Systematics and phylogeography of the genus *Tigriopus* (Copepoda, Harpacticoida, Harpacticidae) in the basin of the Mediterranean Sea

PhD CANDIDATE

LUCA VECCHIONI

PhD COORDINATOR

PROF.SSA MARIA ANTONIETTA GERMANA'

SUPERVISOR

PROF. MARCO ARCULEO

CO-SUPERVISOR

PROF. MIGUEL RODILLA

PROF. EDUARDO J. BELDA

CICLO XXXII

2020

ABSTRACT

The copepod genus *Tigriopus* Norman, 1869 is distributed worldwide in coastal rock-pools and it is currently considered to include 15 valid species. *Tigriopus fulvus* (Fischer, 1860), with its subspecies *Tigriopus fulvus adriaticus* Van Douwe, 1913 and *Tigriopus fulvus algiricus* Monard, 1935, are currently reported to occur in the Mediterranean area, but the actual diversity of the genus is currently unknown. We aimed to assess the actual identity of Mediterranean *Tigriopus* populations and to elucidate their taxonomy and pattern of genetic diversity. In order to reach these goals, we use two different approaches. The first, based on morphology, where the possible morphological differences among topotypical samples of *Tigriopus fulvus* s.s. and topotypical samples of the two subspecies were investigated. A second, on a molecular basis, where fragments of two mitochondrial DNA genes (cytochrome c oxidase subunit I, COI and small ribosomal RNA subunit, 12S) and a nuclear DNA gene (28S) were sequenced to be used as a reference marker. In this frame, molecular taxonomical approaches, such as Automatic Barcode Gap Discovery (ABGD), bPTP (bayesian Poisson Tree Processes) and K/Θ ratio, were used in order to investigate the existences of the alleged subspecies of *Tigriopus* through the identification of Operational Taxonomic Units (OTUs). Our data suggest the presence of a single species characterized by a noteworthy geographically-based genetic structure in the whole study area. The observed diversity pattern is tentatively ascribed here to a strong monopolization of the rock pools by the first immigrants that reach them. However, such a monopolization is periodically disrupted by local extinction events, which are frequent in the intrinsically unstable rock pool habitats. We propose the name "clockwork monopolization" for this pattern.

KEY WORDS: genetic structuring, clockwork monopolization, rocky shore communities, cryptic species, DNA taxonomy.

RESUMEN

El género de copépodos *Tigriopus* Norman, 1869 se distribuye en todo el mundo en piscinas costeras de rocas y actualmente se considera que incluye 15 especies válidas. *Tigriopus fulvus* (Fischer, 1860), con su subespecie *Tigriopus fulvus adriaticus* Van Douwe 1913 y *Tigriopus fulvus algiricus* Monard 1935, actualmente se informa que ocurren en el área mediterránea, pero actualmente se desconoce la diversidad real del género. Nuestro objetivo fue evaluar la identidad real de las poblaciones mediterráneas de *Tigriopus* y dilucidar su taxonomía y patrón de diversidad genética. Para alcanzar estos objetivos, utilizamos dos enfoques diferentes. El primero, basado en la morfología, donde las posibles diferencias morfológicas entre muestras topotípicas de *Tigriopus fulvus* s.s. y muestras topotípicas de las dos subespecies fueron investigadas. Un segundo, sobre una base molecular, donde se secuenciaron fragmentos de dos genes de ADN mitocondrial (subunidad I de citocromo c oxidasa, COI y subunidad de ARN ribosómico pequeño, 12S) y un gen de ADN nuclear (28S) para ser usados como marcador de referencia. En este marco, se utilizaron enfoques taxonómicos moleculares, como el descubrimiento automático de brechas de código de barras (ABGD), bPTP (procesos de árbol de Poisson bayesianos) y la relación K / Θ, para investigar la existencia de las supuestas subespecies de *Tigriopus* a través de la identificación de Operacional Unidades Taxonómicas (OTU).

Nuestros datos sugieren la presencia de una sola especie caracterizada por una notable estructura genética basada en la geografía en toda el área de estudio. El patrón de diversidad observado se atribuye tentativamente aquí a una fuerte monopolización de los estanques de rocas por parte de los primeros inmigrantes que los alcanzan. Sin embargo, tal monopolización se ve interrumpida periódicamente por los eventos de extinción locales, que son frecuentes en los hábitats intrínsecamente inestables de las piscinas de rocas Aquí proponemos para este patrón el nombre de "monopolización periódica" ("Clockwork monopolization").

RESUM

El género de copépodos *Tigriopus* Norman, 1869 se distribuix en tot el mon en piscines costeres de roques i actualment se considera que inclou 15 especies valides. *Tigriopus fulvus* (Fischer, 1860), en la seua subespecie *Tigriopus fulvus adriaticus* Van Douwe 1913 i *Tigriopus fulvus algiricus* Monard 1935, actualment s'informa que ocorren en l'area mediterranea, pero actualment se desconeix la diversitat real del género. Nostre objectiu fon evaluar l'identitat real de les poblacions mediterranees de *Tigriopus* i dilucidar la seua taxonomia i patrón de diversitat genetica. Per a alcançar estos objectius, utilisem dos enfocaments diferents. El primer, basat en la morfologia, a on les possibles diferencies morfologiques entre mostres topotípicas de *Tigriopus fulvus* s.s. i mostres topotípicas dels dos subespecies foren investigades. Un segon, sobre una base molecular, a on se secuenciaron fragments de dos gens d'adn mitocondrial (subunidad i de citocromo c oxidasa, coi i subunidad d'arn ribosómico menut, 12s) i un gen d'adn nuclear (28s) per a ser amprats com marcador de referencia. En este marc, s'utilisaren enfocaments taxonomics moleculars, com el descobriment automatic de breches de codic de barres (ABGD), bPTP (processos d'arbre de poisson bayesianos) i la relacio K/θ, per a investigar l'existencia de les supostes subespecies de *Tigriopus* a través de l'identificacio d'operacional unitats taxonomiques (OTU).

Nostres senyes sugerixen la presencia d'una sola especie caracterisada per una notable estructura genetica basada en la geografia en tota l'area d'estudi. El patrón de diversitat observat s'atribuix tentativament aci a una forta monopolisacio dels safarejos de roques per part dels primers immigrants que els alcancen. No obstant, tal monopolisacio se veu interrompuda periodicament pels events d'extincio locals, que son freqüents en els hábitats intrinsecament inestables de les piscines de roques aci proponem per a este patrón el nom de "Monopolisacio Periodica" ("Clockwork Monopolization").

1. INTRODUCTION

1.1 Diversity and distribution of the genus *Tigriopus*

Tigriopus Norman, 1869 is a genus of harpacticoid copepods widespread throughout the world (Figure 1). The genus currently includes 15 valid species (Walter & Boxshall, 2019). *T. californicus* (Baker, 1912), distributed along the Pacific coasts of North America; *T. incertus* (Smirnov, 1932), from the Land of Francis Joseph, an archipelago located in the Barents Sea, and in the Aleutian Islands, in the Pacific Ocean; *T. angulatus* Lang, 1933, and *T. raki* Bradford, 1967, from New Zealand; *T. japonicus* Mori, 1938, from Japan; *T. kerguelenensis* Soyer, Thiriot-Quievreux & Colomines, 1987, from the Kerguelen Islands in the southern Indian Ocean; *T. igai* Itô, 1977, and *T. kingsejongensis* Park, S. Lee, Cho, Yoon, Y. Lee & W. Lee, 2014, in Antarctica; *T. crozettensis* Soyer, Thiriot-Quievreux & Colomines, 1987, from the Crozet Islands, in the southern Indian Ocean; *T. thailandensis* Chullasorn, Ivanenko, Dahms, Kangtia & Yang, 2012, and *T. sirindhornae* Chullasorn, Dahms & Klangsin, 2013 from Thailand.

In the Atlantic-Mediterranean area, there are four species of *Tigriopus* to date known to occur: *T. brevicornis* (Müller, 1776), spread from Northern Europe and Iceland to the Atlantic coasts of Spain (personal observation of the Author) and Nova Scotia (Canada) (Handschumacher et al., 2010); *T. fulvus* (Fischer, 1860), in the Mediterranean Sea, partly in the African Atlantic coast and in Madeira, where it was described for the first time; *T. minutus* Božić, 1960, in Senegal, and *T. brachydactylus* Candeias, 1959, that occurs in Africa (Angola) (Figure 2).
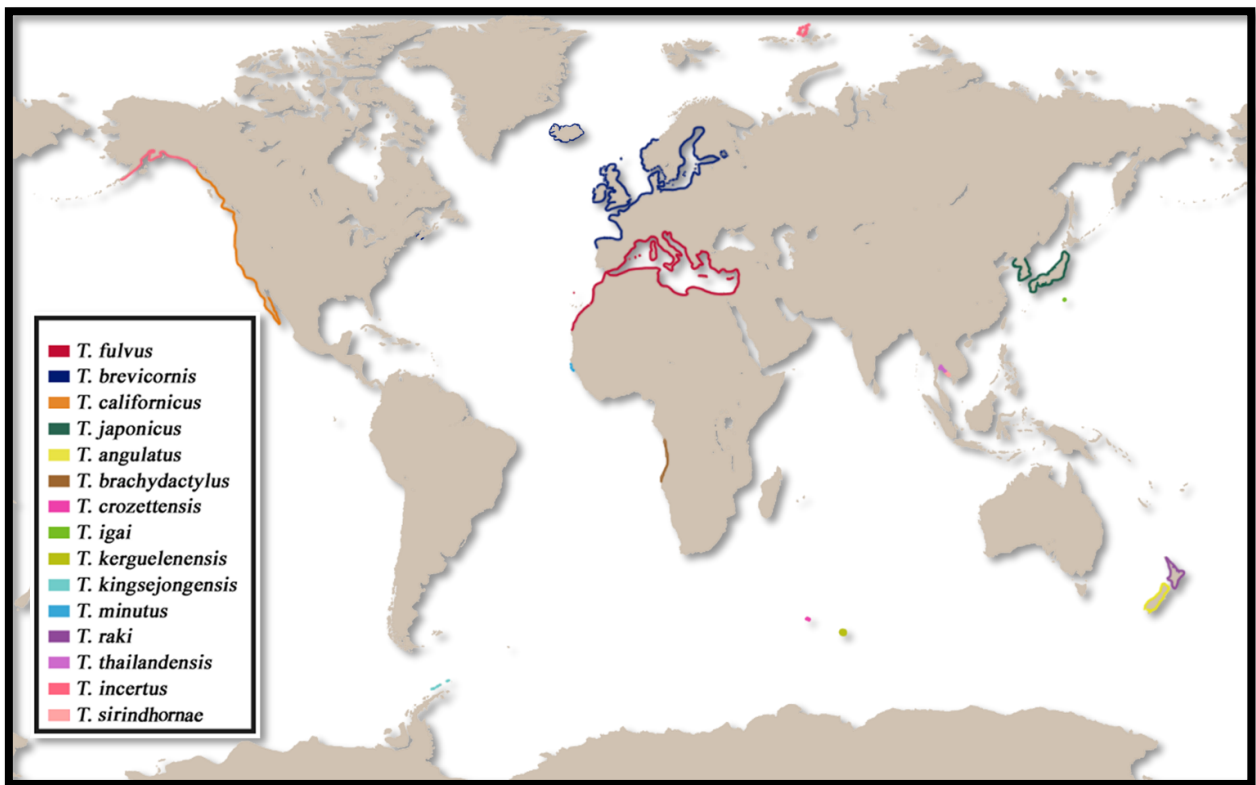
Figure 1. Worldwide map of the known distribution of the genus *Tigriopus*.
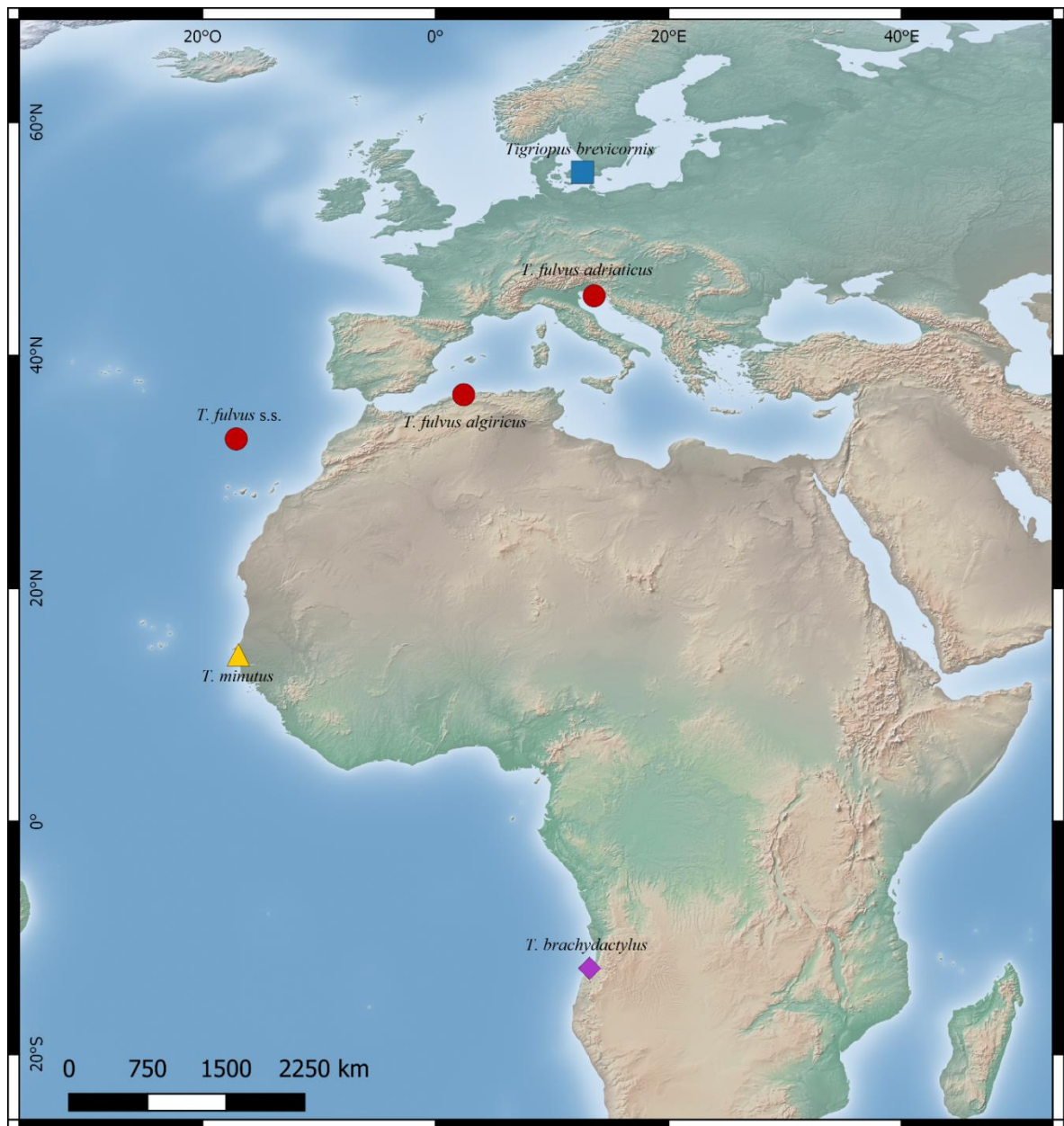
Figure 2. *Loci typici* of *Tigriopus* spp. in the Atlantic-Mediterranean area.

1.2 The environment of the rock-pools

Rock-pools are environments mainly populated by diatoms, flagellates, and some animal taxa, such as rotifers, crustaceans (e.g. *Tigriopus* spp.), molluscs and insects (e.g. hydraenid beetles and culicid flies) (Issel, 1994; Antonini et al., 2009; Mastrantonio et al., 2015).

The rock-pools environment is very dynamic and subject to extreme chemical and physical variations (McAllen, 1999). During summer, the evaporation of water in the pools causes a considerable increase in salinity and, eventually, the rock-pools can dry up

completely. Conversely, during the winter the abundant and frequent precipitations reduce the salinity of the water in the rock-pools and the intense storm surges can cause their washout. Furthermore, low temperatures can freeze almost entirely the rock-pools. In addition, during the year, and even during a single day, there are considerable variations in temperature, salinity, amount of water, pH and oxygen content (Powlik, 1999). These phenomena can cause high mortality or even extinction of the animal communities of a given rock-pool.

Tigriopus is a copepod genus typically related to the rock-pools (Figure 3) occurring in the supratidal and the uppermost intertidal zones (Powlik, 1999; McAllen, 1999), although it is also known to occur subtidally in the Antarctic Peninsula (Waller et al., 2006; Park et al., 2014), Mexico (Ganz & Burton, 1995; Edmands, 2001), Southern Asia (Jung et al., 2006; Ki et al., 2009), and Sweden (Lang, 1948).

This harpacticoid genus is present with active stages throughout the year, with some exception due to extremely cold temperatures (Issel, 1994). In addition, *Tigriopus* has a generation time (from egg to adult) of 30 days *circa* and its life span could be up to five weeks (Powlik, 1997, 1998). During the unfavourable phases, the pools do not remain uninhabited: the *in situ* survival of some specimens is sufficient to colonize it again (McAllen, 1999). In fact, *Tigriopus* females can steadily produce a large number of nauplii. For example, *T. californicus* can produce up to 12-14 broods, each containing up to about 150 eggs (Brown, 1991). As thermal tolerance is concerned, Issel (1914) studied *T. fulvus* in rock-pools near Genova (Italy) over a period of two years, recording a maximum water temperature of 35.7° C and a minimum water temperature of 3.8° C in which he found alive specimens of *T. fulvus*. However, assuming temperature values close to or equal to zero during the night, he extrapolated the existence of a temperature excursion greater than 35° C. Analogous values were observed from *T. brevicornis* (Damgaard & Davenport, 1994).

Figure 3. Typical rock-pool environments.

1.3 Studies on the resistance of the genus *Tigriopus*

Organisms that live in the rock-pools have developed mechanisms that allow them to escape or face adverse conditions. Organisms capable of flying, like some beetles, can actively move between pools during periods of drought; instead, other taxa develop stages of resistance to overcome, *in situ*, the adverse periods (Williams, 2006). The adult copepods of the genus *Tigriopus* are able to enter in a state of quiescence and the environmental conditions in which the phenomenon occurs have been the subject of several studies (e.g. Issel, 1914; Ranade, 1957; Vittor, 1971; Powlik & Lewis, 1996). Issel (1914) reported that *T. fulvus* can survive in a state of quiescence for up to 22 days, at temperatures between 22° C and 31° C. The phenomenon of quiescence is induced in *Tigriopus* by the increase in the concentration of salts dissolved in water and by the presence of hypoxic conditions. The transition to the state of quiescence takes place gradually, with an initial loss of vivacity which ends with a state of complete immobility. As the water density decreases, organisms can resume their normal activity. Issel (1914) noted that the best conditions for awakening are at densities slightly

higher than those of sea water, with values of around 1,050 g/cm^3. However, the longer the time spent in the state of quiescence, the longer the time required for awakening, an observation confirmed also by Ranade (1957) in his studies on the similar species *T. brevicornis*. Furthermore, the number of awakening individuals may decrease if the period of inactivity is prolonged, showing the presence of a growing mortality rate depending on the duration of the quiescence period. The increase in temperature has the effect of reducing the period of immobility, thus accelerating awakening. These harpacticoids show a high resistance to high temperatures. In fact, they are more active during the hot season. Issel (1914) observed that some specimens manage to tolerate temperatures of 39.4° C surviving them, analogous to what reported for *T. brevicornis* (Damgaard & Davenport, 1994). However, the lethal temperatures depend directly on the salinity and can vary between 32° C and 41.8° C (Ranade, 1957). The concentration of salts dissolved in water, therefore, is an important factor, since it regulates both the maximum temperatures that *Tigriopus* specimens can tolerate, and the onset and duration of the state of quiescence. Ranade (1957) reported that *T. brevicornis* can normally live at salinities between 4.2 and 90 ‰, a limit beyond which the state of quiescence occurs. Moreover, Issel (1914) exposed the organisms to sudden changes of salinity, which resulted in accelerating the appearance of the quiescence, with effects that are all the more accentuated the greater is the difference in density to which they are subjected. Conversely, the abrupt passage from freshwater to seawater is not well tolerated, with a high mortality rate (Issel, 1994). In nature, however, these abrupt changes in salinity generally do not occur, even in the presence of intense climatic events, because the water of the rock-pools stratifies with a density gradient, with a higher concentration of NaCl in the deeper layers than the superficial ones that are diluted by precipitation. Issel's experiments also showed that adult specimens are more resistant than nauplii to salinity fluctuations.

In addition to the quiescence stage as a resistance mechanism, *Tigriopus* can also adopt different behavioural adaptations that allows to avoid desiccation. Both the nauplii and the

adults, who are able to dig, can find a humid refuge in the sediment at the bottom of the pools (McAllen, 1999). McAllen reported that he found two adult specimens of *T. brevicornis* inside an exuvia of the amphipod *Apohyale prevostii* (indicated in the article as *Hyale prevostii*), within which a certain degree of humidity had been maintained even when the rockpool was dry. But even more significant is the finding of several hundred specimens, both adult and immature, within the cavity of a single thallus of *Ulva intestinalis* (referred to as *Enteromorpha intestinalis*) (McAllen, 1999), a green alga sometimes presents in the rock-pools together with *Tigriopus* (Davenport, 1997; Handschumacher et al., 2010). The internal cavity of the alga could therefore provide a moist and hydrated environment for several weeks. From the study by Powlik & Lewis (1996) it appears that the presence of *Ulva* thalli increases the resistance of *T. californicus* to desiccation.

1.4 Main dispersal mechanisms

To date, the main dispersal mechanism of this genus is unknown, but several hypotheses have been proposed. Davenport et al. (1997) and Handschumacher et al. (2010) suggested that groups of floating algae, such as uprooted *Ulva* thalli, could act as means of transport for hypothetical colonies of copepods present within them. However, Powlik (1999) stated that the presence of *Tigriopus* appears to be independent of the presence of algae or marine plants in the rock-pools, which are generally poor in macroflora. Also, the sea currents and the tides could constitute valid dispersing vectors through hydrocoria. Furthermore, rock pools are occasionally frequented by birds, that can disperse organisms for long distances, transporting them on the plumage or on the legs dirty with sediment residues (Incagnone et al., 2015). Although no cases of *Tigriopus* transport have yet been documented through avifauna, other microcrustaceans have been routinely found in bird plumage (Swanson, 1984; Powlik, 1999; Incagnone et al., 2015). Anemochory is another dispersal mode, as wind can transport organisms from one rock-pool to another in the period in which they are dry (Powlik, 1999; Incagnone et al., 2015). Finally, humans could also be a vector of dispersion,

considering the widespread use of species of the *Tigriopus* genus in aquaculture as live food for fish and invertebrates, although to date no ascertained cases of human-mediated introductions of the genus *Tigriopus* are known (Handschumacher et al., 2010). In addition, it is important to remember that after dispersal, successful establishment is needed in order to determining the colonization of a viable population in the newly colonized rock-pool.

1.5 The genus *Tigriopus* in the Atlantic-Mediterranean area

Lazzaretto & Libertini (1986), comparing the morphology of chromosomes of some *T. fulvus* and *T. brevicornis* populations, suggested that the Trapani *Tigriopus* population investigated by them could represent a different species, i.e. *Tigriopus minutus*. However, no further evidences were found to corroborate this hypothesis.

Over the years, the systematic of the genus has undergone some changes, mostly concerning the species *T. brevicornis* and *T. fulvus*. In 1776 Müller, based on specimens collected along the Danish coasts, described *Cyclops brevicornis*. In 1860 Fisher described a harpacticoid, native of Madeira, which he named *Harpacticus fulvus*. A few years later, Norman (1869) described the English copepod *Tigriopus lilljeborgii*, which was later regarded as a junior synonym of *H. fulvus* by Brady (1872) and by Sars (1911). The latter, moreover, in his study on Norwegian harpacticoids, accepted the genus *Tigriopus* recognizing *Tigriopus fulvus* as its only valid species. Using the description of Sars (1911) as a comparison, in 1913 Van Douwe described an Adriatic "variety" of *Tigriopus fulvus* (*T. fulvus* var. *adriatica*), based on animals collected in Rovinj, Croatia. Monard (1935), based on the same description of Sars, described a second "variety", *T. fulvus* var. *algirica*, from the town of Tipaza (currently Tipasa), in Algeria. The reader should here note that the description made by Sars under *Tigriopus fulvus* in fact referred to *Tigriopus brevicornis* so that the two alleged varieties of *T. fulvus* were in fact established through a comparison with a different species, i.e. *Tigriopus brevicornis* (Božić, 1960). Considering the species described by Müller (1776) and Fischer (1860) as synonyms and following the principle of priority of the ICZN, Lang (1948) established as

valid the binomen *Tigriopus brevicornis* (Müller, 1776), placing *T. fulvus* in synonymy and maintaining however the varieties described by Van Douwe (1913) and Monard (1935) as varieties of *T. brevicornis*. Božić (1960), studying the morphology of the southern and northern European *Tigriopus* populations and performing hybridization experiments, established belonged to two different species: *T. brevicornis* (Müller, 1776) and *T. fulvus* (Fischer, 1860), corresponding, respectively, the former to the northern populations and the latter to the southern ones. Carli & Fiori (1977) investigated the morphological differences of the two species, analysing further micro-characters that allowed a unique identification of the two taxa.

According to the Article 45.6.4 of the International Code of Zoological Nomenclature (ICZN.org), if an infrasubspecific taxon was established before 1961, it is currently to be considered as a subspecies; accordingly, the two varieties described within *Tigriopus fulvus sensu* Sars are nowadays accepted as subspecies. *T. fulvus* is therefore considered a polytypic species and includes the subspecies: *T. fulvus fulvus* (Fischer, 1860), from Madeira; *T. fulvus adriaticus* Van Douwe, 1913, from Rovinj, and *T. fulvus algiricus* Monard, 1935, from Tipasa. In the Atlantic-Mediterranean area are thus currently reported four *Tigriopus fulvus*, *T. brevicornis*, *T. minutus* and *T. brachydactylous* (Figure 2).

1.6 Scope of the research

Currently, despite the frequent use of *T. fulvus* and other species of the genus in ecological and physiological studies, the actual diversity of the genus *Tigriopus* in the Mediterranean area is almost unknown and needs to be further investigated. The purpose of this study was to explore the morphological and genetic diversity of *T. fulvus* and its alleged three subspecies, verifying their validity based on morphological and genetic data.

