardigrades that have a high dispersal potential the ability to reproduce asexually might represent a strong advantage because these species are not limited by the necessity of finding a mate. Much of the speciation (based on phenotypic divergence) within *Echiniscus* may be a result of the asexual reproduction resulting in the divergence of asexual lineages. Miller *et*

Tab. 1. Specimens of *Echiniscus testudo* examined in the present study. Moss sampled in 1985 and 1997 are long time stored material.

Species	Locality	Haplotype		Sampling year	GenBank Acc. number	
		COI	ITS2		COI	ITS2
Echiniscus blumi	Greenland, Disko	-	-	2005	EF620382	EF620383
Echiniscus cf. *testudo*	India, Ladakh	Et 1	Et g	1985	EF620367	EF620384
Echiniscus testudo	Denmark, Bornholm	Et 9	Et e	2003	EF620368	EF620385
Echiniscus testudo	Denmark, Bornholm	Et 3	Et e	2005	EF620369	EF620386
Echiniscus testudo	Denmark, Nivå	Et 10	-	2002	EF620377	-
Echiniscus testudo	Denmark, Nivå	Et 10	Et e	2005	EF620378	EF620394
Echiniscus testudo	Egypt, Sinai	Et 7	-	2004	EF620370	-
Echiniscus testudo	Faroe Islands, Torshavn	Et 11	Et f	2006	EF620381	EF620400
Echiniscus testudo	France, Mont Saint Michel	Et 2	Et b/Et e	2005	EF620375	EF620391/EF620392
Echiniscus testudo	Germany, Munich	Et 2	Et c	2005	EF620376	EF620393
Echiniscus testudo	Greece, Crete	Et 10	Et a/Et d	2004	EF620380	EF620395/EF620396
Echiniscus testudo	Greenland, Disko	Et 10	Et b	2002	EF620379	EF620387
Echiniscus testudo	Israel, Golan Heights	Et 4	Et a/Et d	1997	EF620373	EF620388/EF620389
Echiniscus testudo	Italy, Sassomorello	Et 5	Et e	2005	EF620374	EF620390
Echiniscus testudo	Morocco, Tizi-n-Taghatine	Et 6	Et a/Et e	1985	EF620371	EF620397/EF620398
Echiniscus testudo	Morocco, Tizi-n-Talrhemt	Et 8	Et a	1985	EF620372	EF620399

al. (1999) suggested that the *Echiniscus* originated and speciated as sexually reproducing species in Gondwanaland (180 MYA). Though this age of origin might be overestimated many of the *Echiniscus* species could represent examples of ancient asexuals. The parthenogenetic bdelloid rotifers, darwinulid ostracods and some lineages of oribatid mites are regarded as ancient asexuals (Mark-Welch & Meselson 2000; Schön & Martens 2003; Maraun *et al*. 2004). However, this ancient asexual status has recently been questioned for the mites and ostracods (Schaefer *et al*. 2006; Smith *et al*. 2006).

Tardigrades have just recently been subject to molecular studies, which are mainly phylogenetic (Garey *et al*. 1996; Giribet *et al*. 1996; Regier & Schultz 2001; Jørgensen & Kristensen 2004; Mallatt *et al*. 2004; Guidetti *et al*. 2005) but also include biodiversity estimation (Blaxter *et al*. 2004) and the transcription of heat shock proteins (Schill *et al*. 2004).

The present study is the first to sample a tardigrade species for a wide geographical comparison of DNA sequence diversity. We investigate the genetic variation of the echiniscid tardigrade *Echiniscus testudo* that has asexual reproduction and evolve in asexual lineages with the aim of elucidating the evolution of different haplotypes and their phylogeography.

2. METHODS

2.1. Specimens

Echiniscus testudo was collected from moss and lichen samples from 13 localities spanning a wide geographical area (two of which were sampled twice with two and three year's interval) (Tab. 1 and Fig. 1). The yellowish-red *E.* cf. *testudo* from Ladakh, India was slightly different from the other *E. testudo*. No males were recorded in our samples. *Echiniscus blumi* Richters, 1903 was included as an outgroup in the phylogenetic analyses. The samples were thoroughly washed through a sieve (mesh size 62 µm) and sorted using a stereomicroscope at 30× magnification. The specimens were identified using a compound microscope and micro slides of voucher specimens have been deposited at the Zoological Museum, University of Copenhagen.

2.2. Molecular methods

The adult *Echiniscus testudo* specimens were 285-340 µm in length. For the DNA extraction between 4 and 20 specimens were pooled from each locality. From Crete, Munich and Nivå single specimen DNA extraction were subsequently performed. These extractions revealed no sequence difference as compared to the pooled material. We mainly used stored (dried out) material, up to 20 years old, which was rehydrated. All specimens returned to a mobile stage. Also non-stored (fresh) Danish moss samples collected in the period 2002-2005 were used. The specimens were carefully grinded during the DNA extraction following the STE-buffer DNA extraction method (Maniatis *et al.* 1982) and the same pool of specimens were used for amplification of both COI and ITS2.

The mitochondrial COI primers and the nuclear ITS2 primers presented in table 2 were used for both PCR amplifications and direct sequencing.

The PCR reaction mix was prepared in a total volume of 20 µl with 0.1 µl Taq polymerase (Roche; 0.5 U / µl), 2 µl 10× buffer (Roche; 15 mM $MgCl_2$), 1 µl dNTP (4 mM), 1 µl of each primer (10 pmol / µl), 1 µl of DNA template, and 14 µl ddH20. Both the LCO-HCOout and the LCO-HCOoutout primer pairs amplified *E. testudo*. The primer pairs LCO-HCOout, LCO-HCOoutout and 3SN-BD2 resulted in bands of approximately 710 bp, 815 bp and 450 bp, respectively. HCOoutout did not amplify as well as HCOout for *E. testudo*, but where possible the accuracy of the sequences was verified by independent amplification and sequencing of both reverse primers.

The PCR settings for all primer pairs were a preheat step at 95 ºC for 5 min, 37 cycles of denaturation at 94 ºC for 10 sec, annealing at 42 ºC for 30 sec and amplifi-

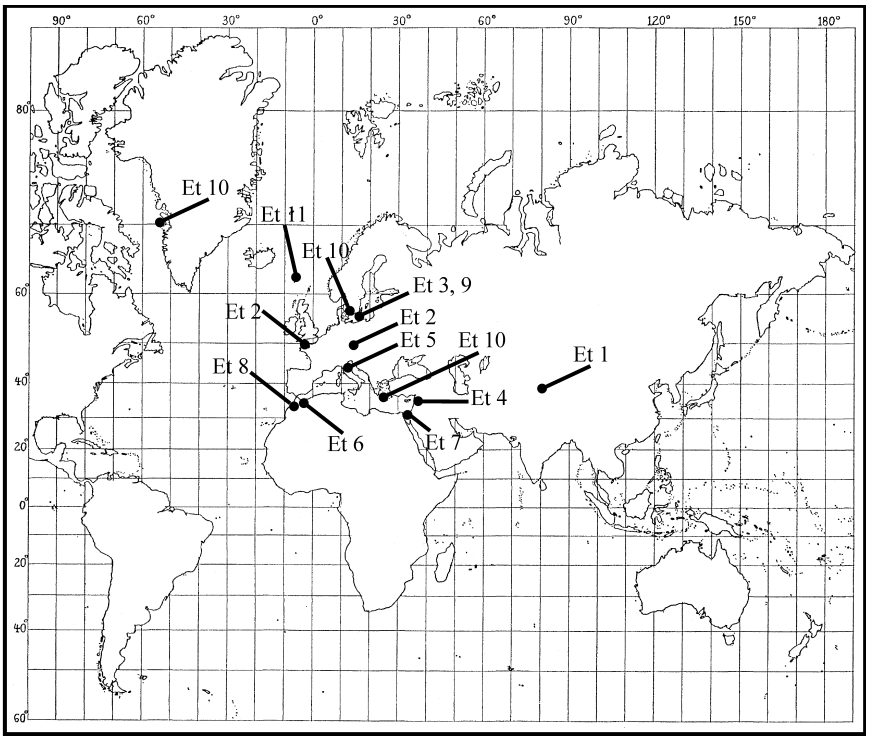

Fig. 1. Sampling localities of *Echinicus testudo*. The sampling localities are indicated on the map with the COI haplotypes.

Tab. 2. Primers used in the present study.