2. MATERIALS AND METHODS

2.1 Sampling and identification

Specimens of *Tigriopus* spp. were collected from 2016 to 2019 in supratidal and intertidal rock-pools from 32 different locations along the coasts of the Mediterranean Sea and the eastern Atlantic Ocean (Table 1 and Figure 4). In addition, some specimens of *T. brevicornis* from Sanxenxo (Spain), and Trondheim (Norway) were collected, and a single sequence of *T. californicus*, of unknown origin, was obtained from a commercial strain of the species kindly provided by Daniel Abed-Navandi (University of Vienna, Austria). The two latter species were used as outgroups in phylogenetic analyses. The sampling sites were geolocated by GPS. The map of the sampling sites was created using the QGIS software v.2.18.2 (http://www.qgis.org).
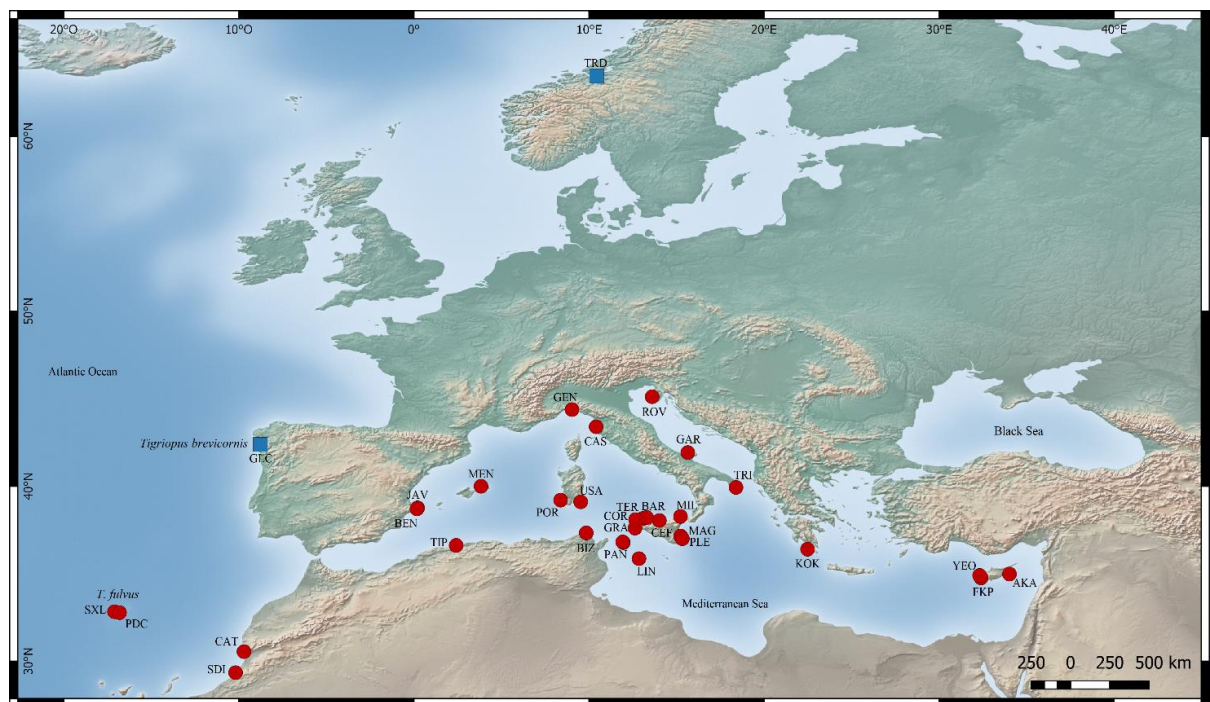
Figure 2. Geographic location of the sampling sites. Circles indicate sites where *Tigriopus fulvus* was sampled and square indicate sampling site for *T. brevicornis*. See Table 1 for the coordinates of the sampling sites and for more information on the collected species. Codes refer to those listed in Table 1.

Harpacticoids were sampled with a 200 μm mesh-sized hand net and a sieve with the same mesh size (Figure 5). Collected specimens were fixed *in situ* in 96% ethanol, sorted out in the laboratory under a stereomicroscope. For the identification of the harpacticoids the dichotomous keys of Wells (2007) and original descriptions of the species (Fischer, 1860;

Müller, 1776; Božić, 1960; Carli & Fiori, 1977), and subspecies (Van Douwe, 1913; Monard, 1935) were used.
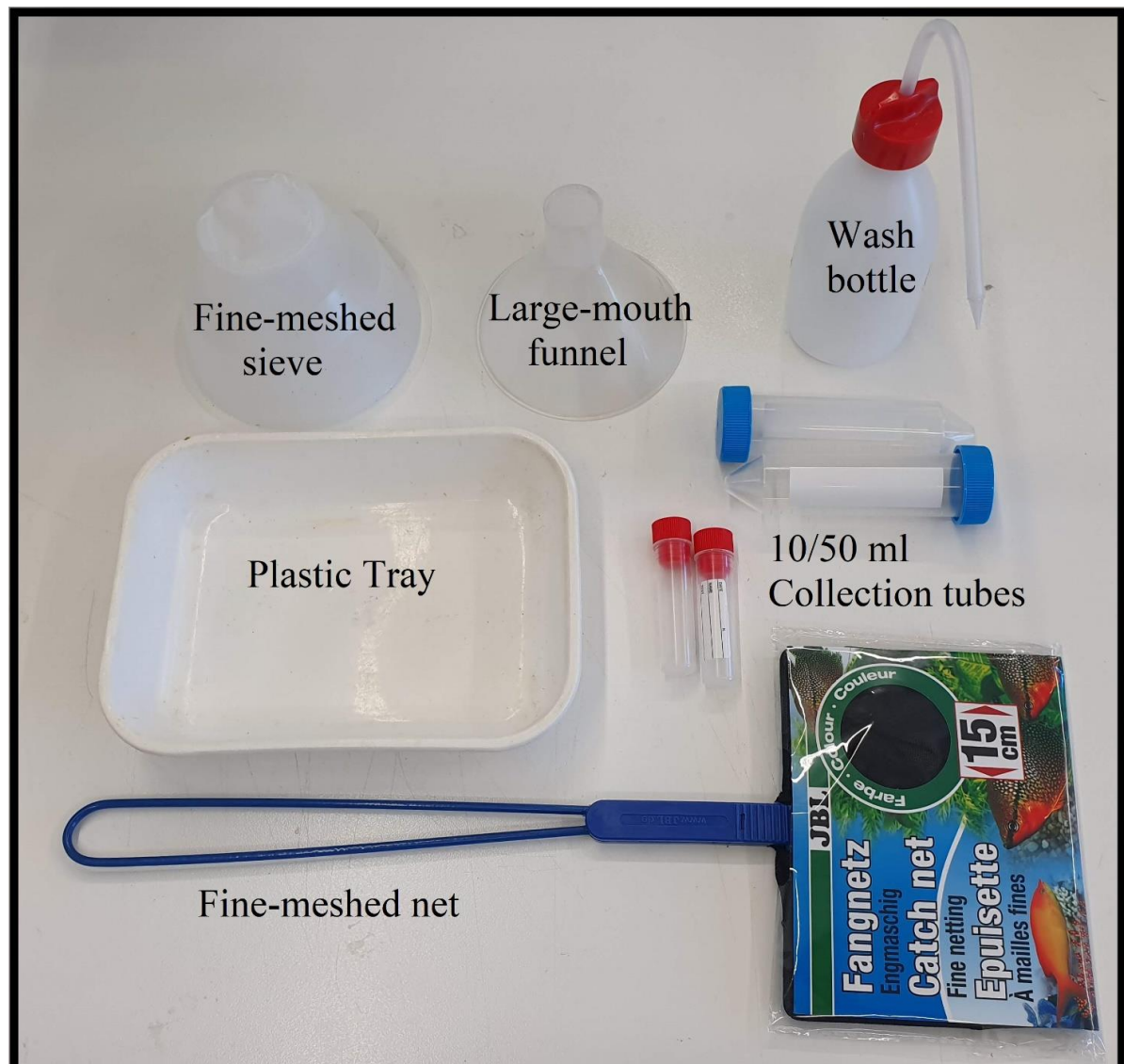
Figure 3. Basic tools used during the sampling activities.

2.2 DNA extraction, amplification and molecular analyses

1-3 Specimens were selected from each sampled population were carefully cleaned of any impurities and soaked in distilled water for 10 minutes. DNA extraction was performed using the BIORON GmbH "Ron's Tissue DNA Mini Kit" following the protocol provided by the manufacturer. The extracted DNA was amplified by polymerase chain reaction (PCR). Fragments of two mitochondrial markers were chosen: the COI (Cytochrome C Oxidase subunit 1) and the 12S srRNA (small ribosomal unit). In addition, a fragment of the nuclear gene 28S was chosen for amplification by PCR. The primer pair "L1384-COI" (5'-GGT CAT

GTA ATC ATA AAG ATA TTG G-3') and "H2612-COI" (5'-AGG CCT AGG AAA TGT ATM GGG AAA-3') (Machida et al., 2004) was used to amplify the COI gene, whereas the primers "L13337-12S" (5'-YCT ACT WTG YTA CGA CTT ATC TC-3') and "H13845-12S" (5'-GTG CCA GCA GCT GCG GTT A-3') (Machida et al., 2002) were used to amplify the small ribosomal RNA 12S; The primer set "28S-F1a" (5'-GCG GAG GAA AAG AAA CTA AC-3') and "28S-R1a" (5'-GCA TAG TTT CAC CAT CTT TCG GG-3') (Ortman, 2008) was used to amplify the nuclear gene 28S .

For the mitochondrial gene COI, the PCR mix included: 18.05 µl of distilled water, 2.5µl of 10X Buffer including 15 mM of MgCl2, 0.25 µl of dNTPs (10 mM each), 0.9 µl of each of the primers (10 µM), 0.4 µl of Taq polymerase (5 U / µl) and 2 µl of DNA template, for a total volume of 25 µl. A thermal cycle was applied consisting of 35 denaturation cycles (95 ° C for 50 s), annealing (48 ° C for 50 s) and extension (72 ° C for 50 s), followed by a cycle of final extension of 7 min at 72 ° C.

The PCR of the mitochondrial gene 12S was carried out with 30 cycles of a 25 µl reaction volume containing 18 µl of distilled water, 3 µl of 10X Buffer including 15 mM of MgCl2, 0.5 µl of dNTPs (10 mM each), 0.5 µl of each of the primers (10 µM), 0.5 µl of Taq polymerase (5 U / µl) and 2 µl of DNA template, for a total volume of 25 µl. The thermal cycle consisted of an initial denaturation phase at 95 ° C, with a duration of 5 min, followed by a denaturation 96 ° C for 15 s, annealing at 45 ° C for 30 s, and extension at 72 ° C for 15 s, plus a final extension cycle of 5 min to 72 ° C.

The composition of the PCR mix for the 28S nuclear gene included: 18.75 µl of distilled water, 2.5 µl of Buffer 10X (including 15 mM of Mg), 0.3 µl of dNTPs (10 mM each), 0.3 µl of each primer (10 µM), 0.35 µl of Taq polymerase (5 U / µl) and 2.5 µl of DNA template, for a total volume of 25 µl. The thermal cycle consisted of an initial denaturation phase at 95 ° C, with a duration of 5 min. This is followed by 35 denaturation cycles (95 ° C, 1 min), annealing (48 ° C, 1 min) and extension (72 ° C, 1 min), plus a final extension cycle

of 8 min to 72 ° C. Subsequently, 5 μl of each PCR product were used to perform electrophoresis on 2% agarose gel, with a voltage of 90 V, for 20 min.

The outcome of the electrophoresis was verified using a UV transilluminator. The samples that showed a single net band, with the expected length for each marker used, were purified using the Exo-SAP-IT® kit (Affymetrix USB). Sequencing was operated by Macrogen Inc. (Seoul, South Korea) via an ABI 3130xL sequencer (Applied Biosystems). The same primers used previously for the PCR were used for the direct sequencing of the PCR products. The quality of the resulting chromatograms was verified by measuring their "Phred score" value (Richterich, 1998). Among these, only the sequences that showed continuous readings of high-quality bases (QV> 20) were kept.

The software Chromas v. 2.6.2 (Technelysium, Pty. Ltd. 1998, Queensland, Australia) was used for chromatogram analysis. Overall, 113 mitochondrial sequences of *Tigriopus fulvus*, eight of *T. brevicornis* and one of *T. californicus* were obtained. In addition, 34 nuclear sequences of *T. fulvus*, four of *T. brevicornis*, and one of *T. californicus* were obtained. All sequences were aligned using the software MEGAX (Kumar et al., 2018). The mtDNA COI sequences were deposited in GenBank (see Table 1 for the Accession Numbers, A.N.). Also, two *T. fulvus* sequences available on GenBank were included in the COI dataset (see Table 1 for the Accession Numbers).

In order to check for the possible presence of frameshift or stop codons, which would indicate the presence of sequencing errors or amplification of pseudogenes, a widespread phenomenon among crustaceans (e.g., Song et al., 2008, Schizas, 2012), the mitochondrial COI sequences were translated into amino acid sequences using the MEGAX software (Kumar et al., 2018), with which the pairwise uncorrected *"p"* distance values were also calculated. For all the sequences, the software MrBayes v. 3.2.6 (Ronquist et al., 2012) and PhyMl v. 3 (Guindon & Gascuel, 2003) were used for molecular identification and reconstruction of the phylogenetic relationships between taxa, through Bayesian Inference (BI) and Maximum

Likelihood (ML) analyses. As support measures for the nodes, bootstrap values were calculated (Felsenstein, 1985) with 1000 replicates in the ML trees, while in the BI tree the posterior probability values were reported. PartitionFinder v. 1.0.1 (Lanfear et al., 2012) was used to choose the best evolutionary model following the "Akaike Information Criterion" (AIC; Akaike, 1974).

For both mitochondrial and nuclear fragments, in the BI and ML analyses, a General Time-Reversible model of sequence evolution was used with a proportion of invariable sites and gamma-distributed rate variation among sites (GTR + I+Γ; nst = 6). In the BI analyses, two independent Markov Chain Monte Carlo analyses were performed with 1 million of generations (temp.: 0.2; default priors). Trees and parameter values were sampled every 100 generations, with the result of 10,000 trees for each analysis. The convergence in the analysis was reached (Effective Sample Size (ESS) greater than 200 in all the analyses performed). The initial 25% of trees were discarded as "burn-in".

The "evolutionary genetic species concept" proposed by Birky et al. (2010) was followed. According to this concept, species are inclusive populations that are evolving independently of each other, either because they are reproductively isolated, or because they are separated by environmental or physical barriers, or both. Those lineages that evolve separately from others were thus considered different taxa of putative species rank. Therefore, we followed different taxonomic approaches to DNA based on different assumptions: a quantitative approach based on a coalescence model ("ABGD"; Puillandre et al., 2012); a phylogenetic criterion based on branching rates ("bPTP"; Zhang et al., 2013) and a population genetic criterion based on genetic isolation ("K/Θ ratio"; Birky et al., 2010; Birky, 2013). The three aforementioned taxonomic approaches were used for mitochondrial sequences only. ABGD and bPTP were performed online (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html and http://species.h-its.org/ptp/). Following Korn and Hundsdoerfer (2016), the K/Θ ratio was computed based on the

uncorrected p-distance matrices within and among the detected clades. This method tests if the reciprocal monophyly of sister lineages is statistically significant, which would suggest that they are independently evolving entities, hence *bonae species sensu* Birky et al. (2010). These taxonomic approaches make it possible to identify Operational Taxonomic Units (OTUs). However, it must be taken into account that these approaches can be influenced by the number of individuals considered in the analysis and by their geographical distribution over- or underestimating the actual number of taxa that occur in the studied dataset (Puillandre et al., 2012; Zhang et al., 2013). Despite this, we decided to use these methods in order to obtain a tentative picture of the distribution of the molecular diversity of the species.

Finally, for mitochondrial and nuclear sequences, haplotype networks were created with PopArt v.1.7 (http://popart.otago.ac.nz) with the "Minimum Spanning Network" method (Kruskal, 1956).

2.3 Phylogenetic reconstruction based on morphological characters

In order to reconstruct the phylogenetic relationships, on a morphological basis, of the species belonging to the genus *Tigriopus*, a table was created (Table 2) showing a series of morphological characters that characterize the different species. For the species for which samples were available (*T. fulvus* and *T. brevicornis*) the status of the characters was determined on the basis of direct observation of the specimens. For the other species, instead, having no specimens to analyse directly, the status of the characters was determined by relying exclusively on the original descriptions and drawings in the literature (Baker, 1912; Lang, 1933; Wilson, 1950; Božić, 1960; Hawkins, 1962; Bradford, 1967; Itô, 1969; Itô, 1970; Itô, 1977; Soyer et al., 1987; Wells, 2007; Chullarsorn et al., 2012; Chullasorn et al., 2013; Park et al., 2014). The Harpacticidae *Zaus wonchoelleei* Kangtia, Dahms, Song, Myoung, Park & Khim, 2014 was included in the analysis as an outgroup. The morphological characters examined have two or more states. The PAUP software (Swofford, 1993) was used for the analysis of the character matrix (Table 3) through a distance analysis (Neighbor-Joining, NJ).

Table 1. Origin and GenBank accession numbers (A.N.) for the analysed *Tigriopus specimens*. Geographic coordinates are expressed as decimal degrees (Map Datum: WGS84). *: specimens from the type locality of *Tigriopus fulvus algiricus*; §: specimen from the type locality of *Tigriopus fulvus adriaticus*; †: specimens from Madeira, the type locality of *Tigriopus fulvus* s.s.

Taxa	Code	Sample	Country	Location	Latitude (N)	Longitude (E)	Accession Number (A.N.)			Source
							COI	12S	28S	
T. fulvus*	TIP	TF44	Algeria	Tipaza	36.6229	02.4081	MK211338	MN625569	-	Present work
T. fulvus*	TIP	TF45	Algeria	Tipaza	36.6229	02.4081	MK211337	MN625570	MN606246	Present work
T. fulvus§	ROV	TF54	Croatia	Rovigno	45.1172	13.6071	MK211332	MN625576	MN606250	Present work
T. fulvus§	ROV	TF53	Croatia	Rovigno	45.1172	13.6071	-	MN625575	-	Present work
T. fulvus§	ROV	TF65	Croatia	Rovigno	45.1172	13.6071	-	MN625577	-	Present work
T. fulvus	TER	TF1	Italy	Terrasini	38.1542	13.0756	MK211350	MN625543	-	Present work
T. fulvus	TER	TF6	Italy	Terrasini	38.1542	13.0756	MK211351	MN625544	-	Present work
T. fulvus	TER	TF7	Italy	Terrasini	38.1542	13.0756	MK211352	MN625542	MN606226	Present work
T. fulvus	LIN	TF104	Italy	Linosa	35.8632	12.8547	-	MN625550	MN606229	Present work
T. fulvus	LIN	TF105	Italy	Linosa	35.8632	12.8547	-	MN625549	MN606230	Present work
T. fulvus	GRA	TF97	Italy	Torretta Granitola	37.6067	12.6257	-	MN625603	MN606259	Present work

Taxa	Code	Sample	Country	Location	Latitude (N)	Longitude (E)	Accession Number (A.N.)			Source
							COI	12S	28S	
T. fulvus	GRA	TF98	Italy	Torretta Granitola	37.6067	12.6257	-	MN625604	-	Present work
T. fulvus	BAR	TF2	Italy	Barcarello	38.2129	13.2916	MK211354	MN625553	-	Present work
T. fulvus	BAR	TF8	Italy	Barcarello	38.2129	13.2916	MK211355	MN625555	-	Present work
T. fulvus	BAR	TF9	Italy	Barcarello	38.2129	13.2916	MK211353	MN625554	MN606227	Present work
T. fulvus	MAG	TF3	Italy	Magnisi	37.1562	15.2369	MK211345	-	-	Present work
T. fulvus	MAG	TF10	Italy	Magnisi	37.1562	15.2369	MK211344	-	MN606232	Present work
T. fulvus	MAG	TF11	Italy	Magnisi	37.1562	15.2369	MK211346	MN625548	-	Present work
T. fulvus	PLE	TF4	Italy	Plemmirio	37.0021	15.3315	-	MN625540	-	Present work
T. fulvus	PLE	TF12	Italy	Plemmirio	37.0021	15.3315	MK211356	MN625539	-	Present work
T. fulvus	PLE	TF13	Italy	Plemmirio	37.0021	15.3315	MK211357	MN625541	MN606238	Present work
T. fulvus	MIL	TF14	Italy	Milazzo	38.2700	15.2245	MK211348	MN625546	MN606239	Present work
T. fulvus	MIL	TF15	Italy	Milazzo	38.2700	15.2245	MK211347	MN625547	-	Present work
T. fulvus	MIL	TF16	Italy	Milazzo	38.2700	15.2245	MK211349	MN625545	-	Present work

Taxa	Code	Sample	Country	Location	Latitude (N)	Longitude (E)	Accession Number (A.N.)			Source
							COI	12S	28S	
T. fulvus	COR	TF17	Italy	Cornino	38.0900	12.6583	MK211343	MN625551	MN606240	Present work
T. fulvus	COR	TF19	Italy	Cornino	38.0900	12.6583	-	MN625552	-	Present work
T. fulvus	UST	TF23	Italy	Ustica	37.7089	13.1963	-	MN625556	MN606241	Present work
T. fulvus	UST	TF24	Italy	Ustica	37.7089	13.1963	-	MN625557	-	Present work
T. fulvus	PAN1	TF26	Italy	Pantelleria	36.7793	11.9541	MK211358	MN625558	-	Present work
T. fulvus	PAN2	TF27	Italy	Pantelleria	36.8158	11.9263	MK211359	MN625559	MN606242	Present work
T. fulvus	TRI	TF32	Italy	Tricase	39.9330	18.3975	-	MN625565	-	Present work
T. fulvus	TRI	TF37	Italy	Tricase	39.9330	18.3975	MK211333	MN625564	-	Present work
T. fulvus	TRI	TF101	Italy	Tricase	39.9330	18.3975	-	MN625566	MN606228	Present work
T. fulvus	CEF	TF38	Italy	Cefalù	38.0415	14.0218	MK211342	MN625567	MN606244	Present work
T. fulvus	CEF	TF39	Italy	Cefalù	38.0415	14.0218	MK211341	MN625568	-	Present work
T. fulvus	POR	TF42	Italy	Portoscuso	39.2065	8.3763	MK211361	-	-	Present work
T. fulvus	POR	TF43	Italy	Portoscuso	39.2065	8.3763	MK211362	MN625605	MN606245	Present work

Taxa	Code	Sample	Country	Location	Latitude (N)	Longitude (E)	Accession Number (A.N.)			Source
							COI	12S	28S	
T. fulvus	CAS	TF46	Italy	Castiglioncello	43.4012	10.4045	MK211339	MN625532	MN606247	Present work
T. fulvus	CAS	TF48	Italy	Castiglioncello	43.4012	10.4045	MK211340	-	-	Present work
T. fulvus	GAR	TF83	Italy	Gargano	41.9264	15.6435	MK211331	MN625588	MN606253	Present work
T. fulvus	GAR	TF84	Italy	Gargano	41.9264	15.6435	-	MN625589	MN606254	Present work
T. fulvus	USA	TF92	Italy	Usai	39.1096	9.5231	-	MN625597	MN606258	Present work
T. fulvus	USA	TF93	Italy	Usai	39.1096	9.5231	-	MN625598	-	Present work
T. fulvus	GEN	TF94	Italy	Genova Nervi	44.3826	9.0261	-	MN625599	-	Present work
T. fulvus	GEN	TF95	Italy	Genova Nervi	44.3826	9.0261	-	MN625600	-	Present work
T. fulvus	GEN	TF99	Italy	Genova Nervi	44.3826	9.0261	-	MN625601	-	Present work
T. fulvus	GEN	TF102	Italy	Genova Nervi	44.3826	9.0261	-	MN625602	-	Present work
T. fulvus	KOK	TF89	Greece	Kokkinoreia	36.4019	22.4873	-	MN625594	MN606257	Present work
T. fulvus	KOK	TF90	Greece	Kokkinoreia	36.4019	22.4873	-	MN625595	-	Present work
T. fulvus	KOK	TF91	Greece	Kokkinoreia	36.4019	22.4873	-	MN625596	-	Present work

Taxa	Code	Sample	Country	Location	Latitude (N)	Longitude (E)	Accession Number (A.N.)			Source
							COI	12S	28S	
T. fulvus	SDI	TF85	Morocco	Sidi Ifni	29.3467	-10.1961	MK211329	MN625590	MN606255	Present work
T. fulvus	SDI	TF86	Morocco	Sidi Ifni	29.3467	-10.1961	-	MN625591	-	Present work
T. fulvus	CPT	TF87	Morocco	Cape Tamry	30.5465	-9.7180	MK211330	MN625592	MN606256	Present work
T. fulvus	CPT	TF88	Morocco	Cape Tamry	30.5465	-9.7180	-	MN625593	-	Present work
T. fulvus[†]	SXL	TF58	Portugal	Seixal	32.8270	-17.1145	MK211326	MN625578	MN606251	Present work
T. fulvus[†]	SXL	TF59	Portugal	Seixal	32.8270	-17.1145	MK211325	MN625580	-	Present work
T. fulvus[†]	SXL	TF72	Portugal	Seixal	32.8270	-17.1145	MK211324	MN625579	-	Present work
T. fulvus[†]	PDC	TF60	Portugal	Porto da Cruz	32.7763	-16.8264	MK211328	MN625581	-	Present work
T. fulvus[†]	PDC	TF61	Portugal	Porto da Cruz	32.7763	-16.8264	-	MN625583	-	Present work
T. fulvus[†]	PDC	TF67	Portugal	Porto da Cruz	32.7763	-16.8264	MK211327	MN625584	MN606252	Present work
T. fulvus[†]	PDC	TF73	Portugal	Porto da Cruz	32.7763	-16.8264	-	MN625582	-	Present work
T. fulvus	JAV	TF49	Spain	Jàvea	38.7635	0.2050	MK211336	MN625571	MN606248	Present work
T. fulvus	JAV	TF50	Spain	Jàvea	38.7635	0.2050	MK211335	MN625572	-	Present work

Taxa	Code	Sample	Country	Location	Latitude (N)	Longitude (E)	Accession Number (A.N.)			Source
							COI	12S	28S	
T. fulvus	BEN	TF51	Spain	Benitachell	38.7080	0.1664	-	MN625573	-	Present work
T. fulvus	BEN	TF52	Spain	Benitachell	38.7080	0.1664	MK211334	MN625574	MN606249	Present work
T. fulvus	MEN	TF76	Spain	Menorca	39.9980	3.8274	-	MN625585	-	Present work
T. fulvus	MEN	TF77	Spain	Menorca	39.9980	3.8274	-	MN625586	-	Present work
T. fulvus	MEN	TF78	Spain	Menorca	39.9980	3.8274	MK211360	MN625587	-	Present work
T. fulvus	AKA	TF109	Cyprus	Ammos tou Kambouri	34.9785	34.0233	-	MN625533	MN606231	Present work
T. fulvus	AKA	TF110	Cyprus	Ammos tou Kambouri	34.9785	34.0233	-	MN625534	MN606233	Present work
T. fulvus	FKP	TF111	Cyprus	Faros Kato Pafou	34.7609	32.4030	-	MN625535	MN606234	Present work
T. fulvus	FKP	TF112	Cyprus	Faros Kato Pafou	34.7609	32.4030	-	MN625536	MN606235	Present work
T. fulvus	YEO	TF113	Cyprus	Ayios Yeorgios	34.9026	32.3170	-	MN625537	MN606236	Present work
T. fulvus	YEO	TF114	Cyprus	Ayios Yeorgios	34.9026	32.3170	-	MN625538	MN606237	Present work
T. fulvus	BIZ	TF28	Tunisia	Bizerte	37.3341	9.8408	-	MN625560	MN606243	Present work
T. fulvus	BIZ	TF29	Tunisia	Bizerte	37.3341	9.8408	-	MN625561	-	Present work

Taxa	Code	Sample	Country	Location	Latitude (N)	Longitude (E)	Accession Number (A.N.)			Source
							COI	12S	28S	
T. fulvus	BIZ	TF30	Tunisia	Bizerte	37.3341	9.8408	-	MN625562	-	Present work
T. fulvus	BIZ	TF34	Tunisia	Bizerte	37.3341	9.8408	-	MN625563	-	Present work
T. fulvus	-	-	France	Banylus Sur Mer	42.4833	3.1333	AF315361	-	-	Edmands, 2001
T. fulvus	-	-	Spain	Blanes	41.6666	2.8000	AF315364	-	-	Edmands, 2001
T. brevicornis	GLC	TF79	Spain	Sanxenxo	42.3898	-8.7767	MK211363	MN625528	MN606222	Present work
T. brevicornis	GLC	TF80	Spain	Sanxenxo	42.3898	-8.7767	MK211364	MN625529	-	Present work
T. brevicornis	GLC	TF81	Spain	Sanxenxo	42.3898	-8.7767	-	MN625530	MN606223	Present work
T. brevicornis	GLC	TF82	Spain	Sanxenxo	42.3898	-8.7767	-	MN625531	-	Present work
T. brevicornis	TRD	TF107	Norway	Trondheim	63.4502	10.4323	-	MN625526	MN606224	Present work
T. brevicornis	TRD	TF108	Norway	Trondheim	63.4502	10.4323	-	MN625527	MN606225	Present work
T. brevicornis	-	-	Spain	Galicia	-	-	-	-	EU370444	Reumont et al., 2009
T. californicus	TCL	TF106	-	-	-	-	-	MN625525	MN606221	Present work

Table 2. Matrix of the characters used on the morphological-based phylogenetic reconstruction analysis.