	Primer sequence	References
COI		
LCO1490 (forw.)	5´-GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer *et al*. 1994
HCOout (rev.)	5´-CCA GGT AAA ATT AAA ATA TAA ACT TC	Schwendinger & Giribet 2005
HCOoutout (rev.)	5´-GTA AAT ATA TGR TGD GCT C	Prendini *et al*. 2005
ITS2		
3SN (forw.)	5´-GCG TCG ATG AAG AGC GCA GC	DeJong *et al*. 2001
BD2 (reverse)	5´-TAT GCT TAA ATT CAG CGG GT	DeJong *et al*. 2001
ITS2-RIXO (rev.)	5'-TTC TAT GCT TAA ATT CAG GGG	Almeyda-Artigas *et al*. 2000

cation at 72 ºC for 30 sec, and a final extension step at 72 ºC for 10 min. The PCR amplifications were visualized with UV light using a 2% agarose gel stained with ethidium bromide. Chromatograms obtained from the automated sequencer (ABI310) were edited and consensus sequences created from the complementary strands using the Staden Package (Staden 1996).

2.3. Data analysis

All sequences were aligned prior to phylogenetic analyses with ClustalX (Thompson *et al*. 1997) using default parameters. Absolute genetic distance and nucleotide composition were calculated using MEGA2.1 (Kumar *et al*. 1993). Evolutionary substitution models to be used in the maximum likelihood analyses were inferred using ModelTest 3.7 (Posada & Crandall 1998). For the *E. testudo* dataset ModelTest inferred the HKY (Hasegawa *et al*. 1985) and the F81 (Felsenstein 1981) substitution models for COI and ITS2, respectively.

Four different methods of inferring phylogeny were used, i.e. distance based (neighbor-joining, NJ), maximum parsimony (MP), maximum likelihood (ML) (PAUP*b4.10; Swofford 2001) and Bayesian Inference (BI) (MrBayes3.1; Huelsenbeck & Ronquist 2001). Bootstrap analyses of 1000 replicates were used to investigate branch support in the NJ, MP and ML analyses. The distance based NJ analysis was conducted using the substitution models HKY (COI) and F81 (ITS2). MP inference was performed via a heuristic search using 1000 replicates of random sequence entry, tree-bisection-reconnection (TBR) branch swapping, and assuming equal weight and unordered character states of all characters. Analyses were done with gaps treated as a fifth character for the ITS2 data matrix. The ML analysis was performed with 1000 replicates on each of the data sets using the substitution models HKY (COI) and F81 (ITS2). The BI analysis was conducted with 1 million generations and a burn-in of 100 genera-