Characters	Description
	A1 ♀
1	Last segment, n. of setae (5 setae: 0; 7 setae: 1; 8 setae: 2)
2	Presence of aesthetasc in the last segment (Absence: 0; Presence: 1)
3	Presence of acrothek in the last segment (Absence: 0; Presence: 1)
4	Presence of aesthetasc in the fourth segment (Absence: 0; Presence: 1)
	A1 ♂
5	N. of segments (7 segments: 0; 8 segments: 1)
6	Segment, excluding the last one, in which the aesthetasc is present (5th: 0; 6th: 1; 7th: 2)
7	Presence of aesthetasc in the last segment (Absence: 0; Presence: 1)
8	Presence of acrothek in the last segment (Absence: 0; Presence: 1)
	A2 ♀, Allobasipodite
9	Presence of ornament (no: 0; yes: 1)
10	Presence of a "tuft" of spinules in the inner margin (no: 0; yes: 1)
11	N. of spinules file (0 row: 0; 1 row: 1; 2 rows: 2; 3 rows: 3; 8 rows: 4)
12	N. of rows of semi-circular spinules (0 row: 0; 1 row: 1; 2 rows: 2)
	A2 ♀, Exopodite
13	N. of segments (3 segments: 0; 4 segments: 1; 2 segments: 2)
14	N. of setae in the first segment (1 seta: 0; 2 setae: 1; 3 setae: 2)
15	N. of setae in the second segment (1 seta: 0; 2 setae: 1; 4 setae: 2)
16	N. of setae in the third segment (1 seta: 0; 2 setae: 1; No setae: 2)
17	N. of setae in the fourth segment (No setae: 0; 0 seta: 1; 2 setae: 2)
18	Plumose setae (Not all the setae are plumose: 0; All setae are plumose: 1)
	P2 ♂, II segment of the endopodite
19	N. of spinous processes (sp) or setae (1 sp + 1 seta: 0; 2 sp: 1; 2 setae: 2)
	P4, III segment of the exopodite
20	Total number of setae and spine (7 setae and spine: 0; 8 setae and spine: 1)
	Mandible (Md)
21	N. setae on the basis (1 seta: 0; 2 setae: 1; 4 setae: 2)
22	N. setae on the exopodite (3 setae: 0; 4 setae: 1; 5 setae: 2; 6 setae: 3; 7 setae: 4)
23	N. setae on the endopodite (5 setae: 0; 6 setae: 1; 8 setae: 2; 9 setae: 3; 10 setae: 4; 7 setae: 5)
	Maxillule (Mxl)
24	Claw (reduced: 0; well developed: 1)
	P5 ♀
25	Shape of the base (reduced: 0; stocky: 1; lengthened: 2)
26	N. setae on the base lobe (4 setae: 0; 5 setae: 1)
	P5 ♂

Table 3. Matrix of the status of the characters listed in Table S1 for the different species examined. Characters with an indeterminate state were indicated with "?".

Taxa	Characters
Tigriopus fulvus	210111101121010000000000000
T. brevicornis	210111101022010000000000000
T. californicus	010101??????02000?1002111111
T. brachydactylus	????????????00110??11000?0??
T. raki	11011000????0101011103210000
T. minutus	2???1???00000101001113201010
T. igai	11?112??10110101000113201000
T. kerguelenensis	2101111?11210101010111211111
T. crozettensis	210111011200100000000000000
T. angulatus	01011??0110011101011????1111
T. japonicus	111111101020010000000000000
T. sirindhornae	111100101030010000000000000
T. kingsejongensis	111101111320100000000000000
T. thailandensis	1111110010400101010?0002411111
T. incertus	????1???????10002?0?????0000
Zaus wonchoelleei	211110111010212000000000000

3. RESULTS

3.1 Morphological identification

The sampling activities led to the collection of 32 *Tigriopus* populations. Overall, 82 specimens from all populations were identified on a morphological basis and included in the molecular analyses. Among these, 76 individuals were identified as *T. fulvus* and six as *T. brevicornis* (Table 1). In addition, six individuals of *T. fulvus* from the populations of Madeira (1 female and 1 male), Rovinj (1 female and 1 male) and Tipasa (1 female and 1 male), and 2 individual of *T. brevicornis*, from Galicia (1 female and 1 male), were used to morphologically compare the topotypical populations of *T. fulvus* s.s. with *T. fulvus adriaticus*, *T. fulvus algiricus* and *T. brevicornis*.

According to the descriptions of Van Douwe (1913), *T. fulvus adriaticus* should be characterized by the following characteristics: baseoendopodite of P5 of the female specimens wider than long and with roundish exopodite; caudal ramus of female specimens adorned with spines; a row of spines on the outer surface of the male P5 exopodite. According to the description of Monard (1935), *T. fulvus algiricus*, on the other hand, is characterized by having the baseoendopodal lobe of female P5 "remarquablement plus large et moins avancé" (larger and less advanced than usual), since it does not reach the level of the distal segment of the exopodite; and the second segment of the male P2 endopodite that always exceeds the distal apex of the third segment of the P2 exopodite . The morphological comparison of the above-mentioned characteristics among the specimens of the different populations analysed in this study has not brought to light substantial morphological differences between the individuals of *Tigriopus fulvus* s.l. (Figures 6-8). The presence of the morphological features considered typical of the two subspecies in Madeira specimens, i.e. the topotypical *T. fulvus* s.s., casts some doubt on the taxonomical relevance of the morphological characters used to allegedly characterize the subspecies *T. fulvus algiricus* and *T. fulvus adriaticus*.
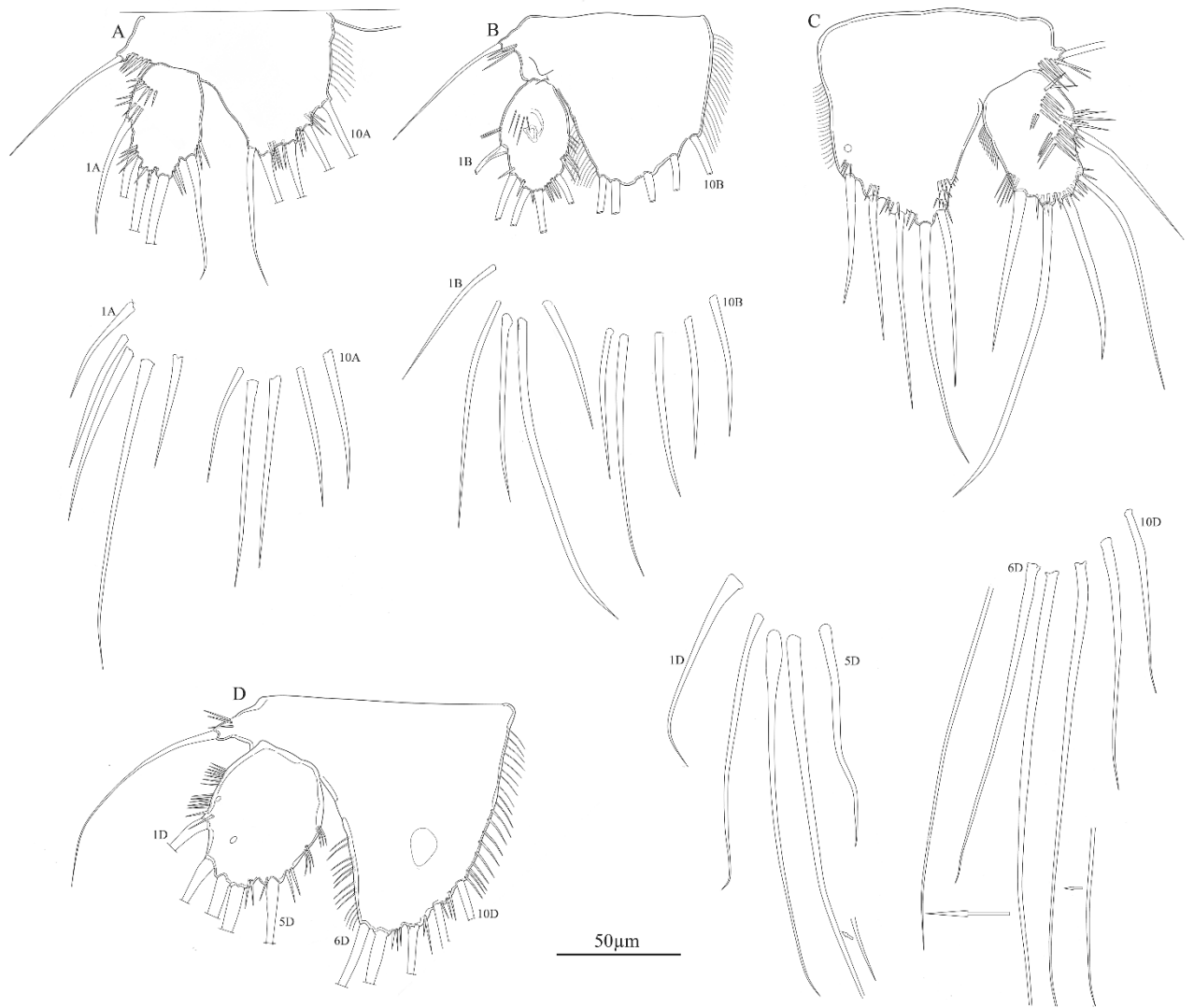
Figure 6. Female. P5 *Tigriopus fulvus adriaticus* (A) P5 *T. fulvus* s.s. (B), P5 *T. fulvus algiricus* (C), and P5 *T. brevicornis* (D).
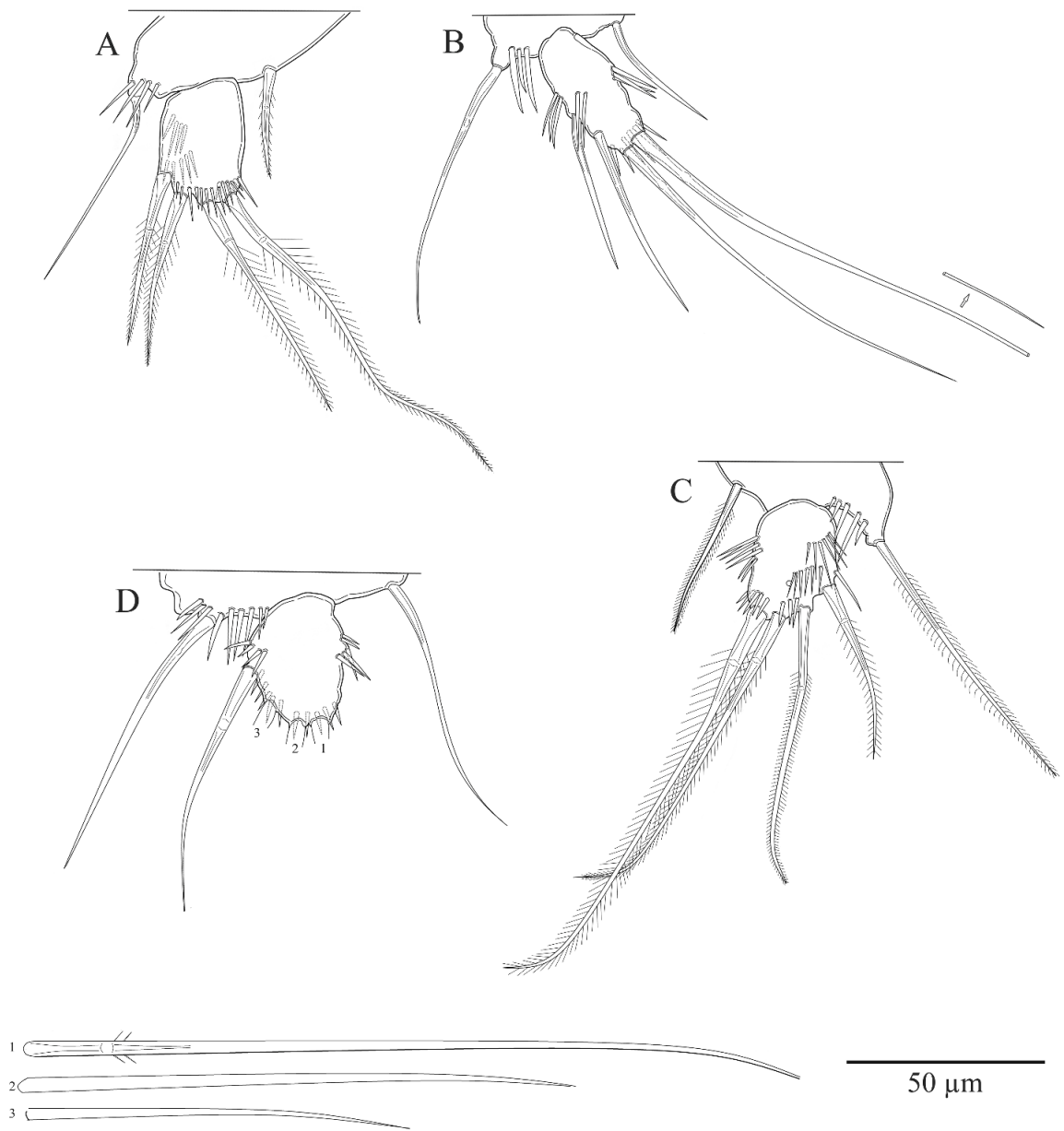
Figure 7. Male. P5 *Tigriopus fulvus adriaticus* (A) P5 *T. fulvus* s.s. (B), P5 *T. fulvus algiricus* (C), and P5 *T. brevicornis* (D).
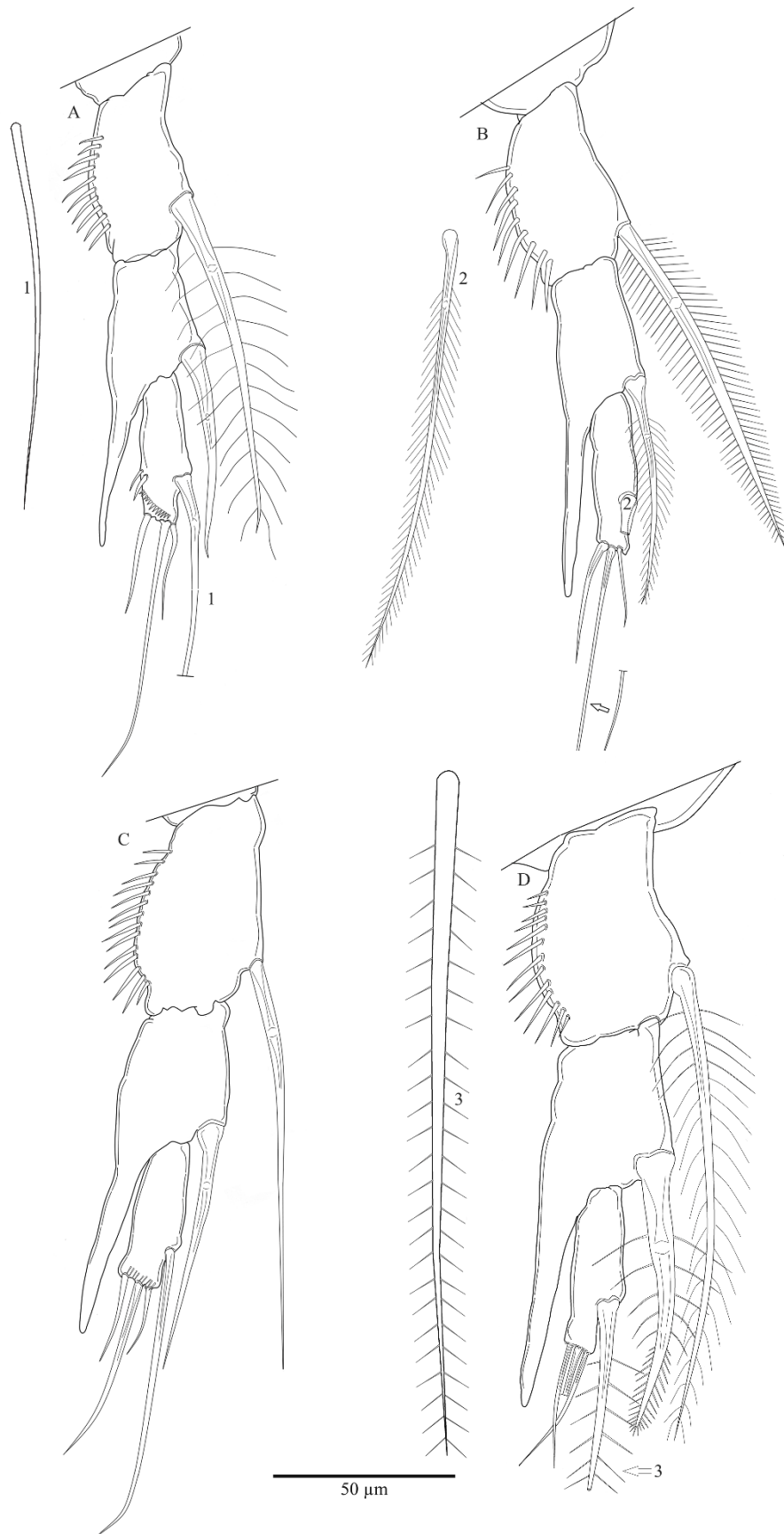
Figure 8. Male. Endopodite P2 *Tigriopus fulvus adriaticus* (A) Endopodite P2 *T. fulvus* s.s. (B), Endopodite P2
T. fulvus algiricus (C), and Endopodite P2 *T. brevicornis* (D).

3.2 Phylogenetic analysis

After having trimmed out the tails of the sequences, properly aligned fragments of 552 and 415 bp-long of the mitochondrial COI and 12S genes, respectively, were obtained, and an aligned fragment of 724 bp-long of the 28S nuclear gene. The BI and ML trees based on the mitochondrial COI fragment and rooted on *T. brevicornis* showed a congruent topology, with a sister–taxa relationship between the Algerian samples from the *T. fulvus algiricus* type locality and the rest of the *T. fulvus* s.l. specimens, whereas the alleged *T. f. adriaticus* sample nested well within the ingroup (Figure 9). Well-supported Moroccan, Madeiran and Mediterranean *T. fulvus* clades stemmed from a basal polytomy. The Mediterranean clade showed noteworthy internal molecular structuring, with intra-clade pairwise uncorrected *"p"* distances ranging from 0% to 19%, i.e. the same exceptionally high diversity observed in the same marker of *T. brevicornis*, *T. californicus* (Edmands, 2001; Handschumacher et al., 2010). Interestingly, the occurrence of private monophyletic haplogroups was observed in different Mediterranean sub-basins (Figure 10), and no haplotypes were shared among different rock pools even within sub-basins.
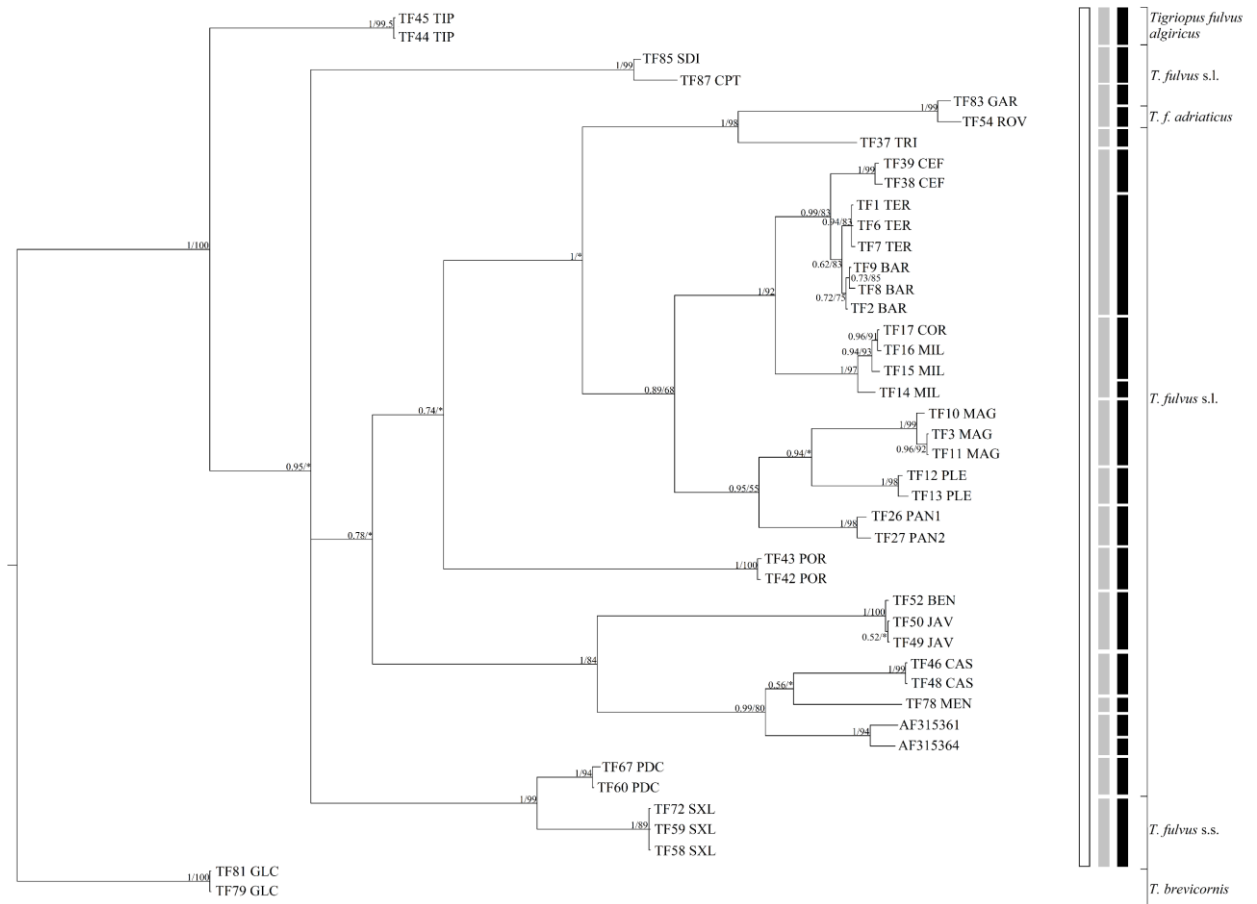
Figure 9. Bayesian phylogram (95% majority rule consensus tree) for *Tigriopus* spp. based on the 552 bp fragment of the mtDNA COI. Samples of *Tigriopus brevicornis* were used as outgroup to root the tree. Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI)/bootstrap values (Maximum Likelihood, ML). Asterisks indicate a bootstrap support value lower than 50. Rectangles refer to MOTUs as indicated by the K/Θ ratio (white rectangle), ABGD (grey rectangles), and bPTP (black rectangles). Square brackets group the samples according to the current taxonomy of the genus. Codes of the analysed specimens are listed in Table 1.
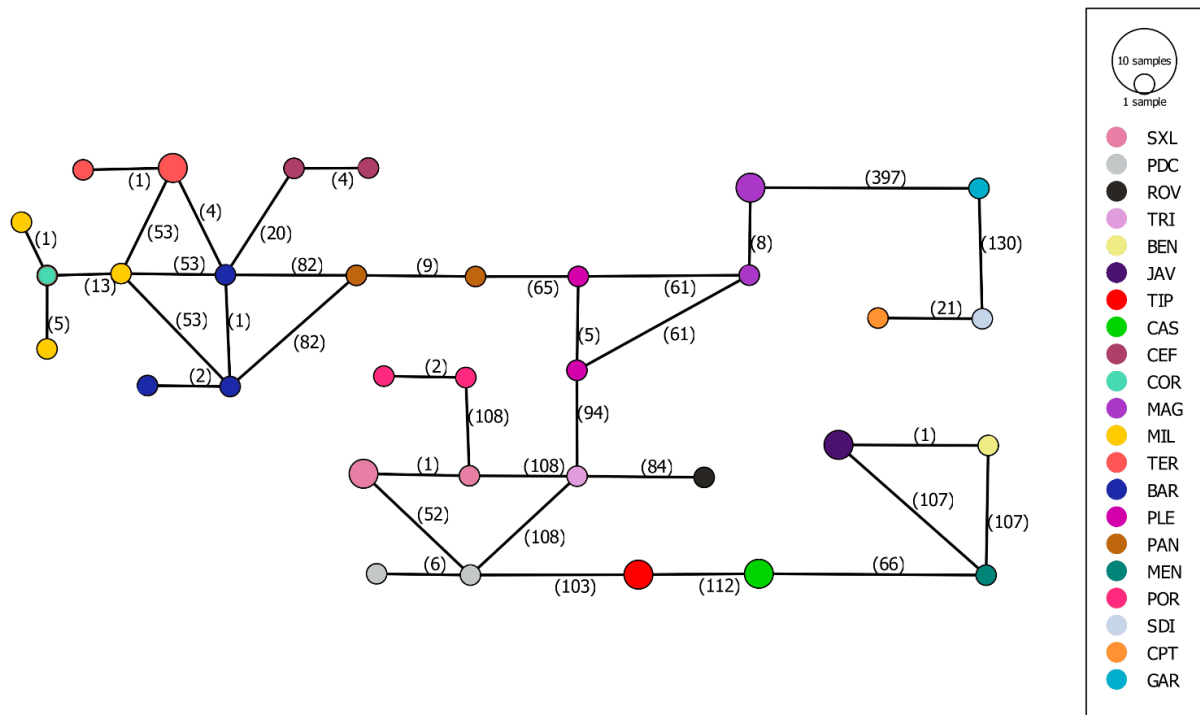
Figure 10. Minimum-spanning haplotype network based on a 552-bp long fragment of the mtDNA COI of *Tigriopus fulvus*. Substitution steps are shown in brackets. Each circle represents a haplotype and its size is proportional to the number of samples where found. Codes of the analysed specimens are listed in Table 1.

The 12S phylogenetic tree obtained from the BI and ML analyses were rooted on *Tigriopus californicus* (Figure 11). Here, after the cladogenetic event that separates specimens of *T. brevicornis* from *T. fulvus*, it is possible to observe a scenario similar to that observed in the tree based on the COI gene, with the main difference that specimens of *T. fulvus algiricus* do not constitute the most divergent clade within *T. fulvus* s.l. Also in this case, it is possible to observe a strong genetic structuring, with pairwise uncorrected *"p"* interclade distance values ranging from 0 to 22%.

Similarly to the COI, the 12S haplotype network shows mostly private haplotypes for each rock-pool with the exception of three shared haplotypes, two of which are from specimens that belong to the populations of Milazzo, Plemmirio and Cornino (Italy) and one haplogroup is from individuals that belong to population of Javea and Benitachell (Spain) (Figure 12).
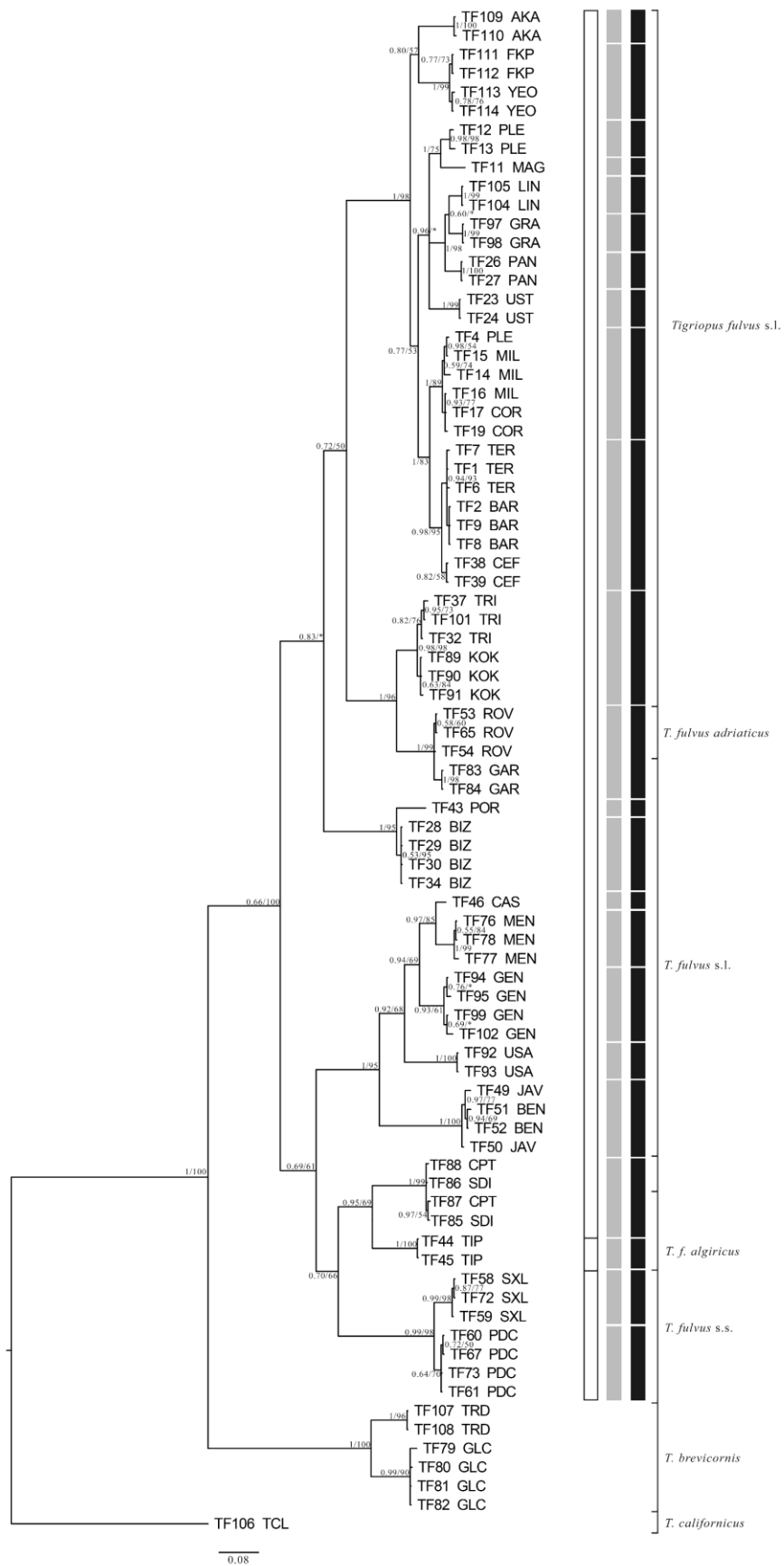
Figure 11. Bayesian phylogram (95% majority rule consensus tree) for *Tigriopus* spp. based on the 415 bp

fragment of the mtDNA 12S. A sample of *Tigriopus californicus* was used as outgroup to root the tree. Node

statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI)/bootstrap values (Maximum Likelihood, ML). Asterisks indicate a bootstrap support value lower than 50. Rectangles refer to MOTUs as indicated by the K/Θ ratio (white rectangle), ABGD (grey rectangles), and bPTP (black rectangles). Square brackets group the samples according to the current taxonomy of the genus. Codes of the analysed specimens are listed in Table 1.
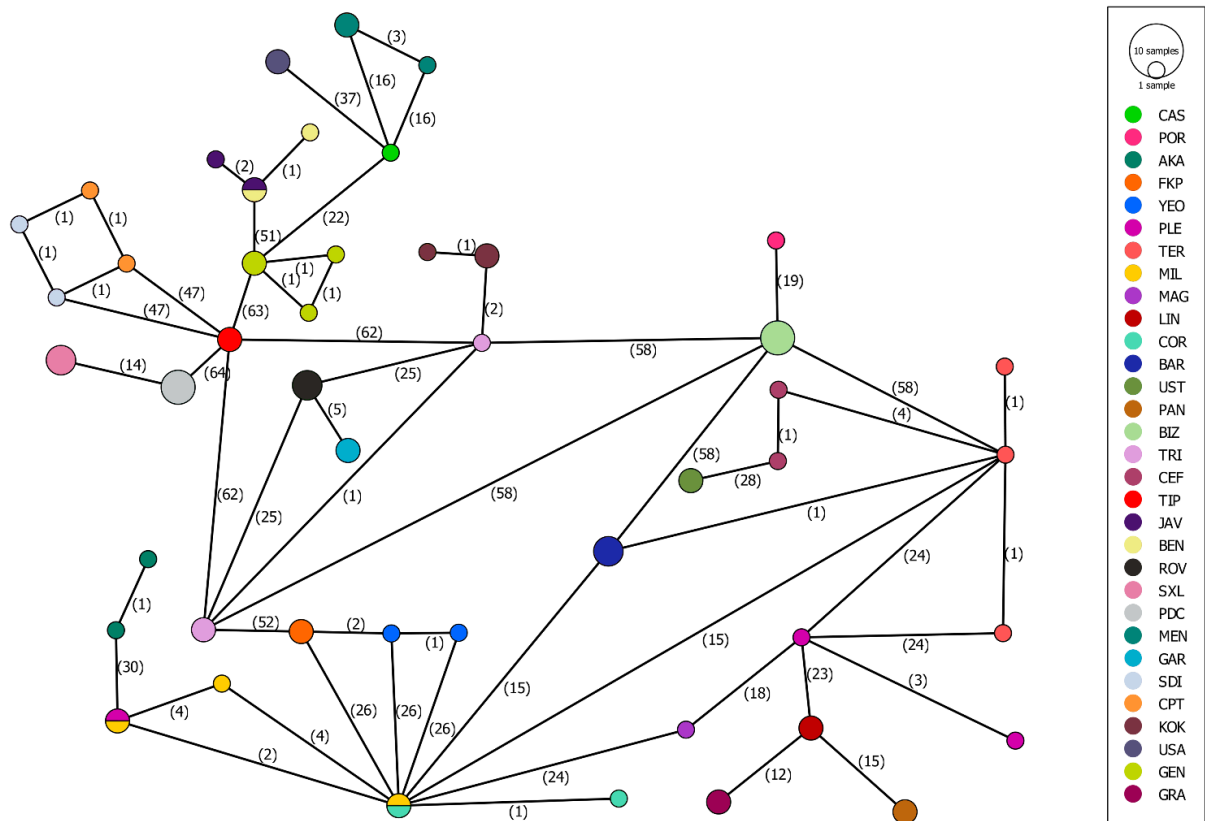
Figure 12. Minimum-spanning haplotype network based on a 415-bp long fragment of the mtDNA 12S of *Tigriopus fulvus*. Substitution steps are shown in brackets. Each circle represents a haplotype and its size is proportional to the number of samples where found. Codes of the analysed specimens are listed in Table 1.

The phylogenetic tree based on BI/ML analyses concerning the 28S nuclear gene was rooted on *Tigriopus californicus* (Figure 13). Here, we find a clear separation between the clade that include specimens of *T. brevicornis* and the one of *T. fulvus*. It is interesting to note that the specimen from Galicia, referred to as *T.* cf. *fulvus* by Reumont et al. (2009) (GenBank A.N. EU370444), it is included into the clade of our *T. brevicornis* samples, whose individuals also come from Galicia. In the light of this, the identification made by Reumont et al. (2009)

42

seems to be wrong and their sequenced specimen should be ascribed to the species *T. brevicornis*. As for the two subspecies of *T. fulvus*, these are nested within the ingroup.
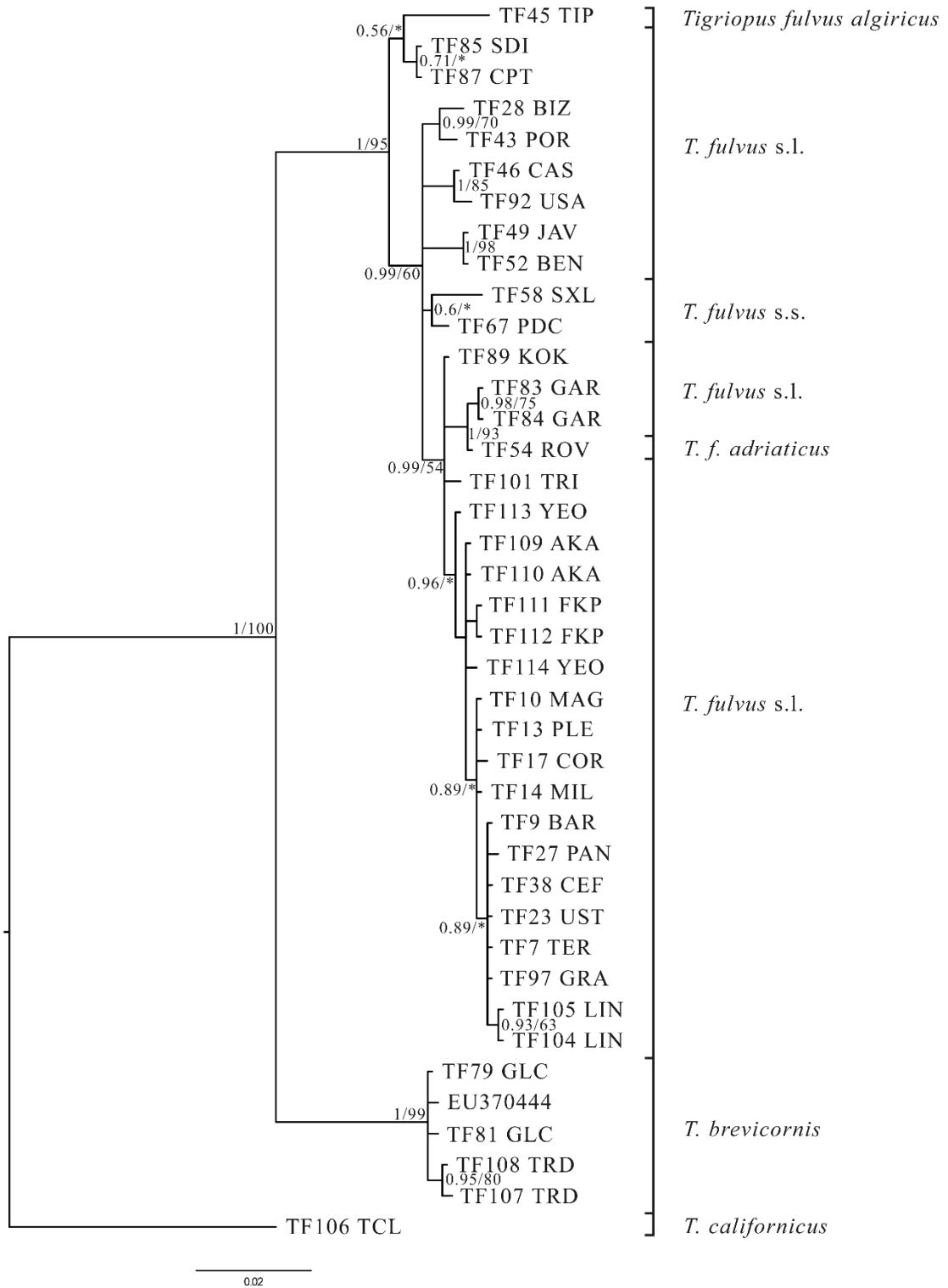
Figure 13. Bayesian phylogram (95% majority rule consensus tree) for *Tigriopus* spp. based on the 724 bp fragment of the nuDNA 28S. A sample of *Tigriopus californicus* was used as outgroup to root the tree. Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI)/bootstrap values (Maximum Likelihood, ML). Asterisks indicate a bootstrap support value lower than 50. Square brackets

group the samples according to the current taxonomy of the genus. Codes of the analysed specimens are listed in Table 1.

The haplotype network for the 28S nuclear marker (Figure 14) shows relationships phylogenetic among taxa similar to those shown with the BI/ML trees, with a maximum number of 14 evolutionary steps within the ingroup, which are needed to separate the Algerian population from the Moroccan one. There are only four shared haplotypes among the populations.

Figure 14. Minimum-spanning haplotype network based on a 724-bp long fragment of the nuDNA 28S of *Tigriopus fulvus*. Substitution steps are shown in brackets. Each circle represents a haplotype and its size is proportional to the number of samples where found. Codes of the analysed specimens are listed in Table 1
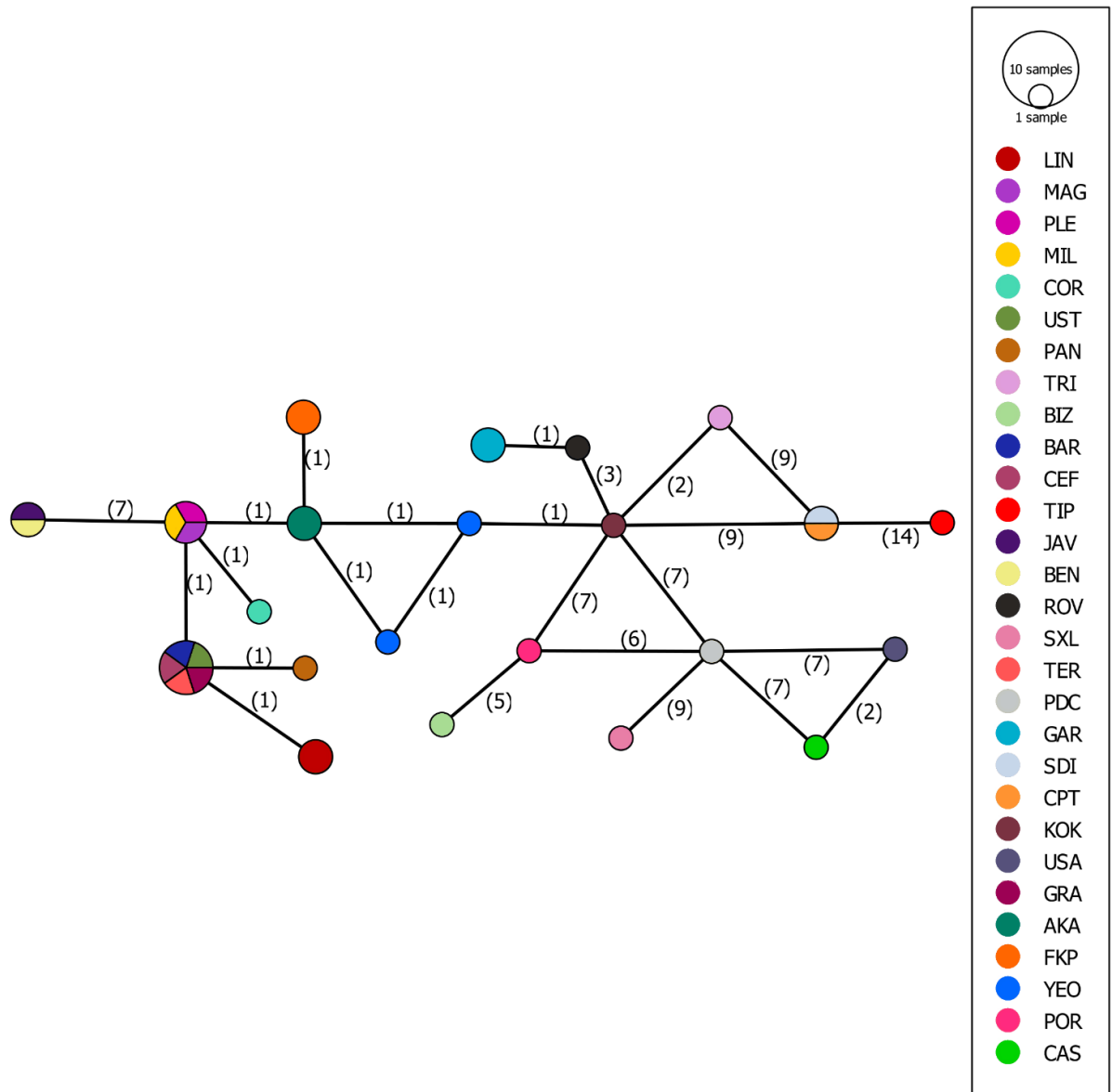
3.3 Results of molecular taxonomy approaches

The analyses of the ABGD, related to the COI fragment, reports the existence of 16 groups of specific rank within the ingroup, with values of *"p"* (prior maximal divergence of intraspecific diversity values) ranging from 0.0129 to 0.0359 (Figures 15). Conversely, the ABGD results based on the mtDNA 12S fragment, suggests the existence of 23 groups of specific rank within the ingroup, with a value of "*p*" of 0.0139 (Figure 16).

The bayesian Poisson Tree Process (bPTP) analysis for the COI dataset, return as a result the existence of 21 groups of specific rank (Figure 17) within of the ingroup, of which 11 are shared with those indicated by the ABGD. Instead, the bPTP analyses related to the 12S gene, report also the existence of 23 groups of specific rank (Figure 18) within the ingroup, in fully agreement with those indicated by the ABGD from the same mtDNA dataset. Interestingly, in both mitochondrial analyses, the Madeira specimens are placed in two/three distinct groups of presumed specific rank.

The K/Θ ratio reported for both the mtDNA datasets values lower than "4" in the intergroup relations, thus suggesting the existence of only one species (Table 4 and 5).
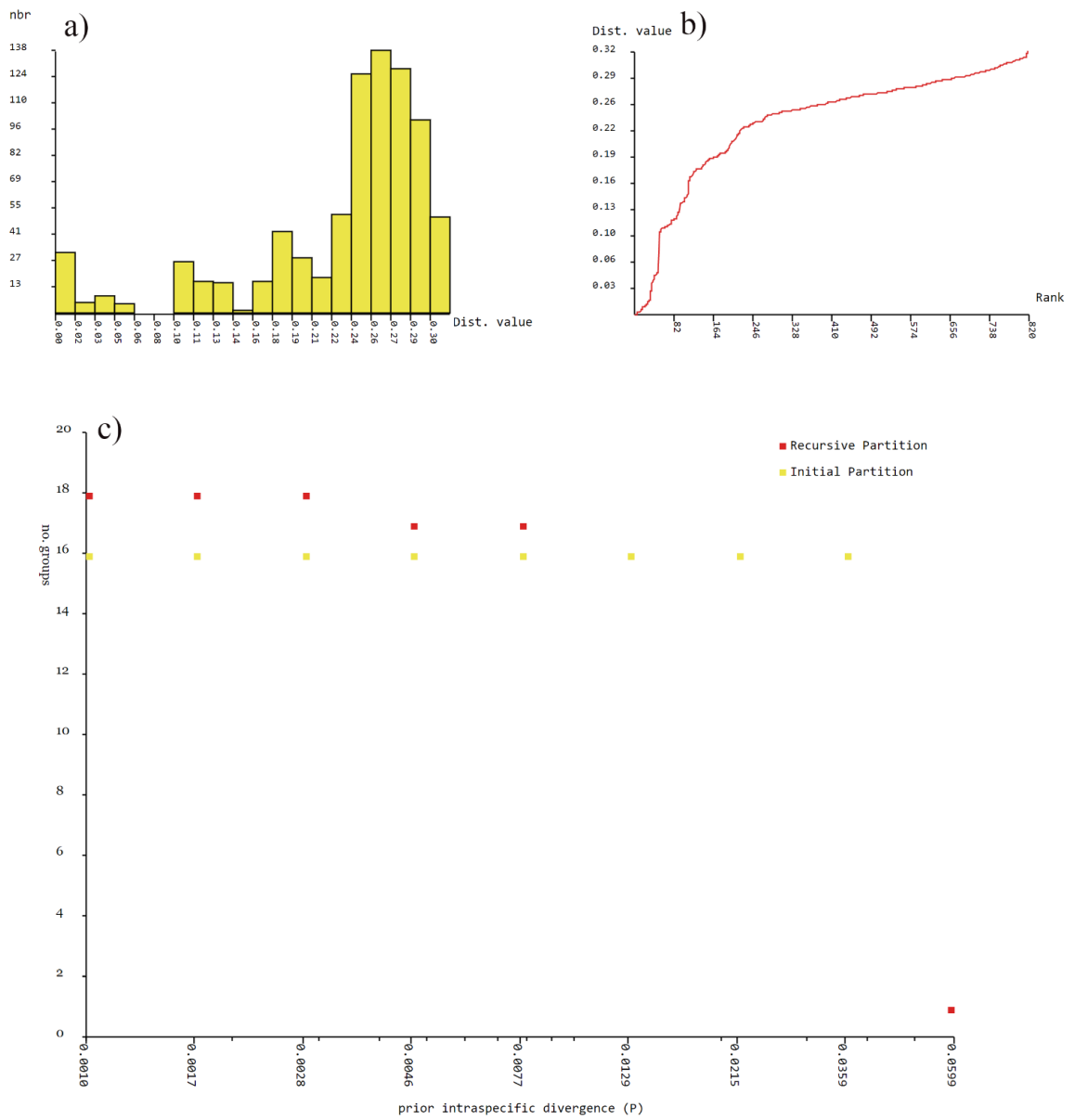
Figure 15. Results of the ABGD analysis using distances based on the K2p model, and the mtDNA COI fragment. (**a**) Hypothetical distribution of pairwise differences; (**b**) ranked pairwise differences; (**c**) automatic partition of the dataset. The number of groups inside the partitions (initial and recursive) are reported as a function of the prior limit between intra- and interspecies divergence.
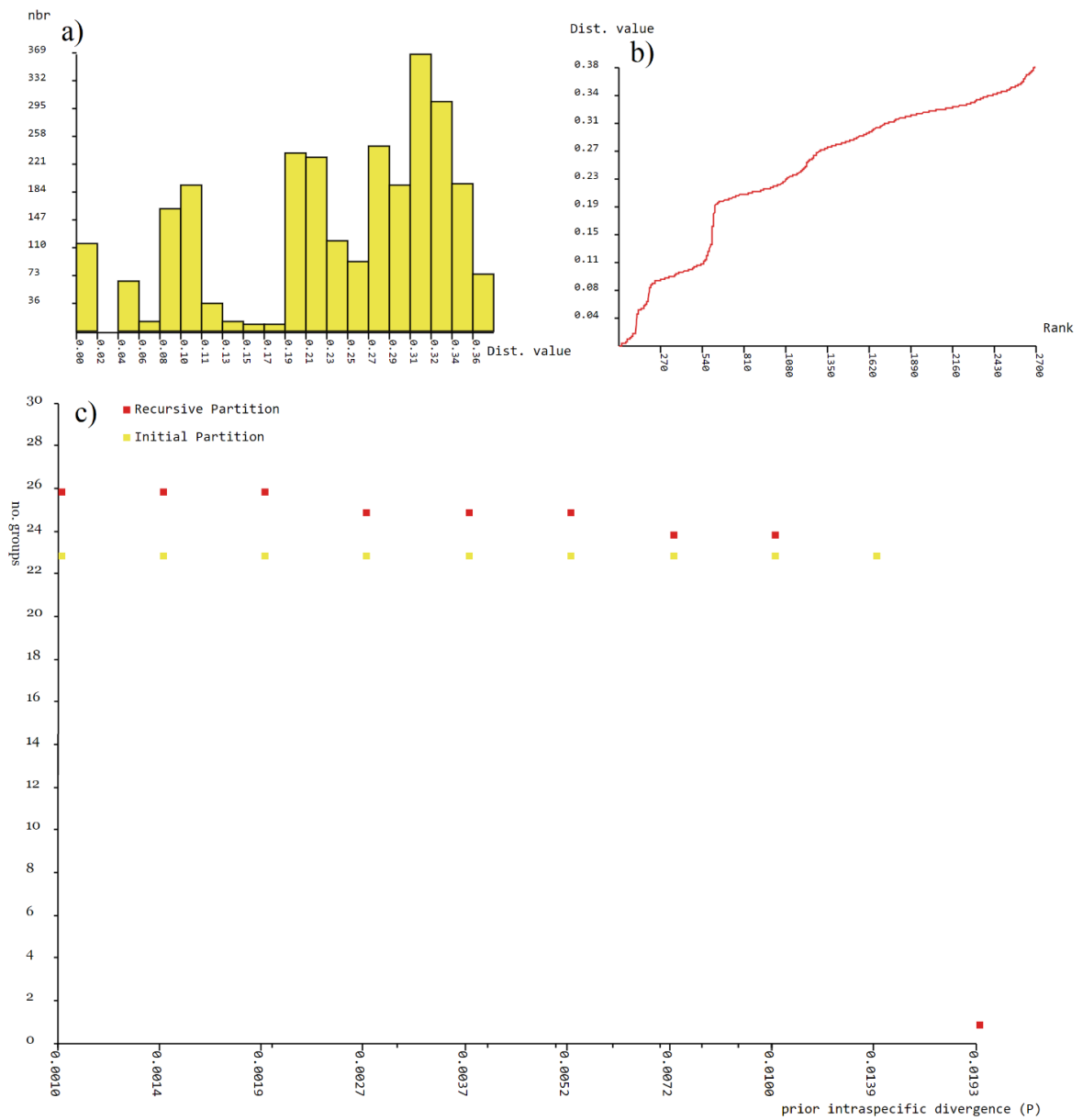
Figure 16. Results of the ABGD analysis using distances based on the K2p model, and the mtDNA 12S fragment. (**a**) Hypothetical distribution of pairwise differences; (**b**) ranked pairwise differences; (**c**) automatic partition of the dataset. The number of groups inside the partitions (initial and recursive) are reported as a function of the prior limit between intra- and interspecies divergence.
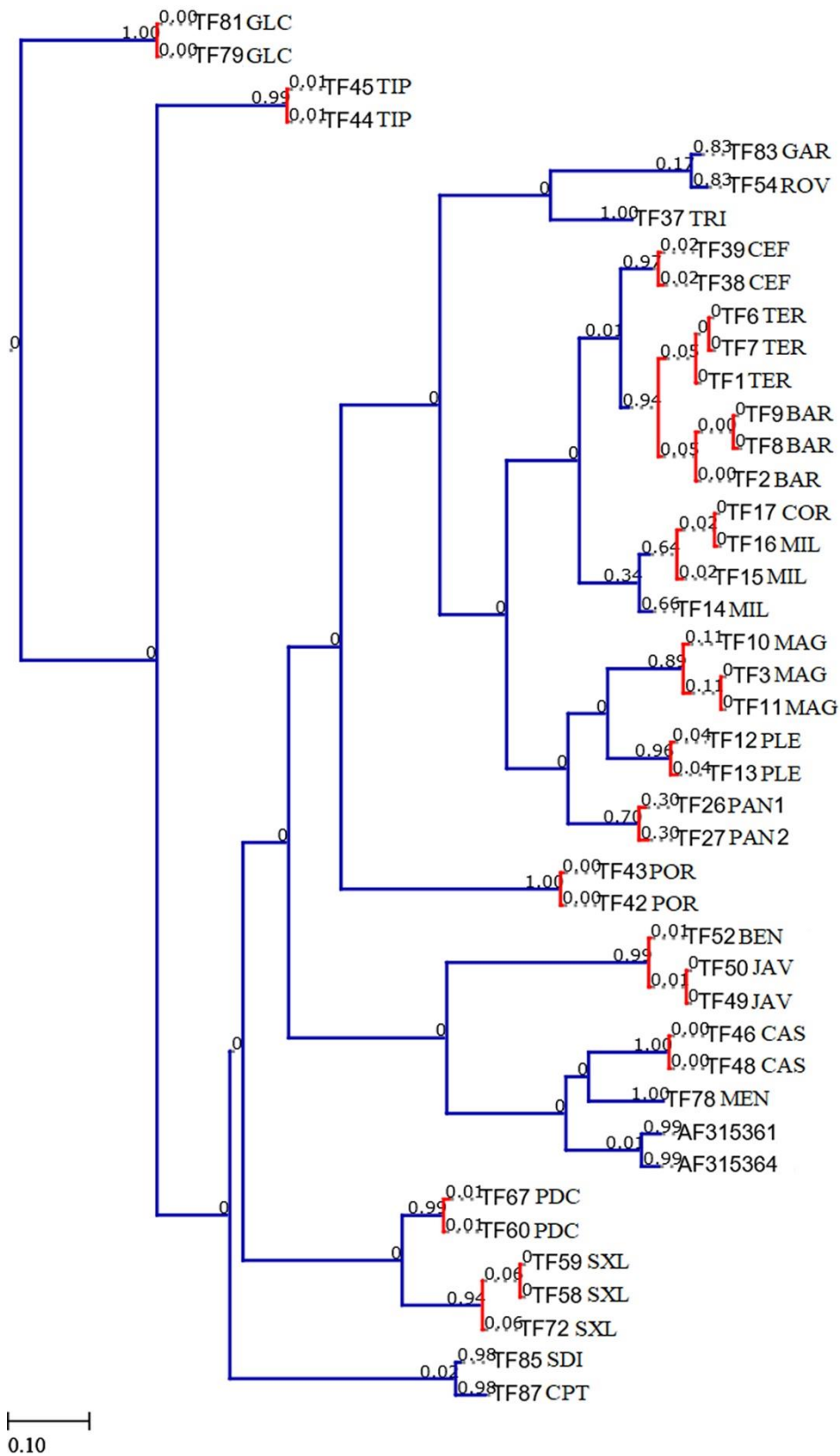
Figure 17. Putative species singled out by the bPTP model based on the mtDNA COI fragment. Codes of the analysed specimens are listed in Table 1.