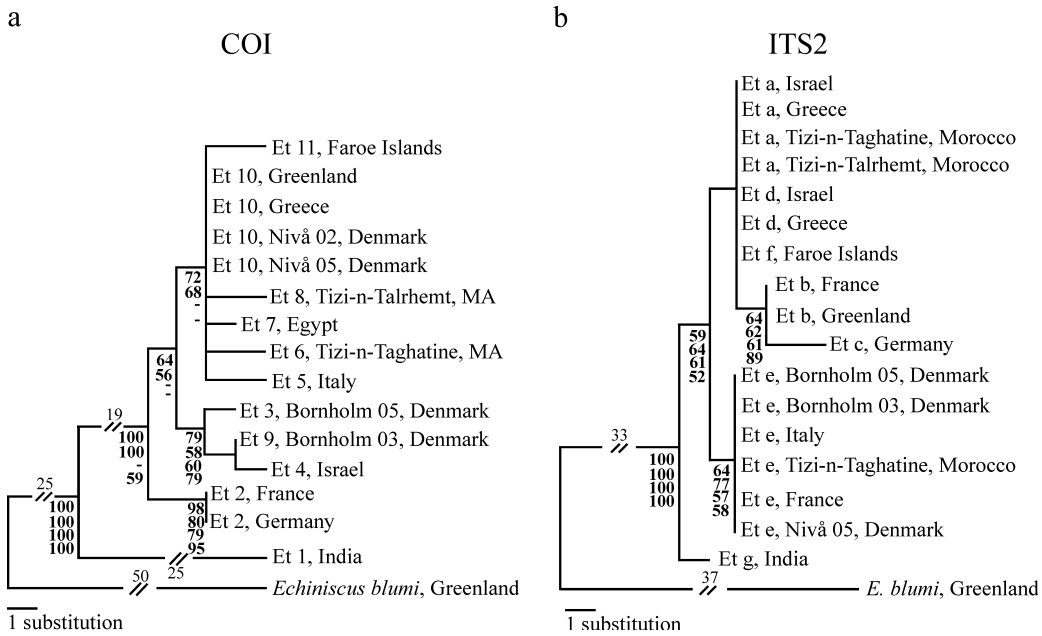

Fig. 2. Neighbor-joining trees for the tardigrade *Echiniscus testudo* from (**a**) cytochrome oxidase subunit I (COI) haplotypes, and (**b**) internal transcribed spacer 2 (ITS2) haplotypes. The bootstrap values of the clades are indicated in the following order NJ, MP, ML and finally posterior probabilities for BI. The number of substitutions (branch length) is stated at the truncated branches.

tions. The analysis was run three times which all resulted in identical topologies within both datasets.

Minimum-spanning network analysis and Mantel tests were conducted in Arlequin 3.01 (Excoffier *et al*. 2005). Mantel tests were used to investigate isolation by distance using matrix correlation analyses of genetic distance (based on COI) and the geographical distance between the sampling sites. Haplotype diversity (h) = $1-\sum p_i^2$, where p*i* is the frequency of the i^{th} haplotype, was calculated for COI and ITS2.

3. RESULTS

3.1. Cytochrome oxidase subunit I

The COI data matrix was 626 bp for *Echiniscus testudo*. The data matrix had 124 variable characters of which 26 were parsimony informative. Of the variable characters 18 and 68 were singletons present in *E.* cf. *testudo* from Ladakh (India) and *E. blumi*, respectively. The average nucleotide composition of COI was A (23.9%), C (14.9%), G (16.2%), T (45.0%) showing an AT bias (AT 68.9%, CG 31.1%). Within *E. testudo* the sequence variation was distributed with four substitutions in the 1st codon position, a single 2nd codon position substitution and 53 substitutions in the 3rd codon position. Two of the 1st codon position substitutions were synonymous and two were non-synonymous changing isoleucine and leucine to valine and phenylalanine, respectively. The single 2nd codon position substitution was non-synonymous changing alanine to valine. All substitutions were point mutations.

The sequence diversity of COI ranged from 0 - 1.28% within *E. testudo* from Greenland, Europe, Middle East and North Africa. The sequence from *E.* cf. *testudo* from Ladakh, India showed a much higher diversity when compared with the other sequences (7.51% - 7.99%). *Echiniscus blumi* showed a sequence diversity of 15.34% - 16.29% when compared to the *E. testudo* sequences.

Eleven haplotypes of COI were recognized within *E. testudo* including *E.* cf. *testudo* Ladakh, India and the haplotype diversity was 0.86. The highest COI sequence diversity within *E. testudo* was present in the specimens from France and Germany sharing haplotype Et 2 (0.64 - 1.28% variation when compared with the other haplotypes). Different haplotypes of COI with a small amount of sequence variation (0.16%) was found between 2003 and 2005 samplings at Bornholm, Denmark. No sequence variation was found between temporal different samplings in 2002 and 2005 at Nivå, Denmark. Identical haplotypes of COI was found in Greenland, Greece and Nivå, Denmark. There is approximately 3400 km between Disko, Greenland and Nivå, Denmark and another approximately 2400 km between Nivå and Crete, Greece. The chromatographs for COI of *E. testudo* indicate single haplotypes during the simultaneous DNA extractions from several specimens.

The phylogenetic inference methods (NJ, MP, ML and BI) inferred four distinct lineages for the COI data set. The NJ analysis inferred the highest resolution of the relationships within and between the clades and is shown in figure 2a. Four main lineages were congruent between different methods of analysis: 1) India, 2) Germany and France, 3) the two haplotypes from Bornholm, Denmark and Israel, and 4) a large clade consisting of the rest of the localities. The bootstrap supports