Figure 18. Putative species singled out by the bPTP model based on the mtDNA 12S fragment. Codes of the analysed specimens are listed in Table 1.

Table 4: Application of the ''K/Ө ratio'' to *Tigriopus fulvus* s.l. mitochondrial COI fragment. n: number of individuals; p-dist: uncorrected p-distance; π: Nucleotide diversity; Ө: intra-clade variation; K: inter-clades distances; Tfulvcm: *T. fulvus* from the central Mediterranean area; Tfadr: *T. 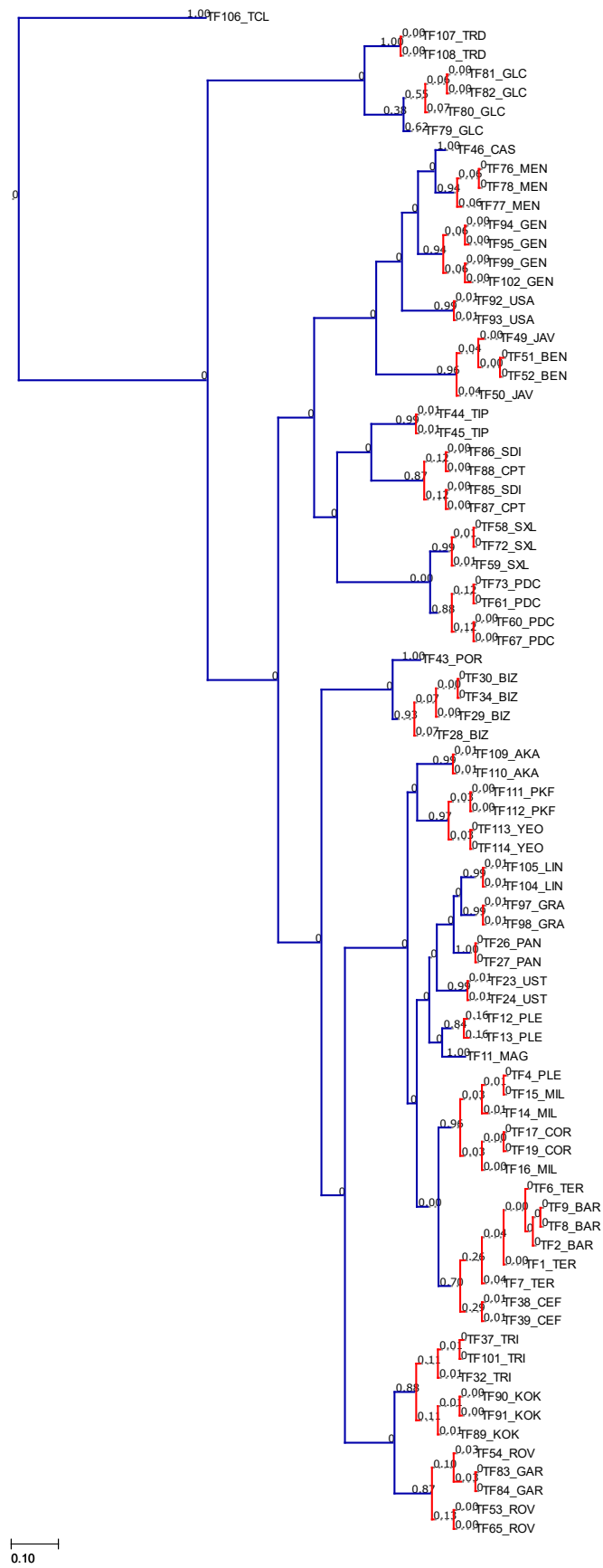f. adriaticus*; Tfalg: *T. f. algiricus*; Tfulvss: *T. fulvus* s.s. from Madeira; Tfulvsl: *T. fulvus* s.l.; *, p-dist corrected using 1/L, where "L" is the length of the fragment (cf. Birky, 2013).

Group	n	p-dist	π	4\3π	Ө	K	K\Ө ratio	Sample
Tfulvcm	19	0.112	0.118	0.157	0.140	0.188	0.868	TF1; TF2; TF3; TF6; TF7; TF8; TF9; TF10; TF11; TF12; TF13; TF14; TF15; TF16; TF17; TF26; TF27; TF38; TF39
Tfadr	3	0.112	0.168	0.224	0.216	0.188	0.868	TF37; TF54; TF83
Tfulvsp1	22	0.062	0.064	0.086	0.071	0.212	2.981	Tfulvcm + Tfadr
Tfulvsp2	2	0.002	0.005	0.006	0.005	0.212	2.981	TF42; TF43
Tfulvsp3	24	0.143	0.149	0.198	0.186	0.228	1.053	Tfulvsp1 + Tfulvsp2
Tfulvsp4	6	0.14	0.168	0.224	0.216	0.228	1.053	TF46; TF48; TF49; TF50; TF52; TF78
Tfulvsp5	30	0.033	0.034	0.045	0.035	0.225	0.357	Tfulvsp3 + Tfulvsp4
Tfulvsp6	2	0.171	0.342	0.456	0.628	0.220	2.545	TF85; TF87
Tfulvss	5	0.062	0.077	0.103	0.086	0.221	0.351	TF58; TF59; TF60; TF67; TF72
Tfulvsl	37	0.187	0.192	0.256	0.258	0.207	0.801	Tfulvsp5 + Tfulvsp6 + Tfulvss
*Tfalg	2	0.001	0.002	0.003	0.002	0.207	0.801	TF44; TF45

Table 5: Application of the ''K/Ө ratio'' to *Tigriopus fulvus* s.l. mitochondrial 12S fragment. n: number of individuals; p-dist: uncorrected p-distance; π: Nucleotide diversity; Ө: intra-clade variation; K: inter-clades distances; Tfulvcm: *T. fulvus* from the central Mediterranean area; Tfadr: *T. f. adriaticus*; Tfalg: *T. f. algiricus*; Tfulvss: *T. fulvus* s.s. from Madeira; Tfulvsl: *T. fulvus* s.l.; *, p-dist corrected using 1/L, where "L" is the length of the fragment (cf. Birky, 2013).

Group	n	p-dist	π	4\3π	Ө	K	K\Ө ratio	Sample
Tfulvcm	25	0.071	0.074	0.098	0.082	0.101	1.230	TF1; TF2; TF4; TF6; TF7; TF8; TF9; TF11; TF12; TF13; TF14; TF15; TF16; TF17; TF19; TF23; TF24; TF26; TF27; TF38; TF39; TF97; TF98; TF105; TF104
Tfadr	11	0.112	0.168	0.224	0.686	0.199	2.431	TF32; TF37; TF53; TF54; TF65; TF83; TF84; TF89; TF90; TF91; TF101
Tfulvsp1	6	0.057	0.069	0.092	0.076	0.189	2.482	TF109; TF110; TF111; TF112; TF113; TF114
Tfulvsp2	42	0.121	0.124	0.165	0.148	0.217	1.463	Tfulvcm + Tfadr + Tfulvsp1
Tfulvsp3	5	0.023	0.029	0.039	0.030	0.217	1.463	TF28; TF29; TF30; TF34; TF43
Tfulvsp4	47	0.144	0.147	0.196	0.183	0.290	1.585	Tfulvsp2 + Tfulvsp3
Tfulvsp5	14	0.127	0.137	0.183	0.168	0.290	1.585	TF49; TF46; TF50; TF51; TF52; TF76; TF77; TF78; TF92; TF93; TF94; TF95; TF99; TF102
Tfulvsp6	4	0.006	0.008	0.010	0.008	0.305	1.665	TF85; TF86; TF87; TF88
*Tfalg	2	0.002	0.004	0.006	0.004	0.159	19.402	TF44; TF45
Tfulvss	7	0.025	0.029	0.038	0.030	0.251	8.341	TF58; TF59; TF60; TF61; TF67; TF72; TF73
Tfulvsl1	47	0.140	0.143	0.190	0.176	0.293	0.943	Tfulvsp4
Tfulvusl2	27	0.212	0.220	0.293	0.311	0.293	0.943	Tfulvsp5 + Tfulvsp6 + Tfalg + Tfulvss

3.4 Morphology-based phylogeny of the genus *Tigriopus*

The tree resulting from the analysis of the matrix of morphological characters of the species of the genus *Tigriopus* is shown in Figure 19. *T. fulvus* and *T. brevicornis* are sister taxa and constitute the sister group of the clade formed by *T. kerguelenensis* and *T. crozettensis*. The presumed *T. fulvus* subspecies are in fact morphologically indistinguishable from *T. fulvus*, and therefore were not included in the analyses. *T. californicus* and *T. angulatus* appear as sister taxa and their clade are included within a monophyletic group which also includes *T. japonicus*, *T. thailandensis* and *T. sirindhornae*. In addition, *T. brachydactylus* and *T. minutus* (both from East Africa) are grouped together as sister taxa. *T. raki* and *T. incertus* (Figure 1) are grouped together, but in this case, the lack of sound information about *T. incertus* could be determinant.
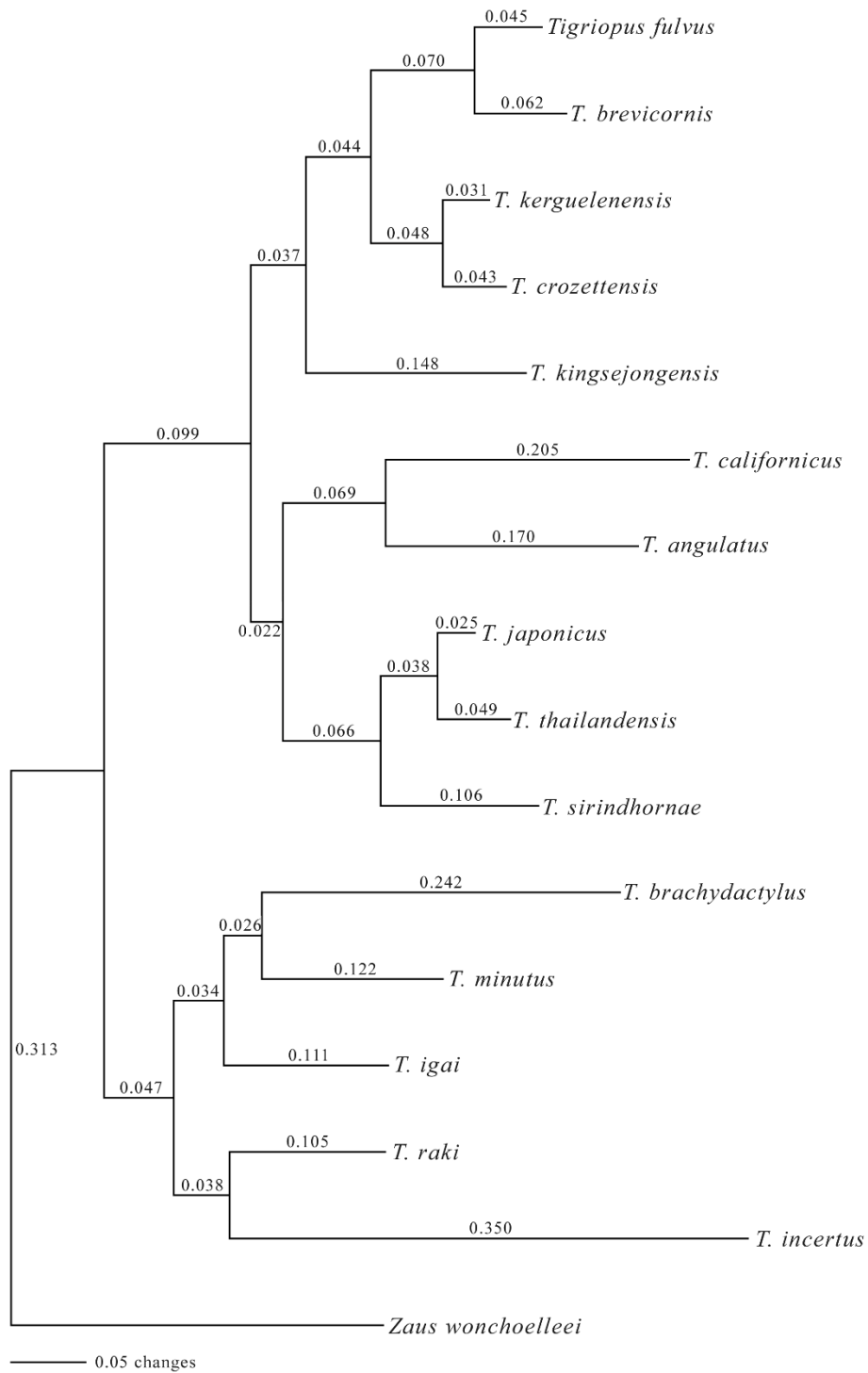
Figure 19. Morphological-based phylogenetic tree of the species of the genus *Tigriopus* based on an NJ analysis.

4. DISCUSSION

4.1 Molecular diversity pattern

The phylogenetic analysis of the studied Atlantic–Mediterranean *Tigriopus fulvus* populations revealed noteworthy geographic structuring of the genetic diversity among the samples based on both the mitochondrial COI and 12S markers, and the nuclear one, 28S. Within *Tigriopus fulvus* s.l. a longitudinal pattern of molecular diversity is sketched, with the Atlantic and the Westernmost Mediterranean populations being well characterized versus the Central and Eastern Mediterranean clades. It is likely that an increased sampling effort might lead to a sharper longitudinal clinal distribution of the molecular diversity. As for the two subspecies of *T. fulvus*, the Algerian population (*T. fulvus algiricus*) appears to be most divergent within the clade of *T. fulvus* s.l.. In particular, in the BI/ML trees based on the mtDNA COI gene, the two Algerian specimens from Tipasa are the first to separate within *T. fulvus* s.l., forming a monophyletic clade together with it. It is likely that this divergence is partly due to the under-sampling of the North African area. A more extensive sampling of the area could show a more gradual diversity pattern, with a clinical distribution of the mtDNA genetic diversity. However, if we look at the BI/ML trees of the 12S mitochondrial gene, we will note that the Algerian population of the alleged *T. fulvus algiricus* falls within the clade containing individuals of the two Atlantic Moroccan populations (Sidi Ifni and Cape Tamry) and of the individuals belonging to the populations of Madeira, including the topotypical specimens of *T. fulvus* s.s.. Regarding individuals coming from Rovinj, the topotypical site of *T. fulvus adriaticus*, both mitochondrial genes are nested inside the clade of *T. fulvus* s.l., forming a monophyletic clade with the specimens of the Adriatic sub-basin.

The pairwise uncorrected "*p*" distance values of the COI (0-19%) and the 12S gene (0-22%) of *T. fulvus* s.l. are much higher than those usually observed within crustaceans species (da Silva et al., 2011) but similar to those obtained within the species *Tigriopus brevicornis* (Handschumacher et al., 2010), *T. japonicus* (Ki et al., 2009) and *T. californicus* (Edmands,

2001), whose divergence values reach up to 23%, indicating a strong genetic structuring. According to Handschumacher et al. (2010), the extensive inter-population divergence observed in *T. brevicornis* could be ascribed to the "paradox of Rockall". This paradox states that species with limited dispersal and scarce inter-population gene flow are, counter-intuitively, very effective in colonizing remote areas after fortuitous long-range passive dispersal events (Johannesson, 1988), thus resulting in the establishment of isolated but widespread populations.

In addition, in *T. californicus* and *T. brevicornis* it was observed that among the southern populations there is a greater genetic diversity than among the northern populations (Edmands, 2001; Handschumacher et al., 2010). This phenomenon is also observed for other invertebrates that live in rock pools, such as the gastropods *Nucella emarginata* and *Nucella ostrina* (Marko, 1998), the beetles of the hydraenid genus *Calobius* and the mosquitos of the genus *Aedes* (Audisio et al., 2010; Mastrantonio et al., 2015). A possible explanation for this type of pattern lies in the paradigm of the "Southern richness vs Northern purity" (Hewitt, 2004; Marrone et al., 2010), according to which, during glaciations, the ice sheets pushed southwards inducing the northernmost populations to migrate towards the south or to take refuge in local refuge-areas, with a consequent bottleneck effect that caused the partial loss of the initial diversity. At the end of the glaciations, the surviving populations in the refuge areas were able to re-colonize the area previously affected by the glacial phenomena, with the consequential loss of genetic diversity through founder-effect. On the contrary, southern populations, which have not undergone the effects of glaciations, retain their original genetic variability. However, the lower molecular diversity of the northern populations could also be the consequence of other phenomena, not necessarily mutually exclusive. For instance, the frequent bottlenecks and founder effect that occur in rock-pools or the presence of possibly higher mutation rates present in southern populations (due to greater exposure to UV radiation) could represent alternative explanations to the phenomenon (Edmands, 2001). Using as

species model the gastropod *Melarhaphe neritoides* (Linnaeus, 1758) (Gastropoda: Littorinidae), Fourdrilis & Backeljau (2019) gave a different interpretation of the unexpectedly high genetic diversity that was observed at least in 43% of animal species and especially in marine rock-dweller. This high genetic diversity observed in the mtDNA is ascribed to a very high mutation rate that may conceal the signal of gene flow. This phenomenon is called "hyperdiversity", and could explain the observed population genetic differentiation patterns. Considering our datasets, we think that a hyperdiversity phenomenon might be in place, but in the absence of proper data, it is currently not possible to corroborate it. However, the strong geographical organization of the genetic diversity of the *Tigriopus* populations in the Atlantic-Mediterranean area is at least in agreement with the paradigm of "non-cosmopolitanism" and with the hypothesis of "Monopolization" of aquatic invertebrates (De Meester et al., 2002; Incagnone et al., 2015; Desiderato et al., 2019; Hupalo et al., 2019), according to which, in a water body not yet inhabited, the first arriving colonists monopolize the resources, preventing or hindering the settlement of successive colonizers. The pattern of diversity observed therefore reflects the historical factors of colonization. Despite all, the present case study slightly differs from this pattern since *Tigriopus* populations are known to be frequently wiped out by physical events (e.g., exceptional droughts, storms, or rainfalls), leading to recurrent local extinctions and recolonizations. Accordingly, those *Tigriopus* haplotypes that colonize a *Tigriopus*-free rock-pool rapidly monopolize it preventing the establishment of other haplotypes, but this monopolization only lasts a relatively short amount of time, i.e., until a new event wipes this population out. Thus, unlike in the monopolization hypothesis, a long-lasting founder effect is not achieved, but rather just a temporary occurrence of monopolizing haplotypes can be observed. Such a pattern could be defined as a "clockwork monopolization", related to the great instability of the rock-pool habitats and to the inability of their inhabitants to produce long-lasting resting stages (Vecchioni et al., 2019).

4.2 Taxonomical remarks and conclusions

Based on the morphological comparison of the specimens coming from the topotypical populations of the two other *T. fulvus* subspecies with those from Madeira, the *terra typica* of *T. fulvus* s.s., no substantial morphological differences emerge. The individuals of the population of Madeira, as well as those from the sampled *Tigriopus fulvus* s.l. Atlantic-Mediterranean sites, share the characters that would define morphologically *T. fulvus adriaticus* and *T. fulvus algiricus*. This is due to the fact that these two subspecies were erroneously characterized using as comparative material specimens of *T. brevicornis* instead of *T. fulvus* (Božić, 1960; Carli & Fiori, 1977). In fact, the morphological features considered diagnostic of these two taxa fall within the morphological variability of *T. fulvus* s.s. from its *terra typica*.

The ABGD and bPTP DNA taxonomy approaches, based on the mitochondrial COI and 12S genes, suggested the presence of an unexpectedly high number of taxa of species rank within the ingroup. Conversely, the K/Θ ratio based on both mtDNA genes, suggests the existence of only one, albeit highly variable, species.

The different approaches of molecular taxonomy taken individually are not, however, sufficient to establish the rank to be attributed to the various lineages, but they are only a "PSH" (Primary Species Hypothesis) to take into account. Therefore, it is preferable to use a combination of different, independent approaches and search for a consensus of results (Fontaneto et al., 2015). In the absence of a consensus among the different molecular taxonomy approaches used in the present work, within and among mtDNA datasets, it was chosen to follow the more conservative approach, namely the K/Θ ratio (Birky & Barraclough, 2009; Bode et al., 2010), whose accuracy, moreover, should not be significantly affected by the sample size (Birky, 2013). However, it should be pointed out that the implementation of inter-lineages crossbreeding experiments as carried out on other *Tigriopus* species (Edmands,

1999; Handschumacher et al., 2010) is desirable since it would provide additional data on the actual conspecificity of the investigated *T. fulvus* s.l. populations.

However, considering the absence of significant morphological differences among the alleged *Tigriopus fulvus* subspecies, and in the light of the outcomes of both phylogenetic analyses and DNA taxonomy approaches, the subspecies T. *fulvus adriaticus* Van Douwe, 1913 and *T. fulvus algiricus* Monard, 1935 should currently not be considered valid taxa and therefore should be considered junior synonyms of *T. fulvus* s.s..

Tigriopus fulvus species shows a high genetic structuring based on both mitochondrial and nuclear markers, as already noticed for other *Tigriopus* species (Edmands, 2001; Handschumacher et al., 2010; Ki et al., 2009). This can be tentatively ascribed to a combination of high mutation rates on mtDNA (Burton, 1998; Willet, 2012) and a strong monopolization of the sites by the first occupants. Since the colonization-extinction dynamics typical of rock-pools should not allow a long-term monopolization of the same site, we proposed the term "clockwork monopolization" to described such a pattern of short-term monopolization due to recurrent local extinctions and recolonizations.

4.3 Taxonomical account and list of synonyms

Systematics

Family: Harpacticidae Dana, 1846

Genus: *Tigriopus* Norman, 1869

Type species: *Tigriopus fulvus* (Fischer, 1860)

Tigriopus fulvus (Fischer, 1860)

Type locality: Madeira (Portugal, Atlantic Ocean)

Synonyms

Harpacticus fulvus Fischer, 1860
Tigriopus lilljeborgii Norman, 1869
Tigriopus fulvus var. *adriatica* Douwe, 1913
Tigriopus fulvus var. *algirica* Monard, 1936

5. REFERENCES

Akaike, H. (1974). A new look at the statistical model identification. Automatic Control, IEEE Transactions on, 19(6), 716-723.

Audisio, P., Trizzino, M., De Biase, A., Rossetti, G., Mancini, E., & Antonini, G. (2010). Molecular and morphological evidence of a new sibling species of *Calobius* (Coleoptera: Hydraenidae) of the *C. quadricollis* complex from peninsular Italy. Italian Journal of Zoology, 77(1), 29-37.

Baker, C. F. (1912). Notes on the Crustacea of Laguna Beach. Reports of the Laguna Marine Laboratory, 1(1).

Birky Jr, C. W. (2013). Species detection and identification in sexual organisms using population genetic theory and DNA sequences. PLoS One, 8(1), e52544.

Birky Jr, C. W., Adams, J., Gemmel, M., & Perry, J. (2010). Using population genetic theory and DNA sequences for species detection and identification in asexual organisms. PLoS one, 5(5), e10609.

Božić, B. (1960). Le Genre *Tigriopus* Norman (Copépodes Harpacticoïdes) et ses forms européennes: recherches morphologiques et expérimentales. Centre National de la Recherche Scientifique.

Bradford, J. M. (1967). Genus *Tigriopus* Norman (Copepoda-Harpacticoida) In New Zealand With A Description Of A New Species. Transactions Of The Royal Society Of New Zealand-Zoology, 10(6), 51.

Brady, G. S. (1872). Contribution to the study of Entomostraca, VII. A list of non-parasitic Copepoda of the northeast coast of England. Natural History. Transaction. Northumbria., 4, 432-445.

Brown, A. F. (1991). Outbreeding depression as a cost of dispersal in the harpacticoid copepod, *Tigriopus californicus*. The Biological Bulletin, 181(1), 123-126.

Burton, R.S. (1998). Intraspecific phylogeography across the point conception biogeographic boundary. Evolution, 52(3), 734-745.

Carli, A., & Fiori, A. (1977). Morphological analysis of the two *Tigriopus* species found along the European coasts (Copepoda Harpacticoida). Natura, 68(1-2), 101-110.

Chullasorn, S., Dahms, H. U., & Klangsin, P. (2013). A new species of *Tigriopus* (Copepoda: Harpacticoida: Harpacticidae) from Thailand with a key to the species of the genus. Journal of natural Natural historyHistory, 47(5-12), 427-447.

Chullasorn, S., Ivanenko, V. N., Dahms, H. U., Kangtia, P., & Yang, W. X. (2012). A new species of *Tigriopus* (Copepoda, Harpacticoida, Harpacticidae) from Thailand with the description of its naupliar development. Helgoland Marine Research, 66(2), 139.

da Silva, J. M., Creer, S., dos Santos, A., Costa, A. C., Cunha, M. R., Costa, F. O., Carvalho, G. R. (2011). Systematic and Evolutionary Insights Derived from mtDNA COI Barcode Diversity in the Decapoda (Crustacea: Malacostraca). PLoS ONE, 6(5): e19449.

Damgaard, R. M., Davenport, J. (1994). Salinity tolerance, salinity preference and temperature tolerance in the high-shore harpacticoid copepod *Tigriopus brevicornis*. Marine Biology, 118, 443-449.

Davenport, J., Barnett, P. R. O., & McAllen, R. J. (1997). Environmental tolerances of three species of the harpacticoid copepod genus *Tigriopus*. Journal of the Marine Biological Association of the United Kingdom, 77(1), 3-16.

De Meester, L., Gómez, A., Okamura, B., & Schwenk, K. (2002). The Monopolization Hypothesis and the dispersal–gene flow paradox in aquatic organisms. Acta Oecologica, 23(3), 121-135.

Desiderato, A., Costa, F. O., Serejo, C. S., Abbiati, M., Queiroga, H., Vieira, P. E. (2019). acaronesian islands as promoters of diversification in amphipods: The remarkable case of the family Hyalidae (Crustacea, Amphipoda). Zoologica Scripta, 48(3), 1-17.

Edmands, S. (1999). Heterosis and Outbreeding Depression in Interpopulation Crosses Spanning a Wide Range of Divergence. Evolution, 53(6), 1757-1768.

Edmands, S. (2001). Phylogeography of the intertidal copepod *Tigriopus californicus* reveals substantially reduced population differentiation at northern latitudes. Molecular ecology, 10(7), 1743-1750.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39(4), 783-791.

Fischer, S. (1860). Beiträgezur Kenntnis der Entomostraceen. Abh Math-Phys Classe der K Bayer Akad Wiss. 8:1–645.

Fontaneto, D., Flot, J. F., & Tang, C. Q. (2015). Guidelines for DNA taxonomy, with a focus on the meiofauna. Marine Biodiversity, 45(3), 433-451.

Guindon, S., & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic biologyBiology, 52(5), 696-704.

Handschumacher, L., Steinarsdóttir, M. B., Edmands, S., & Ingólfsson, A. (2010). Phylogeography of the rock-pool copepod *Tigriopus brevicornis* (Harpacticoida) in the northern North Atlantic, and its relationship to other species of the genus. Marine Biology, 157(6), 1357-1366.

Hawkins, B. L. (1962). The biology of the marine copepod, *Tigriopus californicus* (Baker) (Doctoral dissertation, Humboldt State University).

Hewitt, G. M. (2004). Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 359(1442), 183-195.

Hupało, K., Teixeira, M. A. L., Rewicz, T., Sezgin, M., Iannilli, V., Karaman, G. S., Grabowski, M., Costa, O. F. (2019). Persistence of phylogeographic footprints helps to understand cryptic diversity detected in two marine amphipods widespread in the Mediterranean basin. Molecular Phylogenetics and Evolution, 132, 53-66.

Incagnone, G., Marrone, F., Barone, R., Robba, L., & Naselli-Flores, L. (2015). How do freshwater organisms cross the "dry ocean"? A review on passive dispersal and colonization processes with a special focus on temporary ponds. Hydrobiologia, 750(1), 103-123.

Issel, R. (1914). Vita latente per concentrazione dell'acqua (anabiosi osmotica) e biologia delle pozze di scogliera. R. Friedländer.

Itô, T. (1969). Descriptions and Records of Marine Harpacticoid Copepods from Hokkaido, Ⅱ (With 11 Text-figures). Journal Of The Faculty Of Science Hokkaido University Series ⅤⅠ. Zoology, 17(1), 58-77.

Itô, T. (1970). The Biology of a Harpacticoid Copepod, *Tigriopus japonicus* Mori (With 12 Text-figures, 1 Table and 2 Plates). Journal Of The Faculty Of Science Hokkaido University Series Vi. Zoology, 17(3), 474-500.

Itô, T. (1977). New Species of Marine Harpacticoid Copepods of the Genera *Harpacticella* and *Tigriopus* from the Bonin Islands, with Reference to the Morphology of Copepodid Stages (With 21 Text-figures). Journal Of of The the Faculty Of of Science Hokkaido University Series ⅤⅠ. Zoology, 21(1), 61-91.

Kangtia, P., Dahms, H. U., Song, S. J., Myoung, J. G., Park, J., & Khim, J. S. (2015). On the occurrence of a new species of benthic copepod, *Zaus wonchoelleei* (Harpacticoida,

Harpacticidae), in a macroalgal habitat from Tongyong, Korea. Proceedings of the Biological Society of Washington, 127(4), 585-602.

Ki, J. S., Lee, K. W., Park, H. G., Chullasorn, S., Dahms, H. U., & Lee, J. S. (2008). Phylogeography of the copepod *Tigriopus japonicus* along the Northwest Pacific rim. Journal of Plankton Research, 31(2), 209-221.

Kruskal, J. B. (1956). On the shortest spanning subtree of the graph and the travelling salesman problem. Proceeding of American Math Society, 7, 48–57.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Molecular biology and evolution, 35(6), 1547–1549.

Lanfear, R., Calcott, B., Ho, S. Y., & Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Molecular biology Biology and evolutionEvolution, 29(6), 1695-1701.

Lang, K. (1933). Zwei neue Brackwasserharpacticiden von den Macquarie-inseln. Verlag nicht ermittelbar.

Lang, K. (1948). Monographie der Harpacticiden I+ II, reprint. Otto Koeltz Science Publishers: Königstein.

Lazzaretto, I., & Libertini, A. (1986). Karyological comparison among different Mediterranean populations of the genus *Tigriopus* (Copepoda Harpacticoida). Italian Journal of Zoology, 53(2), 197-201.

Machida, R. J., Miya, M. U., Nishida, M., & Nishida, S. (2004). Large-scale gene rearrangements in the mitochondrial genomes of two calanoid copepods *Eucalanus bungii* and *Neocalanus cristatus* (Crustacea), with notes on new versatile primers for the srRNA and COI genes. Gene, 332, 71-78.

Marko, P. B. (1998). Historical allopatry and the biogeography of speciation in the prosobranch snail genus *Nucella*. Evolution, 52(3), 757-774.

Marrone, F., Brutto, S. L., & Arculeo, M. (2010). Molecular evidence for the presence of cryptic evolutionary lineages in the freshwater copepod genus *Hemidiaptomus* GO Sars, 1903 (Calanoida, Diaptomidae). Hydrobiologia, 644(1), 115-125.

Mastrantonio, V., Porretta, D., Bellini, R., Nascetti, G., & Urbanelli, S. (2015). Molecular systematics and origin of the Mediterranean Sea rock-pool mosquitoes of the *Aedes mariae* (Diptera: Culicidae) complex. Annals of the Entomological Society of America, 108(4), 593-599.

McAllen, R. (1999). *Enteromorpha intestinalis* a refuge for the supralittoral rockpool harpacticoid copepod *Tigriopus brevicornis*. Journal of the Marine Biological Association of the United Kingdom, 79(6), 1125-1126.

Monard, A. (1935). Note préliminaire sur la faune des Harpacticoïdes marins d'Alger. Bulletin de la Station d'Aquiculture et de Pêche Castiglione, 1935(1), 45-85.

Müller, O. F. (1776). Zoologiae Danicae prodromus: seu Animalium Daniae et Norvegiae indigenarum characteres, nomina, et synonyma imprimis popularium. typis Hallageriis.

Norman, A. M. (1869). Shetland final dredging report. Part II. On the Crustacea, Tunicata, Polyzoa, Echinodermata, Actinozoa, Hydrozoa, and Porifera. Report of the British Association for the Advancement of Science, 38, 247-336.

Ortman, B. D. (2008). DNA barcoding the Medusozoa and Ctenophora. Doctoral Dissertations (AAI3345202). University of Connecticut.

Park, E. O., Lee, S., Cho, M., Yoon, S. H., Lee, Y., & Lee, W. (2014). A new species of the genus *Tigriopus* (Copepoda: Harpacticoida: Harpacticidae) from Antarctica. Proceedings of the Biological Society of Washington, 127(1), 138-154.

Powlik, J. J. (1999). Habitat characters of *Tigriopus californicus* (Copepoda: Harpacticoida), with notes on the dispersal of supralittoral fauna. Journal of the Marine Biological Association of the United Kingdom, 79(1), 85-92.

Powlik, J. J., & Lewis, A. G. (1996). Desiccation Resistance in *Tigriopus californicus* (Copepoda, Harpacticoida). Estuarine, Coastal and Shelf Science, 43(4), 521-532.

Powlik, J. J., Lewis, A. G. & Spaeth, M. (1997). Development, body length, and feeding of *Tigriopus californicus* (Copepoda: Harpacticoida) in laboratory culture and field populations. Crustaceana, 70, 324-343

Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Molecular ecology, 21(8), 1864-1877.

QGIS Development Team. (2016) QGIS Geographic Information System. Open Source Geospatial Foundation Project. Available at: http://www.qgis.org/

Ranade, M. R. (1957). Observations on the resistance of *Tigriopus fulvus* (Fischer) to changes in temperature and salinity. Journal of the Marine Biological Association of the United Kingdom, 36(1), 115-119.

Richterich, P. (1998). Estimation of errors in "raw" DNA sequences: a validation study. Genome Research, 8(3), 251-259.

Ride, W. D. J. L. (1999). International code of zoological nomenclature. International Trust for Zoological Nomenclature.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., ... & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic biology, 61(3), 539-542.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology Biology and evolutionEvolution, 4(4), 406-425.

Sars, G. O. (1911). An Account of the Crustacea of Norway: With Short Descriptions and Figures of All the Species. Copepoda. Harpacticoida. Text. Copepoda. Harpacticoida. Plates. Bergen Museum.

Schizas, N. V. (2012). Misconceptions regarding nuclear mitochondrial pseudogenes (Numts) may obscure detection of mitochondrial evolutionary novelties. Aquatic Biology, 17(1), 91–96.

Song, H., Buhay, J. E., Whiting, M. F., Crandall, K. A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proceedings of the National Academy of Sciences, 105(36),13486–13491.

Soyer, J., Thiriot-Quievreux, C., & Colomines, J. C. (1987). Description de deux espéces jumelles du groupe *Tigriopus angulatus* (Copepoda, Harpacticoida) dans les archipels Crozet et Kerguelen (Terres Australes et Antarctiques Frangaises). Zoologica Scripta, 16(2), 143-154.

Swanson, G. A. (1984). Dissemination of amphipods by waterfowl. The Journal of wildlife Wildlife managementManagement, 48(3), 988-991.

Swofford, D. L. (1993). PAUP: phylogenetic analysis using parsimony version 3.11. Computer program distributed by the Illinois Natural History Survey.

Van Douwe, C. (1913). *Tigriopus fulvus* Fisher, var. *adriatica*, ein typischer Rock pools-Copepode. Biol Zentralblatt 33:256–258

Vecchioni, L., Marrone, F., Rodilla, M., Belda, E. J., Arculeo, M. (2019). An account on the taxonomy and molecular diversity of a Mediterranean rock pool-dwelling harpacticoid copepod, *Tigriopus fulvus* (Fischer, 1860) (Copepoda, Harpacticoida). Cientias Marinas, 45(2), 59-75.

Vittor, B. A. (1971). Effects of the environment on fitness related life history characters in *Tigriopus californicus* (Doctoral dissertation, Thesis (Ph. D.) - Oregon, Dept. of Biology).

von Reumont, B. M., Meusemann, K., Szucsich, N. U., Dell'Ampio, E., Gowri-Shankar, V., Bartel, D., & Wägele, J. W. (2009). Can comprehensive background knowledge be incorporated into substitution models to improve phylogenetic analyses? A case study on major arthropod relationships. BMC Evolutionary Biology, 9(1), 119.

Waller, C. L., Worland, M. R., Convey, P., & Barnes, D. K. A. (2006). Ecophysiological strategies of Antarctic intertidal invertebrates faced with freezing stress. Polar Biology, 29(12), 1077-1083.

Walter, T.C. & Boxshall, G. (2019). World of Copepods database. *Tigriopus* Norman, 1869. Accessed through: World Register of Marine Species at: http://marinespecies.org/aphia.php?p=taxdetails&id=115351 on 2019-01-20.

Wells, J. B. J. (2007). An annotated checklist and keys to the species of Copepoda Harpacticoida (Crustacea). Zootaxa, 1568(1), 1-872.

Willet, S. W. (2012). Quantifying the Elevation of Mitochondrial DNA Evolutionary Substitution Rates Over Nuclear Rates in the Intertidal Copepod *Tigriopus californicus*. Journal of Molecular Evolution, 74, 310-318.

Williams, D. D. (2006). The biology of temporary waters. Oxford University Press.

Wilson, C. B. (1950). Contributions to the biology of the Philippine Archipelago and adjacent regions, vol. 14, pt. 4: Copepods Gathered by the United States Fisheries Steamer Albatross from 1887 to 1909, Chiefly in the Pacific Ocean. Bulletin of the United States National Museum.

Zhang, J., Kapli, P., Pavlidis, P., & Stamatakis, A. (2013). A general species delimitation method with applications to phylogenetic placements. Bioinformatics, 29(22), 2869-2876.

6. APPENDIX

6.1 PhD Research Production

In the frame of this work, the research led to the production of:

1) one paper published in an ISI-indexed journal (doi: https://doi.org/10.7773/cm.v45i2.2946)

2) a talk presented in the 79° congress of the "Unione Zoologica Italiana".

3) a second manuscript that is currently in preparation that will include both the morphological and molecular parts.

An account on the taxonomy and molecular diversity of a marine rock-pool dweller, *Tigriopus fulvus* (Copepoda, Harpacticoida)

Una revisión de la taxonomía y diversidad molecular de un habitante de las charcas litorales *Tigriopus fulvus* (Copepoda, Harpacticoida)

Luca Vecchioni[1]*, Federico Marrone[1], Miguel Rodilla[2], Eduardo J Belda[2], Marco Arculeo[1]

[1] Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Università degli Studi di Palermo, via Archirafi, 18, I-90123 Palermo, Italy.
[2] Research Institute for Integrated Management of Coastal Areas, Universitat Politècnica de València, C/Paranimf, no. 1, E-46730, Gandía (Valencia), Spain.

* Corresponding author. E-mail: luca.vecchioni@unipa.it

ABSTRACT. The copepod genus *Tigriopus* Norman, 1869 is distributed worldwide in coastal rock pools and it is currently considered to include 14 valid species. *Tigriopus fulvus* (Fischer 1860), with its subspecies *Tigriopus fulvus adriaticus* Van Douwe 1913 and *Tigriopus fulvus algiricus* Monard 1935, and *Tigriopus minutus* Bozic 1960 are currently reported to occur in the Mediterranean area, but the actual diversity of the genus is currently unknown. We aimed to assess the actual identity of Mediterranean *Tigriopus* populations and to elucidate their taxonomy and pattern of genetic diversity. In order to reach these goals, a fragment of a mitochondrial DNA gene (cytochrome c oxidase subunit I, COI) was sequenced to be used as a reference marker. Our data suggest the presence of a single species characterized by a noteworthy geographically based genetic structure in the whole study area. The observed diversity pattern is tentatively ascribed here to a strong monopolization of the rock pools by the first immigrants that reached them. However, such a monopolization is periodically disrupted by local extinction events, which are frequent in the intrinsically unstable rock pool habitats. We propose the name "clockwork monopolization" for this pattern.

Key words: genetic structuring, clockwork monopolization, rocky shore communities, cryptic species, DNA taxonomy.

RESUMEN. El género de copépodos *Tigriopus* Norman, 1869 se distribuye en todo el mundo en charcas de rocas costeras y se considera que actualmente incluye 14 especies válidas. *Tigriopus fulvus* (Fischer 1860), con sus subespecies *Tigriopus fulvus adriaticus* Van Douwe 1913 y *Tigriopus fulvus algiricus* Monard 1935, y *Tigriopus minutus* Bozic 1960 han sido descritos para el área del Mediterráneo, pero la diversidad real del género es desconocida actualmente. El objetivo de este estudio fue evaluar la identidad real de las poblaciones mediterráneas de *Tigriopus* y dilucidar su taxonomía y patrón de diversidad genética. Con este fin, se secuenció un fragmento del gen de ADN mitocondrial (citocromo c oxidasa subunidad I, COI) como marcador de referencia. Los resultados sugieren la presencia de una sola especie caracterizada por una estructuración genética con una notable base geográfica en toda el área de estudio. El patrón de diversidad observado aquí se atribuye tentativamente a una fuerte monopolización de las charcas de las costas rocosas por parte de los primeros inmigrantes que las alcanzan. Sin embargo, tal monopolización se interrumpe periódicamente por los eventos de extinción local, los cuales son frecuentes en los hábitats de charcas de rocas que son intrínsecamente inestables. Aquí proponemos para este patrón el nombre de "monopolización periódica" ("*clockwork monopolization*").

Palabas clave: estructuración genética, monopolización periódica, comunidades de costas rocosas, especies crípticas, taxonomía basada en ADN.

INTRODUCTION

Tigriopus Norman, 1869 is a copepod genus typically linked to marine rock pools in the supratidal and the uppermost intertidal zones (McAllen 1999), although it is also known to occur subtidally off the Antarctic Peninsula (Waller et al. 2006, Park et al. 2014), Mexico (Ganz and Burton 1995, Edmands 2001), Southern Asia (Jung et al. 2006, Ki et al. 2009), and Sweden (Lang 1948). The supratidal and intertidal coastal rock pools are harsh habitats characterized by the large instability of physical (e.g., temperature or water amount) and chemical conditions (e.g., oxygen content, pH, salinity) (Ganning and Wulff 1970, Underwood and

INTRODUCCIÓN

Tigriopus Norman, 1869 es un género de copépodos típicamente vinculado a las charcas que se forman en las zonas de rocas en las zonas supramareales y partes superiores del área intermareal (McAllen 1999), aunque también se sabe que ocurre de manera submareal frente a la península Antártica (Waller et al. 2006, Park et al. 2014), México (Ganz y Burton 1995, Edmands 2001), Asia meridional (Jung et al. 2006, Ki et al. 2009) y Suecia (Lang 1948). Las charcas de roca costeras en las zonas supramareales e intermareales son hábitats hostiles caracterizados por una gran inestabilidad de las condiciones físicas (por ejemplo, la temperatura o la cantidad

Open Access

Skilleter 1996). To cope with such continuously changing habitats, behavioral and\or physiological mechanisms aimed at avoiding or overcoming adverse conditions were developed by the rock-pool-inhabiting fauna, which thus became a focal point for studies of these extreme environments (Dethier 1980, Raisuddin et al. 2007).

Like some species of the beetle family Hydraenidae (Antonini et al. 2010) and mosquitoes of the family Culicidae (Mastrantonio et al. 2015) occurring in the same habitat type, *Tigriopus* developed adaptations to survive these stresses. Adult beetles and mosquitoes are able to fly away from drying pools, switching to nearby filled pools, and in other taxa, late instar larvae can enter into resting stages, or resistant eggs might be produced (Williams 2007). Conversely, *Tigriopus* spp. survive desiccation as adults in a dormant state (Battaglia 1982) and are not able to produce resistant cysts or diapausing eggs. Moreover, adult *Tigriopus californicus* may persist in dried pools for some days or weeks, even without entering the "dormancy phase", retaining themselves in small habitable habitat patches awaiting better conditions to recolonize the same rock pool (Powlik 1998). The means of dispersal of *Tigriopus* spp. are still unknown, although the passive dispersal of the species mediated by birds, floating algae, and water currents has been proposed (see discussion in Handschumacher et al. 2010)

The genus *Tigriopus* includes 14 species (Walter and Boxshall 2018), and the following are known to occur in the Atlantic–Mediterranean area: *Tigriopus brevicornis* (Müller 1776) and *Tigriopus brachydactylus* (Candeias 1959) from Africa, *Tigriopus fulvus* (Fischer 1860) from the Mediterranean Sea and the Atlantic island of Madeira, and *Tigriopus minutus* Bozic 1960 from Senegal; this last species has also been dubitatively reported to occur in the Mediterranean Sea by Lazaretto and Libertini (1986), although no other records of *T. minutus* are available for this area. The species occurring in the Atlantic–Mediterranean area can be morphologically distinguished based on a few characters whose intra- and interspecific variability has not been exhaustively examined to date, and *T. brevicornis* was not recognized as taxonomically distinct from *T. fulvus* until the late 1970s (Carli and Fiori 1977). Moreover, in the early XX century, 2 "varieties" of *T. fulvus* were described, i.e., *T. fulvus* var. *adriatica* by Van Douwe (1913) from specimens collected in Rovinj (Croatia) and *T. fulvus* var. *algirica* by Monard (1935) from specimens collected in Tipasa (currently Tipaza, Algeria); oddly, these taxa were characterized based on a morphological comparison of these populations with the Atlantic species *T. brevicornis* instead of with *T. fulvus* s.s. (Carli and Fiori 1977). According to article 45.6.4 of the International Code of Zoological Nomenclature (https://www.iczn.org/), if a taxon of infra-subspecific rank was established before 1961, it has to be considered of subspecific rank (ICZN 1999); accordingly, *T. fulvus* is currently to be considered a polytypic

de agua) y químicas (por ejemplo, el contenido de oxígeno, pH, salinidad) (Ganning y Wulff 1970, Underwood y Skilleter 1996). Para hacer frente a estos hábitats en constante cambio, la fauna que habita en las charcas de roca desarrolló mecanismos de comportamiento y/o fisiológicos destinados a evitar o superar condiciones adversas, lo que se convirtió así en un punto focal para los estudios de estos ambientes extremos (Dethier 1980, Raisuddin et al. al. 2007).

Al igual que algunas especies de escarabajos de la familia *Hydraenidae* (Antonini et al. 2010) y mosquitos de la familia *Culicidae* (Mastrantonio et al. 2015) que aparecen en el mismo tipo de hábitat, *Tigriopus* desarrolló adaptaciones para sobrevivir a estas tensiones. Los escarabajos adultos y los mosquitos pueden volar y escapar de las charcas que se están secando a otras charcas llenas cercanas y, en otros taxones, las larvas de estadios tardíos pueden entrar en las etapas de reposo, o pueden producirse huevos resistentes (Williams 2007). Por el contrario, *Tigriopus* spp. sobreviven a la desecación cuando son adultos en un estado latente (Battaglia 1982), y no pueden producir quistes de resistencia ni huevos de diapausa. Además, se ha observado que los adultos de *Tigriopus californicus* pueden sobrevivir en charcas secas durante algunos días o semanas, incluso sin entrar en "fase de latencia", manteniéndose en pequeños parches de hábitat habitables esperando mejores condiciones para recolonizar la misma charca de roca (Powlik 1998). Hasta la fecha, se desconocen los mecanismos de dispersión de *Tigriopus* spp., aunque se ha propuesto que es por dispersión pasiva mediada por aves, algas flotantes y corrientes de agua (ver discusión en Handschumacher et al. 2010).

El género *Tigriopus* incluye 14 especies (Walter y Boxshall 2018), y se sabe que las siguientes ocurren en el área del Atlántico-Mediterráneo: *Tigriopus brevicornis* (Müller 1776) y *Tigriopus brachydactylus* (Candeias 1959) en África, *T. fulvus* (Fischer 1860) en el mar Mediterráneo y la isla atlántica de Madeira, y *Tigriopus minutus* (Bozic 1960) en Senegal. Para esta última especie, Lazaretto y Libertini (1986) reportaron su ocurrencia en el mar Mediterráneo, aunque de manera dubitativa ya que hasta la fecha no hay otros registros de *T. minutus* disponibles para esta área. Las especies que se encuentran en el área atlántico-mediterránea pueden distinguirse morfológicamente en función de unos pocos caracteres cuya variabilidad intraespecífica e interespecífica no se ha examinado exhaustivamente hasta la fecha, y solo hasta finales de la década de los setenta, *T. brevicornis* fue reconocida como taxonómicamente distinta de *T. fulvus* (Carli y Fiori 1977). Además, a principios del siglo XX, se describieron 2 "variedades" de *T. fulvus*, es decir, *T. fulvus var. adriatica* por Van Douwe (1913) a partir de especímenes recolectados en Rovinj (Croacia) y *T. fulvus var. algirica* por Monard (1935) a partir de especímenes recolectados en "Tipasa" (actualmente Tipaza, Argelia). Curiosamente, estos taxones se caracterizaron con base en una comparación morfológica de estas poblaciones con la especie del Atlántico *T. brevicornis* en lugar de con *T. fulvus* s.s. (Carli y Fiori 1977). Según el

species including the 3 subspecies *Tigriopus fulvus fulvus* (Fischer 1860), *Tigriopus fulvus algiricus* Monard 1935, and *Tigriopus fulvus adriaticus* Van Douwe 1913. As stressed by Lazzaretto and Libertini (1986), the actual genetic diversity pattern of Mediterranean *Tigriopus* populations is still unknown and yet to be properly explored.

In light of the paucity of data currently available, and of the taxonomic uncertainties affecting the genus *Tigriopus* in the Mediterranean area, the goal of this work is to explore the genetic variability of *T. fulvus* and its alleged subspecies along the coasts of the Mediterranean Sea, and to contribute to the clarification of their taxonomy. Moreover, we aimed to evaluate if the noteworthy interpopulation genetic diversity observed in several Mediterranean rock-pool-inhabiting taxa (e.g., Antonini et al. 2010, Audiso et al. 2010, Mastrantonio et al. 2015) also characterizes the harpacticoid copepods of the genus *Tigriopus*.