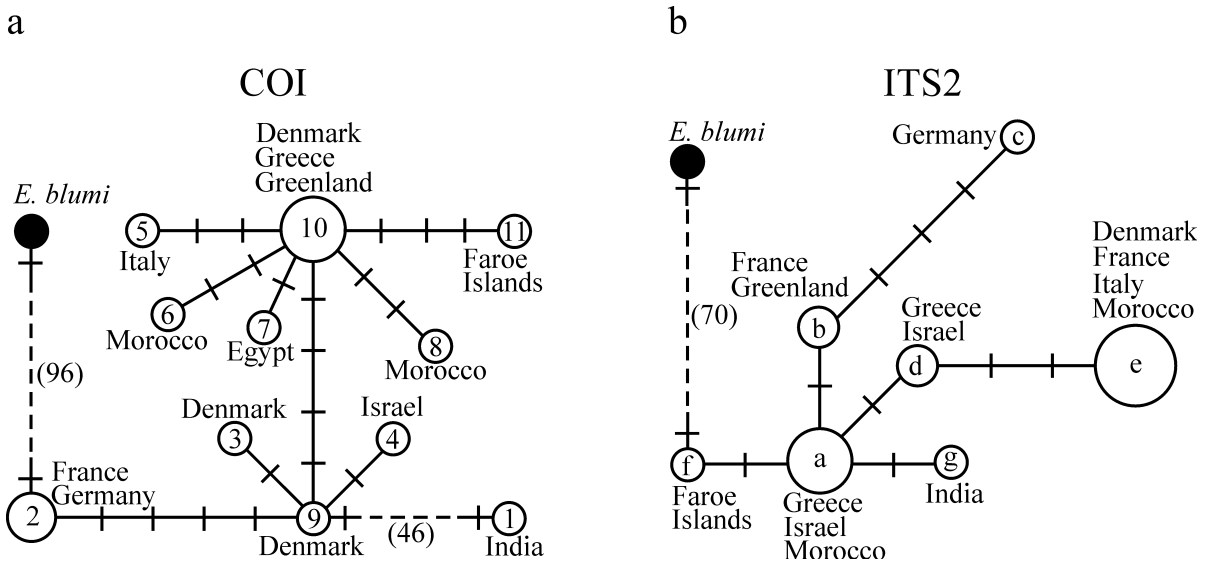

Fig. 3. Minimum-spanning trees for the tardigrade *Echiniscus testudo* from (**a**) COI, and (**b**) ITS2. The haplotypes are indicated by circles with the area being proportional to the number of localities sharing that haplotype. The outgroup *Echiniscus blumi* is emphasized with a filled black circle.

for the clades were generally weak except for the France/Germany clade. No clear phylogeographical patterns are present in the inferences.

The minimum spanning network for the 11 COI haplotypes revealed two possible founder haplotypes (Et 9 and Et 10; Fig. 3a). The MSN analysis showed that the haplotypes Et 3 and Et 9 from the temporal different samplings at Bornholm, Denmark were closely related with Et 9 ancestral to Et 3. The haplotype from India (Et 1) and the outgroup *E. blumi* showed distant relationships with the rest of the haplotypes. The most widespread haplotype Et 10 gave rise to a predominantly Mediterranean radiation of haplotypes Et 5 (Italy), Et 6 and Et 8 (Morocco) and Et 7 (Egypt), but also to an Atlantic haplotype (Et 11, Faroe Islands). No significant evidence of isolation by distance was detected by the Mantel tests in *E. testudo* both including ($p = 0.066$) and excluding ($p = 0.723$) the Indian haplotype of *E.* cf. *testudo*.

3.2. Internal transcribed spacer 2

The ITS2 data matrix consisted of 416 bp for *E. testudo* (426 bp with *E. blumi* as an outgroup). The data matrix had three variable parsimony uninformative characters and four parsimony informative characters. The nucleotide composition in *E. testudo* of ITS2 was A (23.1%), C (20.2%), G (21.8%), T (34.9%); AT (58.0%), CG (42.0%). The substitutions within *E. testudo* were point mutations and a single insertion/deletion substitution. *E. blumi* possessed seven deletions ranging from 1-10 bases in length and five insertions ranging from 1-3 bases in length compared to *E. testudo*. The *E. blumi* sequence was only 402 bp compared to 416 in *E. testudo*.

The sequence diversity of ITS2 ranged from 0-1.20% within *E. testudo* (including the specimens from Ladakh, India). *E. blumi* had a sequence diversity of 17.86-18.88% compared with *E. testudo*.