MATERIALS AND METHODS

In order to investigate the genetic diversity occurring among *T. fulvus* populations, copepods were collected from intertidal and supratidal rock pools in 21 different locations across the Mediterranean Sea and the eastern Atlantic Ocean (Table 1, Fig. 1). In addition, specimens of the North Atlantic species, *T. brevicornis*, were collected in Galicia (Spain) to be used as outgroup. Latitude and longitude for each locality was determined with a geographical positioning system (GPS). The map of the sampling sites was done using QGIS software v. 2.18.2 (http://www.qgis.org).

Harpacticoids were sampled with a 200-μm mesh hand net. Collected specimens were fixed *in situ* in 96% ethanol, sorted out under a stereomicroscope and identified according to Wells (2007). In an attempt to identify the alleged *T. fulvus* subspecies, the identification keys provided by Monard (1935) and Van Douwe (1913) were used.

After morphological identification, specimens were dipped in double distilled water for 15 min and processed for DNA extraction using the BIORON GmbH Ron's Tissue DNA Mini Kit following the manufacturer's instructions. The selective amplification of a cytochrome c oxidase subunit I (COI) fragment was carried out by the polymerase chain reaction (PCR) using the primers L1384-COI and H2612-COI (Machida et al. 2004).

The PCR mix consisted of 18.05 μL double-distilled water, 2.5 μL Buffer 10X including 15 mM MgC_{12} solution, 0.25 μL dNTPs (10 mM of each), 0.9 μL of each primer (10 μM), 0.4 μL BIORON DFS-Taq DNA Polymerase 5U/μL, and 2 μL of DNA template, for a total volume of 25 μL. The thermal cycle consisted of 35 cycles of denaturing (95 ºC for 50 s), annealing (48 ºC for 50 s), and extension (72 ºC for 50 s), followed by 7 min at 72 ºC for the final extension step. After PCR, 5 μL of each PCR product were separated by electrophoresis on a

artículo 45.6.4 del Código Internacional de Nomenclatura Zoológica (https://www.iczn.org/), si un taxón de rango infraespecífico se estableció antes de 1961, debe considerarse de rango subespecífico; en consecuencia, *T. fulvus* se considera actualmente una especie politípica que incluye las 3 subespecies *Tigriopus fulvus fulvus* (Fischer 1860), *Tigriopus fulvus algiricus* Monard 1935 y *Tigriopus fulvus adriaticus* Van Douwe 1913. Como subrayaron Lazzaretto y Libertini (1986), el patrón real de la diversidad genética de las poblaciones mediterráneas de *Tigriopus* es hasta la fecha desconocido y debe ser explorado adecuadamente.

En vista de la escasez de datos actualmente disponibles y de las incertidumbres taxonómicas que afectan al género *Tigriopus* en el área del mar Mediterráneo, el objetivo de este trabajo es explorar la variabilidad genética de *T. fulvus* y sus supuestas subespecies a lo largo de las costas del mar Mediterráneo, y contribuir a clarificar su taxonomía. Además, también se evaluó si la notable diversidad genética interpoblacional observada en diversos taxones que habitan las charcas de rocas en el mediterráneo (e.g., Antonini et al. 2010, Audiso et al. 2010, Mastrantonio et al. 2015) también caracteriza a los copépodos harpacticoides del género *Tigriopus*.

MATERIALES Y MÉTODOS

Para investigar la diversidad genética que se produce entre las poblaciones de *T. fulvus*, se recolectaron copépodos de charcas de rocas intermareales y supramareales de 21 lugares diferentes a lo largo del mar Mediterráneo y el océano Atlántico oriental (Tabla 1, Fig. 1). Además, los especímenes de la especie del Atlántico Norte *T. brevicornis* se recolectaron en Galicia (España) para ser utilizados como un grupo externo. La latitud y la longitud para cada localidad se determinaron con un sistema de posicionamiento global (GPS, por sus siglas en inglés). El mapa de los sitios de muestreo se realizó utilizando el *software* QGIS v.2.18.2 (http://www.qgis.org).

Para muestrear los harpacticoides utilizamos una red de mano con una luz de malla de 200 μm. Las muestras recolectadas se fijaron *in situ* con etanol al 96%, se clasificaron bajo un estereomicroscopio y se identificaron según Wells (2007). En un intento por identificar las supuestas subespecies de *T. fulvus*, se utilizaron las claves de identificación proporcionadas por Monard (1935) y Van Douwe (1913).

Tras la identificación morfológica, las muestras se sumergieron en agua bidestilada durante 15 min y se procesaron para la extracción de ADN utilizando el Ron's Tissue DNA Mini Kit de BIORON GmbH siguiendo las instrucciones del fabricante. La amplificación selectiva de un fragmento de la citocromo c oxidasa subunidad I (COI) se llevó a cabo mediante la reacción en cadena de la polimerasa (RCP) utilizando los cebadores L1384-COI y H2612-COI (Machida et al. 2004).

La mezcla para la RCP consistió en 18.05 μL de agua bidestilada, 2.5 μL de tampón 10X que incluyó una

Table 1. Origin and GenBank accession numbers (A.N.) for the analyzed *Tigriopus* specimens. Geographic coordinates are expressed as decimal degrees (Map Datum: WGS84). *: specimens from the type locality of *Tigriopus fulvus algiricus*; §: specimen from the type locality of *Tigriopus fulvus adriaticus*; [†]: specimens from Madeira, the type locality of *Tigriopus fulvus* s.s.
Tabla 1. Origen y números de acceso (A.N.) a GenBank de los especímenes analizados. Las coordenadas geográficas se expresan como grados decimales (Datum del mapa: WGS84). *: especímenes de la localidad tipo de *Tigriopus fulvus algiricus*; §: ejemplar de la localidad tipo de *Tigriopus fulvus adriaticus*; [†]: especímenes de Madeira, la localidad tipo de *Tigriopus fulvus* s.s.

Taxa	Code	Sample	Country	Location	Latitude (N)	Longitude (E)	A.N.	Source
T. fulvus *	TIP	TF44	Algeria	Tipaza	36.6229	2.4081	MK211338	Present work
T. fulvus *	TIP	TF45	Algeria	Tipaza	36.6229	2.4081	MK211337	Present work
T. fulvus	ROV	TF54	Croatia	Rovigno	45.1172	13.6071	MK211332	Present work
T. fulvus	TER	TF1	Italy	Terrasini	38.1542	13.0756	MK211350	Present work
T. fulvus	TER	TF6	Italy	Terrasini	38.1542	13.0756	MK211351	Present work
T. fulvus	TER	TF7	Italy	Terrasini	38.1542	13.0756	MK211352	Present work
T. fulvus	BAR	TF2	Italy	Barcarello	38.2129	13.2916	MK211354	Present work
T. fulvus	BAR	TF8	Italy	Barcarello	38.2129	13.2916	MK211355	Present work
T. fulvus	BAR	TF9	Italy	Barcarello	38.2129	13.2916	MK211353	Present work
T. fulvus	MAG	TF3	Italy	Magnisi	37.1562	15.2369	MK211345	Present work
T. fulvus	MAG	TF10	Italy	Magnisi	37.1562	15.2369	MK211344	Present work
T. fulvus	MAG	TF11	Italy	Magnisi	37.1562	15.2369	MK211346	Present work
T. fulvus	PLE	TF12	Italy	Plemmirio	37.0021	15.3315	MK211356	Present work
T. fulvus	PLE	TF13	Italy	Plemmirio	37.0021	15.3315	MK211357	Present work
T. fulvus	MIL	TF14	Italy	Milazzo	38.2700	15.2245	MK211348	Present work
T. fulvus	MIL	TF15	Italy	Milazzo	38.2700	15.2245	MK211347	Present work
T. fulvus	MIL	TF16	Italy	Milazzo	38.2700	15.2245	MK211349	Present work
T. fulvus	COR	TF17	Italy	Cornino	38.0900	12.6583	MK211343	Present work
T. fulvus	PAN1	TF26	Italy	Pantelleria	36.7793	11.9541	MK211358	Present work
T. fulvus	PAN2	TF27	Italy	Pantelleria	36.8158	11.9263	MK211359	Present work
T. fulvus	TRI	TF37	Italy	Tricase	39.9330	18.3975	MK211333	Present work

62

Table 1 (Cont.)

| Taxa	Code	Sample	Country	Location	Latitude (N)	Longitude (E)	A.N.	Source
T. fulvus	CEF	TF38	Italy	Cefalù	38.0415	14.0218	MK211342	Present work
T. fulvus	CEF	TF39	Italy	Cefalù	38.0415	14.0218	MK211341	Present work
T. fulvus	POR	TF42	Italy	Portoscuso	39.2065	8.3763	MK211361	Present work
T. fulvus	POR	TF43	Italy	Portoscuso	39.2065	8.3763	MK211362	Present work
T. fulvus	CAS	TF46	Italy	Castiglioncello	43.4012	10.4045	MK211339	Present work
T. fulvus	CAS	TF48	Italy	Castiglioncello	43.4012	10.4045	MK211340	Present work
T. fulvus	GAR	TF83	Italy	Gargano	41.9264	15.6435	MK211331	Present work
T. fulvus	SDI	TF85	Morocco	Sidi Ifni	29.3467	−10.1961	MK211329	Present work
T. fulvus	CAT	TF87	Morocco	Cape Tamry	30.5465	−9.7180	MK211330	Present work
T. fulvus[†]	SXL	TF58	Portugal	Seixal	32.8270	−17.1145	MK211326	Present work
T. fulvus[†]	SXL	TF59	Portugal	Seixal	32.8270	−17.1145	MK211325	Present work
T. fulvus[†]	SXL	TF72	Portugal	Seixal	32.8270	−17.1145	MK211324	Present work
T. fulvus[†]	PDC	TF60	Portugal	Porto da Cruz	32.7763	−16.8264	MK211328	Present work
T. fulvus[†]	PDC	TF67	Portugal	Porto da Cruz	32.7763	−16.8264	MK211327	Present work
T. fulvus	JAV	TF49	Spain	Jàvea	38.7635	0.2050	MK211336	Present work
T. fulvus	JAV	TF50	Spain	Jàvea	38.7635	0.2050	MK211335	Present work
T. fulvus	BEN	TF52	Spain	Benitachell	38.7080	0.1664	MK211334	Present work
T. fulvus	MEN	TF78	Spain	Menorca	39.9980	3.8274	MK211360	Present work
T. fulvus	-	-	France	Banylus Sur Mer	42.4833	3.1333	AF315361	Edmands 2001
T. fulvus	-	-	Spain	Blanes	41.6666	2.8000	AF315364	Edmands 2001
T. brevicornis	GLC	TF79	Spain	Sanxenxo	42.3898	−8.7767	MK211363	Present work
T. brevicornis	GLC	TF81	Spain	Sanxenxo	42.3898	−8.7767	MK211364	Present work
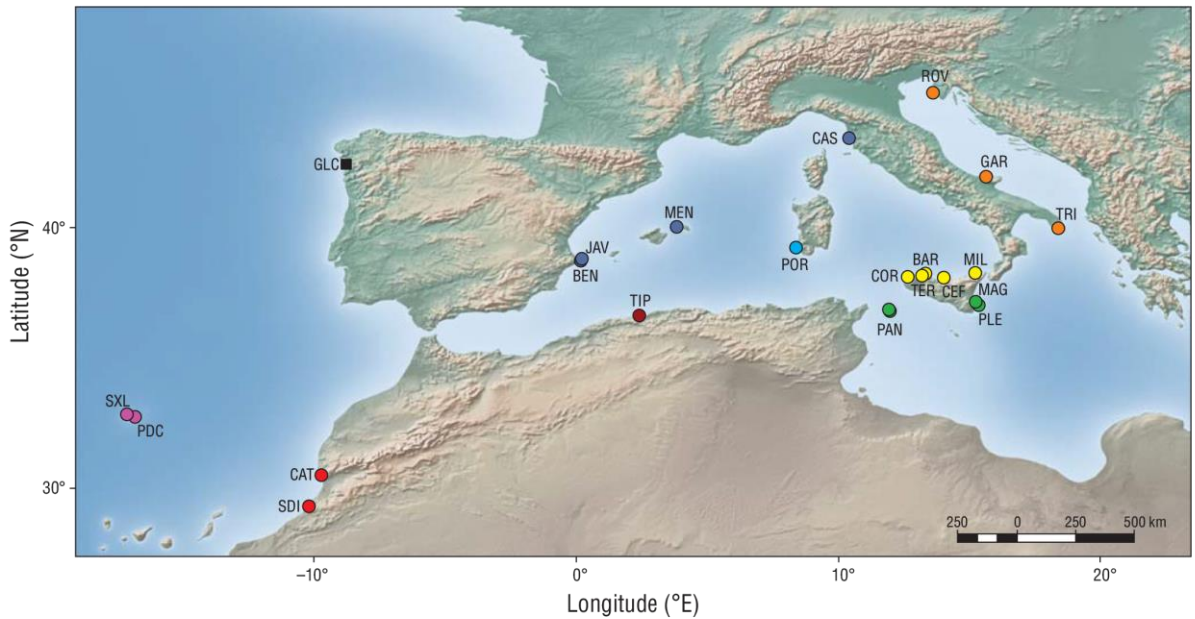
Figure 1. Geographic location of the sampling sites. Circles indicate sites where *Tigriopus fulvus* was sampled and square indicates sampling site for *Tigriopus brevicornis*. See Table 1 for the coordinates of the sampling sites and for more information on the collected species. Codes refer to those listed in Table 1. Color codes refer to those reported in Figure 2.

Figura 1. Localización geográfica de los lugares de muestreo. Los círculos indican puntos de muestreo para *Tigriopus fulvus*, y el cuadrado indica el punto de muestreo para *Tigriopus brevicornis*. Véase la Tabla 1 para las coordenadas de los sitios de muestreo, así como para más información sobre las especies recolectadas. Los códigos se refieren a los indicados en la Tabla 1. Los códigos de colores hacen referencia a la Figura 2.

2% agarose gel at 90 V for 20 min and visualized with a UV Transilluminator. When PCR products showed a clear and single band of the expected length, they were purified using the Exo-SAP-IT kit (Affymetrix USB) and sequenced by Macrogen Inc. (Seoul, South Korea) with an ABI 3130xL (Applied Biosystems) sequencer. The same primers used for the PCR were subsequently used for direct sequencing of the PCR product, and the quality of the obtained chromatograms was checked through the measurement of their Phred scores (Richterich 1998). Only sequences with continuous reads of high-quality bases ($QV > 20$) were used. Chromatograms were analyzed and manually proofread with the software Chromas v.2.6.2 (Technelysium, Pty. Ltd. 1998; Queensland, Australia) and aligned with ClustalX v.2.1 (Larkin et al. 2007).

The 39 novel mitochondrial sequences for *T. fulvus* and the 2 for *T. brevicornis* were deposited in GenBank (see Table 1 for their accession numbers). In addition, the only 2 *T. fulvus* sequences available on GenBank were downloaded and included in the analyses (see Table 1 for their accession numbers).

MEGA v.7.0 (Kumar et al. 2016) was used to translate the mtDNA sequences to amino acids in order to check for the possible presence of frameshifts or stop codons, which would indicate the presence of sequencing errors

solución de MgC_{l2} 15 mM, 0.25 µL de dNTP (10 mM de cada uno), 0.9 µL de cada cebador (10 µM), 0.4 µL de ADN polimerasa BIORON DFS-Taq 5U/µL y 2 µL de plantilla de ADN, para un volumen total de 25 µL. El ciclo térmico consistió en 35 ciclos de desnaturalización (95 ºC por 50 s), alineamiento (48 ºC por 50 s) y extensión (72 ºC por 50 s), seguido de 7 min a 72 ºC para la etapa de extensión final. Después de la RCP, se separaron 5 µL de cada producto de la RCP mediante electroforesis en un gel de agarosa al 2% a 90 V durante 20 min y se visualizaron con un transiluminador UV. Cuando los productos de RCP mostraron una banda clara y única de la longitud esperada, se purificaron utilizando el kit Exo-SAP-IT (Affymetrix USB) y se secuenciaron por Macrogen Inc. (Seúl, Corea del Sur) con un secuenciador ABI 3130xL (Applied Biosystems). Los mismos cebadores utilizados para la RCP se usaron posteriormente para la secuenciación directa del producto de la RCP, y la calidad de los cromatogramas obtenidos se verificó a través de la medición de sus puntuaciones de Phred (Richterich 1998). Solo se utilizaron secuencias con lecturas continuas de bases de alta calidad ($QV > 20$). Los cromatogramas se analizaron y revisaron manualmente con el *software* Chromas v.2.6.2 (Technelysium, Pty. Ltd. 1998; Queensland, Australia) y se alinearon con ClustalX v.2.1 (Larkin et al. 2007).

64

or pseudogenes, a widespread phenomenon among crustaceans (e.g., Song et al. 2008, Schizas 2012), and to calculate the pairwise uncorrected 'p' distance based on the entire mtDNA dataset.

The molecular identification of the studied specimens and the reconstruction of the phylogenetic relationships among the taxa was performed with Bayesian inference (BI) and maximum likelihood (ML) methods as implemented in MrBayes v.3.2.6 (Ronquist et al. 2012) and PhyML v.3 (Guindon and Gascuel 2003), respectively. As a measure of branch support, bootstrap values (Felsenstein 1985) were calculated with 1,000 replicates in the ML tree, and posterior probability values were reported on the BI tree. The choice of the best evolutionary model was made using PartitionFinder v.1.0.1 (Lanfear et al. 2012) according to the Akaike information criterion (AIC, Akaike 1974). The BI and ML analyses were performed using a general time-reversible model of sequence evolution with a proportion of invariant sites (GTR+I; number substitution types = 6). In the BI analyses, two independent Markov chain Monte Carlo analyses were run with 1 million generations (temp.: 0.2; default priors). Trees and parameter values were sampled every 100 generations, resulting in 10,000 saved trees per analysis; in the analysis, convergence was reached (effective sample size above 254.10); 2,500 trees were conservatively discarded as "burn-in".

A haplotype network including all the available *T. fulvus* COI sequences was constructed through the software PopART (v.1.7; http://popart.otago.ac.nz), using the minimum spanning method (Kruskal 1956) (Fig. S1).

In this study, the "evolutionary genetic species concept" proposed by Birky et al. (2010) was followed. According to this concept, species are inclusive populations that are evolving independently of each other, either because they are reproductively isolated, or because they are separated by environmental or physical barriers, or both. Those lineages that evolve separately from others were thus considered different taxa of putative species rank. In order to single out evolutionary lineages of species rank, DNA taxonomy approaches based on different assumptions were implemented, i.e., a quantitative approach based on coalescent model (ABGD, Puillandre et al. 2012), a phylogenetic criterion based on branching rates (bPTP, Zhang et al. 2013), and a genetic population criterion based on genetic isolation (K/Θ ratio; Birky and Barraclough 2009, Birky et al. 2010, Birky 2013). Both ABGD and bPTP methods were implemented through their online interfaces (http://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html and http://species.h-its.org/ptp/). Following Korn and Hundsdoerfer (2016), the K/Θ ratio was computed based on the uncorrected p-distance matrices within and among the detected clades. This method tests if the reciprocal monophyly of sister lineages is statistically significant, which would suggest

Las 39 nuevas secuencias mitocondriales de *T. fulvus* y las 2 de *T. brevicornis* se depositaron en GenBank (ver la Tabla 1 para conocer sus números de acceso). Además, las únicas 2 secuencias de *T. fulvus* disponibles en GenBank se descargaron e incluyeron en los análisis (ver la Tabla 1 para conocer sus números de acceso).

Para traducir las secuencias de ADN mitocondrial a aminoácidos se usó el *software* MEGA v.7.0 (Kumar et al. 2016) para, así, poder evaluar la posible presencia de mutaciones con cambios del marco de lectura o codones de parada, lo que indicaría la presencia de errores de secuenciación o pseudogenes, un fenómeno generalizado en los crustáceos (e.g., Song et al. 2008, Schizas 2012), y para calcular la comparación por pares de la distancia 'p' no corregida en función del conjunto de datos del ADN mitocondrial.

La identificación molecular de los especímenes estudiados y la reconstrucción de las relaciones filogenéticas entre los taxones se realizaron con los métodos de inferencia bayesiana (IB) y de máxima verosimilitud (MV) implementados con el *software* MrBayes v.3.2.6 (Ronquist et al. 2012) y PhyML v.3 (Guindon y Gascuel 2003), respectivamente. Para medir el soporte de rama, los valores de *bootstrap* (Felsenstein 1985) se calcularon con 1,000 repeticiones en el árbol de MV y los valores de probabilidad posteriores se reportaron en el árbol IB. La elección del mejor modelo evolutivo se realizó utilizando PartitionFinder v.1.0.1 (Lanfear et al. 2012) de acuerdo con el criterio de información de Akaike (CIA, Akaike 1974). Los análisis de IB y MV se realizaron utilizando un modelo general de tiempo reversible de evolución de secuencia con una proporción de sitios invariantes (GTR+I; número de tipo de sustituciones = 6). En los análisis de IB, se ejecutaron 2 análisis independientes de cadenas de Markov de Monte Carlo con 1 millón de generaciones (temp: 0.2; estimador previo usado por defecto). Los árboles y los valores de los parámetros se muestrearon cada 100 generaciones, lo que dio como resultado 10,000 árboles guardados por análisis; en el análisis, se alcanzó la convergencia (tamaño de muestra efectivo por encima de 254.10); 2,500 árboles fueron descartados de forma conservadora como "*burn-in*".

Se construyó una red de haplotipos que incluyó todas las secuencias COI de *T. fulvus* disponibles usando el *software* PopART (v.1.7; http://popart.otago.ac.nz), utilizando el método del árbol de expansión mínima (Kruskal 1956) (Fig. S1).

En el marco de este estudio se siguió el concepto de "especie genética evolutiva" propuesto por Birky et al. (2010). Según este concepto, las especies son poblaciones inclusivas que están evolucionando independientemente unas de otras, ya sea porque están aisladas reproductivamente, o porque están separadas por barreras ambientales o físicas, o ambas cosas. Aquellos linajes que evolucionan por separado de otros se consideraron, por lo tanto, taxones diferentes con rango de especie putativa. Con el fin de señalar los linajes evolutivos del rango de especies, se implementaron enfoques

65

that they are independently evolving entities, hence *bonae species sensu* Birky et al. (2010).

RESULTS

Overall, 41 *Tigriopus* specimens belonging to *T. fulvus* s.l. and *T. brevicornis* were analyzed, and included in the analyses (Table 1). According to the morphological study of the collected samples, the Algerian and Adriatic samples coming from the type localities of the alleged *T. fulvus* subspecies showed no consistent morphological differences when compared to the samples coming from Madeira Island, where the type locality of *T. fulvus* s.s. occurs, thus casting some doubts on the actual subspecific status of these populations. No specimens morphologically ascribable to the poorly characterized *T. minutus* were collected in this survey.

After having trimmed out the sequences, a properly aligned fragment 552 bp long of the COI mtDNA gene was obtained. All the sequences were deposited in GenBank (accession numbers MK211324–MK211364).

The BI and ML trees based on the mitochondrial COI fragment and rooted on *T. brevicornis* showed a congruent topology, with a sister–taxa relationship between the Algerian samples from the *T. fulvus algiricus* type locality and the rest of the *T. fulvus* s.l. specimens, whereas the alleged *T. f. adriaticus* sample was nested well within the ingroup (Fig. 2). Well-supported Moroccan, Madeiran, and Mediterranean *T. fulvus* clades stemmed from a basal polytomy. The Mediterranean clade showed noteworthy internal molecular structuring, with intra-clade pairwise uncorrected *p* distances ranging from 0% to 19%. Interestingly, the occurrence of private monophyletic haplogroups was observed in different Mediterranean subbasins (see Figs. 1, 2), and no haplotypes were shared among different rock pools even within subbasins.

The initial and recursive partitions of the ABGD analysis found 16 groups of putative species rank within the ingroup, with prior maximal divergence of intraspecific diversity values (*p*) ranging from 0.0129 to 0.0359 (Fig. S2). Among these 16 groups, two correspond to the Madeiran clade of *T. fulvus* s.s., as shown in the mtDNA based tree (Fig. 2). bPTP analysis estimated the presence of 21 putative species in the ingroup, i.e., finding 11 of the groups highlighted by ABGD analysis, and further splitting the other ones. Madeiran *T. fulvus* samples were ascribed to two different groups also by bPTP, as shown in Figure 2.

The K/Θ ratio showed the presence of a single species in the ingroup, with inter-clade distances (K) much lower than 4Θ, i.e., 4 times the average sequence divergence among individuals of each clade (Table 2). The K/Θ ratio thus does not support the existence of independently evolving lineages of species rank within the studied dataset.

de taxonomía de ADN basados en diferentes supuestos, es decir, un enfoque cuantitativo basado en el modelo coalescente (ABGD, por sus siglas en inglés; Puillandre et al. 2012), un criterio filogenético basado en tasas de ramificación (bPTP, Zhang et al. 2013) y un criterio de población genética basado en el aislamiento genético (relación K/Θ; Birky y Barraclough 2009, Birky et al. 2010, Birky 2013). Los métodos ABGD y bPTP se implementaron a través de sus interfaces en línea (http://www.abi.snv.jussieu. fr/public/abgd/abgdweb.html y http://species.h-its.org/ptp/). Siguiendo a Korn y Hundsdoerfer (2016), se calculó la relación K/Θ con base en las matrices de distancia *p* no corregidas dentro y entre los clados detectados. Este método prueba si la monofilia recíproca de linajes hermanos es estadísticamente significativa, lo que sugeriría que son entidades que evolucionan independientemente, y por tanto *bonae especies sensu* Birky et al. (2010).

RESULTADOS

En conjunto se recolectaron, analizaron e incluyeron en los análisis 41 especímenes de *Tigriopus* pertenecientes a *T. fulvus* s.l. y *T. brevicornis* (Tabla 1). De acuerdo con el estudio morfológico de las muestras recolectadas, las muestras argelinas y adriáticas provenientes de las localidades tipo de las supuestas subespecies de *T. fulvus* no mostraron diferencias morfológicas consistentes en la comparación con las muestras provenientes de la isla de Madeira, donde la localidad tipo de *T. fulvus* s.s. ocurre, lo que arroja algunas dudas sobre el status real de las diferentes subespecies de estas poblaciones. En este estudio no se recolectaron especímenes morfológicamente atribuibles a la especie *T. minutus*.

Después de haber recortado las secuencias, se obtuvo un fragmento correctamente alineado de 552 pb de longitud del gen COI de ADN mitocondrial. Todas las secuencias se depositaron en GenBank (números de acceso MK211324–MK211364).

Los árboles IB y MV basados en el fragmento de COI mitocondrial y basados en *T. brevicornis* mostraron una topología congruente, con una relación de taxón hermano entre las muestras argelinas de la localidad tipo de *T. fulvus algiricus* y el resto de especímenes de *T. fulvus* s.l, mientras que la supuesta muestra de *T. f. adriaticus* se enclavo bien dentro de su grupo (Fig. 2). Los clados de Marruecos, Madeira y del Mediterraneo de *T. fulvus* parten de una politomía basal bien fundamentada. El clado mediterráneo mostró una estructura molecular interna notable, con distancias *p* no corregidas por pares intra-clado que oscilaron entre 0% y 19%. Resulta interesante la aparición de haplogrupos monofiléticos privados observada en diferentes subcuencas del Mediterráneo (ver Figs. 1, 2); no se compartieron haplotipos entre diferentes charcas a nivel de subcuenca.