Seven nuclear genotypes of ITS2 were recognized within *E. testudo* and the nuclear genotype diversity was low-moderate at 0.56. The genetic diversity of ITS2 was 1.20% between the most divergent nuclear genotypes Et c from Germany and Et e from various countries. The most common nuclear genotypes were Et a and Et e with four and six localities, respectively. Apart from a single substitution our *Echiniscus* cf. *testudo* from India (Et g) shared a general nuclear genotype pattern with specimens from Greece, Israel, and Morocco (Et a). The most variable ITS2 sequence belonged to a nuclear genotype (Et c) from *E. testudo* in Germany. Four localities had two nuclear genotypes in the same sample, i.e. France (Et b / Et e), Greece (Et a / Et d), Israel (Et a / Et d) and Tizi-n-Taghatine, Morocco (Et a / Et e).

The topological resolution of the ITS2 tree was poor and the different inference methods were only fully congruent with regard to the clade consisting of nuclear genotype Et e (Bornholm and Nivå (Denmark), France, Italy and Morocco), which was poorly supported by bootstrap analysis (Fig. 2b). No clear phylogeographical patterns are present in the inferences.

The minimum spanning network for the seven ITS2 nuclear genotypes showed an almost linear relationship between the nuclear genotypes (Fig. 3b). Only nuclear genotype Et a gave rise to more than one nuclear genotype lineage. The MSN analysis of ITS2 showed that the nuclear genotype present at the localities with two nuclear genotypes could either be evolved from each other (Et a / Et d) or be more distantly related (Et b / Et e; Et a / Et e).

4. DISCUSSION

Our data reveal the presence of low sequence diversity (COI and ITS2) and low-moderate (ITS2; 0.56) to high (COI; 0.86) nuclear genotype/haplotype diversity. The sequence diversity displayed in the COI gene within the tardigrade *Echiniscus testudo* (0.16-1.28%) is low for a geographically widespread taxon. *Echiniscus* cf. *testudo* (Ladakh, India) showed a much higher diversity (7.51% - 7.99%), which suggest that it is a distinct taxon (cryptic species).

The presence of shared haplotypes of COI in Greenland, Greece and Nivå, Denmark (Et 10) separated by a geographical distance of approximately 5800 km is evidence of long range dispersal in *E. testudo*. Shared COI haplotypes are also found in France and Germany (Et 2), but the remaining seven sample sites have unique haplotypes. Although identical COI haplotypes (Et 10) were present in Greenland, Greece and Nivå, Denmark all three localities had different haplotypes of ITS2 Et b, Et a/Et d, and Et e, respectively. The same was found at France and Germany that shared the COI haplotype (Et 2), but had the ITS2 haplotypes Et b / Et e and Et c, respectively. Morgan-Richards and Trewick (2005) found similar incongruence between COI and ITS haplotypes in parthenogenetic New Zealand stick insects and suggested that it could be evidence of the very rare occurrence of males introducing new nuclear genotypes into the asexual lineages by rarely occurring hybridizations (sexual reproduction).

Echiniscus males have been reported occasionally, indicating the presence of sexual as well as asexual reproduction within the genus (Miller *et al*. 1999). The presence of more than one nuclear genotype of the multi-copy ITS2 could be interpreted as evidence of past sexual reproduction leading to the heterozygotic condition in the diploid or polyploid *E. testudo*. These data could also be explained by the pooling of two or more asexual lineages or simply by individual variability in the multi-copy gene.

The minimum-spanning network for COI indicates a pattern characteristic of two expanding populations or haplotypes (Et 9 and Et 10) (Slatkin & Hudson 1991). The position of the COI haplotype Et 1 (*E.* cf. *testudo* from Ladakh, India) as a descendant of haplotype Et 9 might be an artifact caused by the large genetic diversity, which is not consistent with intraspecific variation or reticulate evolution. Also ITS2 nuclear genotype Et a shows the pattern of an expanding haplotype. The MSN analysis of COI reveals some geographic structure that could not be interpreted from the phylogenetic inference methods.

5. CONCLUSIONS

The present study finds low DNA sequence variation and moderate (ITS2) to high (COI) nuclear genotype/haplotype diversity within geographically widely separated localities. These findings indicate a recently evolved species with high dispersal potential. Different phylogeny inference methods revealed little topological resolution, but minimum spanning networks showed some phylogeographic patterns. The COI and ITS2 minimum spanning networks show patterns that indicate dispersal of several haplotypes from founding populations.

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