Las particiones iniciales y recursivas del análisis ABGD encontraron 16 grupos de especies putativas dentro del grupo, con divergencia máxima previa de valores de diversidad
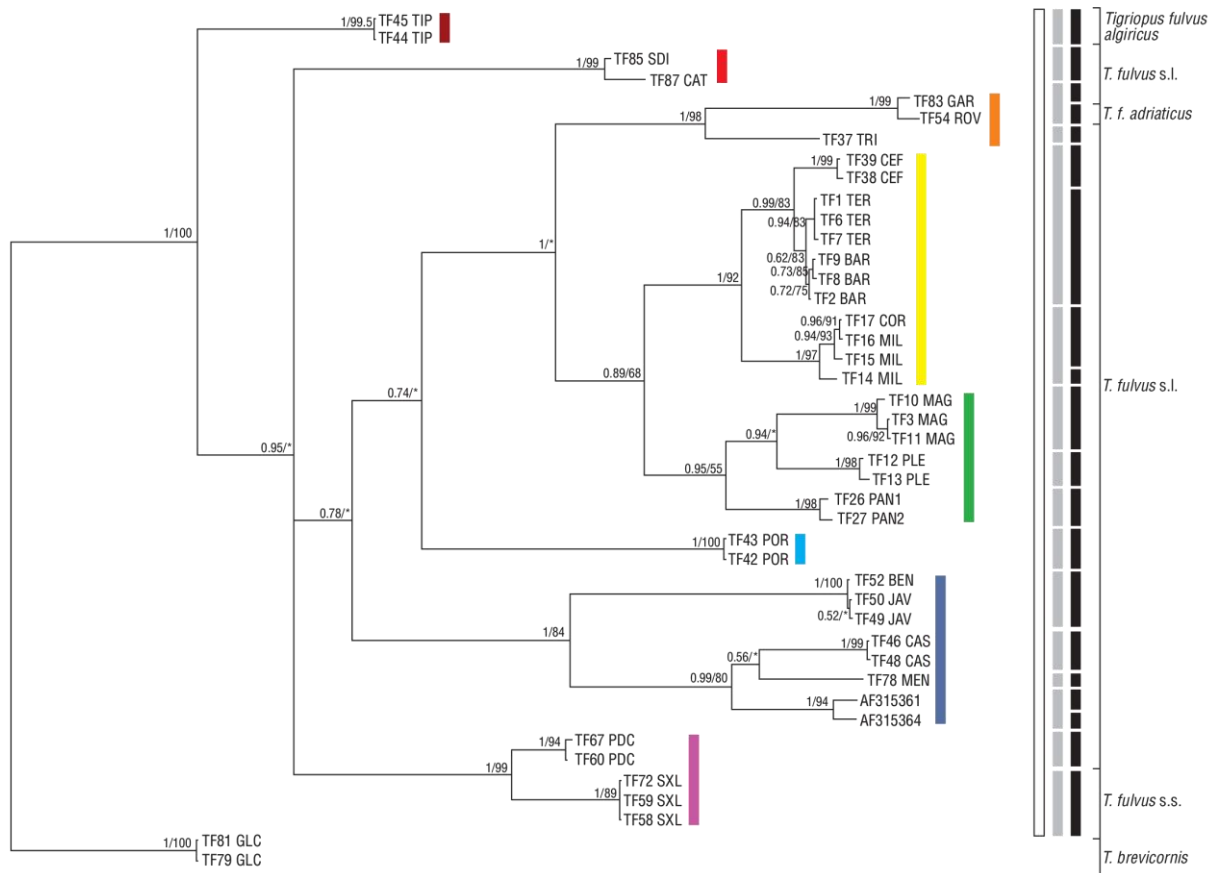
Figure 2. Bayesian phylogram (95% majority rule consensus tree) for *Tigriopus* spp. based on the 552 bp fragment of the mtDNA COI. Samples of *Tigriopus brevicornis* were used as outgroup to root the tree. Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI)/bootstrap values (Maximum Likelihood, ML). Asterisks indicate a bootstrap support value lower than 50. Rectangles refer to MOTUs as indicated by the K/Θ ratio (white rectangle), ABGD (gray rectangles), and bPTP (black rectangles). Square brackets group the samples according to the current taxonomy of the genus. The analyzed specimens are reported using the codes listed in Table 1.

Figura 2. Filograma bayesiano (árbol de consenso de la regla de mayoría al 95%) de *Tigriopus* spp. basado en el fragmento de 552 pb del ADNmt COI. Se utilizaron muestras de *Tigiropus brevicornis* como grupo externo. El soporte estadístico de los nodos se informa como probabilidades nodales posteriores (inferencia bayesiana de la filogenia, BI)/valores bootstrap (verosimilitud máxima, ML). Los asteriscos indican un valor de soporte de bootstrap inferior a 50. Los rectángulos se refieren a los MOTU indicados por la relación K/Θ (rectángulo blanco), ABGD (rectángulos grises) y bPTP (rectángulos negros). Los corchetes agrupan las muestras según la taxonomía actual del género. Los especímenes analizados se indican utilizando los códigos que se enumeran en la Tabla 1.

DISCUSSION

Taxonomical notes

The ABGD and bPTP DNA taxonomy approaches used in the frame of this work suggested the presence of an unexpectedly high number of species within the ingroup. Conversely, according to the same dataset, the K/Θ ratio identified the presence of a single species in the whole study area, namely *T. fulvus*. However, sample size is known to differently affect the accuracy of the molecular taxonomy approaches implemented in our analyses as ABGD and bPTP are more sensitive

intraespecífica (*p*) de 0.0129 a 0.0359 (Fig. S2). Entre estos 16 grupos, dos corresponden al clado de Madeira de *T. fulvus* s.s., como se muestra en el árbol basado en ADN mitocondrial (Fig. 2). El análisis de bPTP estimó la presencia de 21 especies putativas dentro del grupo, es decir, encontrando 11 de los grupos destacados por el análisis ABGD, y dividiendo los otros. Las muestras de *T. fulvus* de Madeira se asignaron a 2 grupos diferentes también por bPTP, como se muestra en la Figura 2.

La relación K/Θ mostró la presencia de una sola especie dentro del grupo, con distancias entre clados (K) mucho más bajas que 4Θ, es decir, 4 veces la divergencia de secuencia

to the size of the sample (Pulliandre et al. 2012, Zhang et al. 2013), whereas the K/Θ ratio should be not significantly influenced by the sample size (cf. Birky 2013). Moreover, it should be kept in mind that DNA taxonomy approaches just delimit primary species hypotheses, i.e., indications that, taken alone, are not sufficient to clarify the taxonomical rank to be attributed to the studied clades. It is thus necessary to adopt an integrative approach and search for a consensus between their outputs before delineating species (Fontaneto et al. 2015). For these reasons, lacking a consensus among the outputs of the different DNA taxonomy approaches implemented and pending a wider sampling coverage of *Tigriopus* molecular diversity in the whole Atlantic–Mediterranean area, we opted to rely on the results of the K/Θ ratio approach, which is considered the most conservative method among the implemented molecular taxonomy approaches (Birky and Barraclough 2009, Bode et al. 2010). Moreover, the morphological study of the samples highlighted that the morphological features used to distinguish the subspecies fall, in fact, within the internal variability of the species (L Vecchioni in prep.); this, coupled with the branching pattern of the phylogenetic tree (Fig. 2), suggests that the currently described *T. fulvus* subspecies are to be considered as junior synonyms of *T. fulvus* s.s., although a wider sampling of the overall morphological and molecular diversity of Atlantic–Mediterranean *Tigriopus* populations is desirable. Currently available morphological and molecular data therefore suggest that only *T. fulvus* occurs in the study area, but more surveys aimed at obtaining a clearer picture of its overall diversity are needed.

promedio entre los individuos de cada clado (Tabla 2). Por lo tanto, la relación K/Θ no admite la existencia de linajes que evolucionan a rango de especie de manera independiente dentro del rango de datos estudiado.

DISCUSIÓN

Notas taxonómicas

Los enfoques de taxonomía de ADN ABGD y bPTP utilizados en este trabajo sugirieron la presencia de un número inesperadamente alto de especies dentro del grupo. Por el contrario, basándose en el mismo conjunto de datos, la relación K/Θ identificó la presencia de una sola especie en toda el área de estudio, *T. fulvus*. Sin embargo, se sabe que el tamaño de la muestra afecta de manera diferente la precisión de los enfoques de taxonomía molecular implementados en nuestros análisis, ya que ABGD y bPTP son más sensibles al tamaño de la muestra (Pulliandre et al. 2012, Zhang et al. 2013), mientras que la relación K/Θ no debe verse afectada significativamente por el tamaño de la muestra (cf. Birky 2013). Además, debe tenerse en cuenta que la taxonomía basada en ADN solo delimita las hipótesis de las especies primarias, es decir, indicaciones que, tomadas por sí solas, no son suficientes para aclarar el rango taxonómico que se debe atribuir a los clados estudiados. Por lo tanto, es necesario adoptar un enfoque integrador y buscar un consenso entre sus resultados antes de delimitar las especies (Fontaneto et al. 2015). Por estas razones, al carecer de un consenso

Table 2. Application of the K/Θ ratio to *Tigriopus fulvus* s.l. mitochondrial lineages. *n*: number of individuals; *p*-dist.: uncorrected *p* distance; π: nucleotide diversity; Θ: intra-clade variation; K: inter-clade distance; Tfulvcm: *T. fulvus* from the central Mediterranean area; Tfadr: *T. f. adriaticus*; Tfalg: *T. f. algiricus*; Tfulvss: *T. fulvus* s.s. from Madeira; Tfulvsl: *T. fulvus* s.l.
Tabla 2. Aplicación de la relación K/Θ a los linajes mitocondriales de *Tigriopus fulvus* s.l.. *n*: número de individuos; *p*-dist: distancia *p* no corregida; π: diversidad de nucleótidos; Θ: variación intraclado; K: distancias interclados; Tfulvcm: *T. fulvus* del área central del Mediterráneo; Tfadr: *T. f. adriaticus*; Tfalg: *T. f. algiricus*; Tfulvss: *T. fulvus* s.s. de Madeira; Tfulvsl: *T. fulvus* s.l.

Group	n	*p*-dist.	π	4\3π	Θ	K	K\Θ ratio	Sample
Tfulvcm	19	0.112	0.118	0.157	0.140	0.188	0.868	TF1, TF2, TF3, TF6, TF7, TF8, TF9, TF10, TF11, TF12, TF13, TF14, TF15, TF16, TF17, TF26, TF27, TF38, TF39
Tfadr	3	0.112	0.168	0.224	0.216	0.188	0.868	TF37, TF54, TF83
Tfulvsp1	22	0.062	0.064	0.086	0.071	0.212	2.981	Tfulvcm + Tfadr
Tfulvsp2	2	0.002	0.005	0.006	0.005	0.212	2.981	TF42, TF43
Tfulvsp3	24	0.143	0.149	0.198	0.186	0.228	1.053	Tfulvsp1 + Tfulvsp2
Tfulvsp4	6	0.140	0.168	0.224	0.216	0.228	1.053	TF46, TF48, TF49, TF50, TF52, TF78
Tfulvsp5	30	0.033	0.034	0.045	0.035	0.225	0.357	Tfulvsp3 + Tfulvsp4
Tfulvsp6	2	0.171	0.342	0.456	0.628	0.220	2.545	TF85, TF87
Tfulvss	5	0.062	0.077	0.103	0.086	0.221	0.351	TF58, TF59, TF60, TF67, TF72
Tfulvsl	37	0.187	0.192	0.256	0.258	0.207	0.801	Tfulvsp5 + Tfulvsp6 + Tfulvss
Tfalg	2	0.001	0.002	0.003	0.002	0.207	0.801	TF44, TF45

Pattern of molecular diversity

The phylogenetic analysis of the studied Atlantic–Mediterranean *T. fulvus* populations revealed noteworthy geographic structuring of the genetic diversity among the samples (Figs. 1, 2), with inter-population mitochondrial divergence values reaching up to 19%. This is in accordance with the known occurrence of high genetic diversity in the North Atlantic *T. brevicornis* (0–21%, Handschumacher et al. 2010), the Pacific Japanese *T. japonicus* Mori 1938 (0–23%, Ki et al. 2009), and the Pacific North American *T. californicus* (Baker 1912) (0–23%, Edmands 2001). According to Handschumacher et al. (2010), the extensive inter-population divergence observed in *T. brevicornis* could be ascribed to the "paradox of Rockall". This paradox states that species with limited dispersal and scarce inter-population gene flow are very effective in colonizing remote areas after fortuitous long-range passive dispersal events (Johannesson 1988), thus resulting in the establishment of isolated but widespread populations.

Southern populations of *T. californicus* and *T. brevicornis* have been found to be genetically distinct from each other, while the northernmost populations appear to show substantially lower inter-population divergences (Edmands 2001, Handschumacher et al. 2010). This pattern is in accordance with the "Southern richness vs. Northern purity" paradigm (Hewitt 2004, Marrone et al. 2010), where the "northern purity" is attributed to the post-glacial recolonization of the northern area by a subset of the diversity survived in peripheral or southern refugia during glacial events, and the "southern richness" to the continuous persistence of the populations in these areas.

A similar pattern was observed in other species inhabiting rock pools (e.g., in the dipteran genus *Aedes*, Mastrantonio et al. 2015), whereas the high genetic structure in rock-pool dwelling coleopterans of the genus *Calobius* was considered in good accordance with the theory of the "refugia within refugia" (Gómez and Lunt 2006, Antonini et al. 2010). However, the role that recent bottlenecks and/or founder effects might have on the genetic structuring of rock–pool dwelling organisms should also be considered (cf. Audisio et al. 2010).

The high level of geographically based genetic structure observed in the Atlantic–Mediterranean *Tigriopus* populations seems in good accordance with the non-cosmopolitanism paradigm of aquatic taxa and the monopolization hypothesis (De Meester et al. 2002, Incagnone et al. 2015). This hypothesis predicts that the monopolization of water bodies by the first colonizers leads to a long-lasting persistent founder effect; this generates a structured pattern of genetic diversity that mirrors the history of colonization rather than deterministic environmental factors (e.g., Ventura et al. 2014, but see also Kappas et al. 2017). However, the present case study slightly differs from this pattern since *Tigriopus* populations are known to be frequently wiped out by physical events

entre los resultados de los diferentes enfoques de taxonomía basada en ADN implementados y en espera de una cobertura de muestreo más amplia de la diversidad molecular de *Tigriopus* en toda el área atlántico-mediterránea, optamos por confiar en los resultados del enfoque de la relación K/Θ, que se considera el método más conservador entre los enfoques de taxonomía molecular implementados (Birky y Barraclough 2009, Bode et al. 2010). Además, el estudio morfológico de las muestras destacó que las características morfológicas utilizadas para distinguir las subespecies se encuentran, de hecho, dentro de la variabilidad interna de la especie (L Vecchioni en preparación); esto, junto con el patrón de ramificación del árbol filogenético (Fig. 2), sugiere que las subespecies de *T. fulvus* descritas actualmente se consideran como un sinónimo menor de *T. fulvus* s.s., aunque un muestreo más amplio de la morfología general y de la diversidad molecular de las poblaciones de *Tigriopus* atlántico-mediterráneas es deseable. Los datos morfológicos y moleculares disponibles actualmente sugieren que solo se encuentra una especie, *T. fulvus*, en el área de estudio, aunque se necesitan más muestreos para obtener una imagen más clara de su diversidad general.

Patrón de diversidad molecular

El análisis filogenético de las poblaciones atlántico-mediterráneas de *T. fulvus* estudiadas reveló una estructuración geográfica notable de la diversidad genética entre las muestras (Figs. 1, 2), con valores de divergencia mitocondrial interpoblacional que alcanzaron hasta el 19%. Esto concuerda con la conocida presencia de una alta diversidad genética en la especie del Atlántico Norte *T. brevicornis* (0–21%, Handschumacher et al. 2010), en la especie japonesa del Pacífico *Tigriopus japonicus* Mori 1938 (0–23%, Ki et al. 2009), y en la especie norteamericana del Pacífico *T. californicus* (Baker 1912) (0–23%, Edmands 2001). Según Handschumacher et al. (2010), la gran diversidad interpoblacional observada en *T. brevicornis* podría atribuirse a la "paradoja de Rockall". La paradoja indica que las especies con una capacidad de dispersión limitada y escaso flujo genético entre poblaciones son muy efectivas en la colonización de áreas remotas después de eventos fortuitos de dispersión pasiva de largo alcance (Johannesson 1988), lo que resulta en el establecimiento de poblaciones aisladas, pero con una distribución extensa.

Se ha encontrado que las poblaciones del sur de *T. californicus* y *T. brevicornis* son genéticamente distintas unas de otras, mientras que las poblaciones más al norte parecen presentar divergencias interpoblaciónales sustancialmente más bajas (Edmands 2001, Handschumacher et al. 2010). Este patrón concuerda con el paradigma de "riqueza del sur frente a la pureza del norte" (Hewitt 2004, Marrone et al. 2010), donde la "pureza del norte" se atribuye a la recolonización postglacial de la zona norte por un subconjunto de la diversidad que sobrevivió en refugios periféricos

(e.g., exceptional droughts, storms, or rainfalls), leading to recurrent local extinctions and recolonizations. Accordingly, those *Tigriopus* haplotypes that colonize a *Tigriopus*-free rock pool rapidly monopolize it preventing the establishment of other haplotypes, but this monopolization only lasts a relatively short amount of time, i.e., until a new event wipes this population out. Thus, unlike in the monopolization hypothesis, a long-lasting founder effect is not achieved, but rather just a temporary occurrence of monopolizing haplotypes can be observed. Such a pattern could be defined as a "clockwork monopolization", related to the great instability of the rock pool habitats and to the inability of their inhabitants to produce long-lasting resting stages.

REFERENCES

Akaike H. 1974. A New look at the statistical model identification. IEEE Trans. Automat. Contr. 19(6): 716–723.
https://doi.org/10.1109/TAC.1974.1100705

Antonini G, Audisio P, Mancini E, De Biase A, Tronci C, Rossetti G, Trizzino M. 2010. Molecular phylogeography of two Italian sibling species of *Calobius* (Coleoptera, Hydraenidae, Ochthebiinae) inhabiting mediterranean marine rock-pools. Mar. Biol. 157(2): 371–381.
https://doi.org/10.1007/s00227-009-1324-9

Audisio P, Trizzino M, De Biase A, Rossetti G, Mancini E, Antonini G. 2010. Molecular and morphological evidence of a new sibling species of *Calobius* (Coleoptera: Hydraenidae) of the *C. quadricollis* complex from peninsular Italy. Ital. J. Zool. 77(1): 29–37.
https://doi.org/10.1080/11250000902845738

Battaglia B. 1982. Genetic variation and speciation events in marine copepods. In: Liss AR (ed.), Mechanisms of Speciation. New York: A.R. Liss Inc.; pp. 377–392.

Birky CW, Adams J, Gemmel M, Perry J. 2010. Using population genetic theory and DNA sequences for species detection and identification in asexual organisms. PLoS ONE 5(5). e1069.
https://doi.org/10.1371/journal.pone.0010609

Birky CW Jr, Barraclough TG. 2009. Asexual Speciation. In: Schön I, Martens K, Dijk P (eds.), Lost Sex. Dordrecht (Netherlands): Springer.
https://doi.org/10.1007/978-90-481-2770-2_10

Birky CW Jr. 2013. Species detection and identification in sexual organisms using population genetic theory and DNA Sequences. PLoS ONE 8. e52544.
https://doi.org/10.1371/journal.pone.0052544

Bode SNS, Adolfsson S, Lamatsch DK, Martins MJF, Schmit O, Vandekerkhove J, Mezquita F, Namiotko T, Rossetti G, Schön I et al. 2010. Exceptional cryptic diversity and multiple origins of parthenogenesis in a freshwater ostracod. Mol. Phyl. Evol. 54(2): 542–552.
https://doi.org/10.1016/j.ympev.2009.08.022

o del sur durante los periodos glaciares, y la "riqueza del sur" a la persistencia continua de las poblaciones en estas áreas.

Un patrón similar se ha encontrado en otras especies que habitan en las charcas de roca (e.g., en el género díptero *Aedes*, Mastrantonio et al. 2015), en tanto que la alta estructuración genética de los coleópteros del género *Calobius* que hábitan en estas charcas de rocas fue considerada por Antonini et al. (2010) en concordancia con la teoría de los "refugios dentro de los refugios" (Gómez y Lunt 2006, Antonini et al. 2010). No obstante, también debe considerarse el papel que los cuellos de botella recientes y/o el efecto fundador podrían tener en la estructuración genética de los organismos que habitan en las charcas de las rocas (cf. Audisio et al. 2010).

El alto nivel de estructuración genética a nivel geográfico observada en las poblaciones atlántico-mediterráneas de *Tigriopus* parece concordar con el paradigma de no cosmopolitismo de los taxones acuáticos y la hipótesis de monopolización (De Meester et al. 2002, Incagnone et al. 2015). La hipótesis mencionada predice que la monopolización de los cuerpos de agua por parte de los colonizadores primarios conduce a un efecto fundador persistente y duradero; esto genera un patrón estructurado de diversidad genética que refleja la historia de la colonización en lugar de factores ambientales deterministas (e.g., Ventura et al. 2014, pero vea también Kappas et al. 2017). Sin embargo, los resultados del presente estudio difieren ligeramente de este patrón, ya que se sabe que las poblaciones de *Tigriopus* desaparecen con frecuencia por eventos físicos (e.g., sequías excepcionales, tormentas o lluvias) que conducen a extinciones locales y recolonizaciones recurrentes. En consecuencia, los haplotipos de *Tigriopus* que colonizan una charcas de rocas libre de *Tigriopus* la monopolizan rápidamente impidiendo el establecimiento de otros haplotipos, pero esta monopolización solo dura un tiempo relativamente corto, es decir, hasta que un nuevo evento borre a esta población. Por lo tanto, a diferencia de la hipótesis de monopolización, no se logra un efecto fundador de larga duración, sino que solo se puede observar una ocurrencia temporal de haplotipos monopolizantes. Tal patrón podría definirse como una "monopolización periódica" ("*clockwork monopolization*", relacionada con la gran inestabilidad de los hábitats de las charcas de roca y la incapacidad de sus habitantes para producir etapas de resistencia de larga duración.

Carli A, Fiori A. 1977. Morphological analysis of the two *Tigriopus* species found along the European coasts. Soc. Ital. Sci. Nat. Mus. Civ. Stor. Nat. e Acquar. Civ. Milano. 68: 101–110.

De Meester L, Gómez A, Okamura B, Schwenk K. 2002. The monopolization hypothesis and the dispersal–gene flow paradox in aquatic organisms. Acta Oecologica. 23(3): 121–135. https://doi.org/10.1016/S1146-609X(02)01145-1

Dethier MN. 1980. Tidepools as refuges: Predation and the limits of the harpacticoid copepod *Tigriopus californicus* (Baker). J. Exp. Mar. Bio. Ecol. 42(2): 99–111. https://doi.org/10.1016/0022-0981(80)90169-0

Edmands S. 2001. Phylogeography of the intertidal copepod *Tigriopus californicus* reveals substantially reduced population differentiation at northern latitudes. Mol. Ecol. 10(7): 1743–1750. https://doi.org/10.1046/j.0962-1083.2001.01306.x

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 39(4): 783–791. https://doi.org/10.2307/2408678

Fontaneto D, Flot JF, Tang CQ. 2015. Guidelines for DNA taxonomy, with a focus on the meiofauna. Mar. Biodivers. 45(3): 433–451. https://doi.org/10.1007/s12526-015-0319-7

Ganning B, Wulff F. 1970. Measurements of community metabolism in some baltic brackish water rockpools by means of diel oxygen curves. Oikos, Nord Soc. 21(2): 292–298. https://doi.org/10.2307/3543686

Ganz HH, Burton RS. 1995. Genetic differentiation and reproductive incompatibility among Baja California populations of the copepod *Tigriopus californicus*. Mar. Biol. 123(4): 821–827. https://doi.org/10.1007/BF00349126

Gómez A, Lunt DH. 2006. Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: Weiss S, Ferrand N (eds.), Phylogeography of Southern European Refugia. Netherlands: Springer, pp. 155–188. https://doi.org/10.1007/1-4020-4904-8_5

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52(5): 696–704. https://doi.org/10.1080/10635150390235520

Handschumacher L, Steinarsdóttir MB, Edmands S, Ingólfsson A. 2010. Phylogeography of the rock-pool copepod *Tigriopus brevicornis* (Harpacticoida) in the northern North Atlantic, and its relationship to other species of the genus. Mar. Biol. 157(6): 1357–1366. https://doi.org/10.1007/s00227-010-1415-7

Hewitt GM. 2004. Genetic consequences of climatic oscillations in the Quaternary. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 359(1442): 183–95. https://doi.org/10.1098/rstb.2003.1388

Incagnone G, Marrone F, Barone R, Robba L, Naselli-Flores L. 2015. How do freshwater organisms cross the "dry ocean"? A review on passive dispersal and colonization processes with a special focus on temporary ponds. Hydrobiologia 750(1): 103–123. https://doi.org/10.1007/s10750-014-2110-3

[ICZN] International Commission on Zoological Nomenclature. 1999. International Code of Zoological Nomenclature. 4th edition). London (United Kingdom): International Trust for Zoological Nomenclature; 306 pp.

Johannesson K. 1988. The paradox of Rockall: why is a brooding gastropod (*Littorina saxatilis*) more widespread than one having a planktonic larval dispersal stage (*L. littorea*)? Mar. Biol. 99(4): 507–513. https://doi.org/10.1007/BF00392558

Jung SO, Lee YM, Park TJ, Park HG, Hagiwara A, Leung KMY, Dahms HU, Lee W, Lee JS. 2006. The complete mitochondrial genome of the intertidal copepod *Tigriopus* sp. (Copepoda, Harpactidae) from Korea and phylogenetic considerations. J. Exp. Mar. Bio. Ecol. 333(2): 251–262. https://doi.org/10.1016/j.jembe.2005.12.047

Kappas I, Mura G, Synefiaridou D, Marrone F, Alfonso G, Alonso M, Abatzopoulos TJ. 2017. Molecular and morphological data suggest weak phylogeographic structure in the fairy shrimp *Streptocephalus torvicornis* (Branchiopoda, Anostraca). Hydrobiologia 801(1): 21–32. https://doi.org/10.1007/s10750-017-3203-6

Ki JS, Lee KW, Park HG, Chullasorn S, Dahms HU, Lee JS. 2009. Phylogeography of the copepod *Tigriopus japonicus* along the Northwest Pacific rim. J. Plankton. Res. 31(2): 209–221. https://doi.org/10.1093/plankt/fbn100

Korn M, Hundsdoerfer AK. 2016. Molecular phylogeny, morphology and taxonomy of Moroccan *Triops granarius* (Lucas, 1864) (Crustacea: Notostraca), with the description of two new species. Zootaxa 4178(3): 328–346. https://doi.org/10.11646/zootaxa.4178.3.2

Kruskal JB. 1956. On the shortest spanning subtree of the graph and the travelling salesman problem. Proc. Amer. Math. Soc. 7(1): 48–50. https://doi.org/10.1090/S0002-9939-1956-0078686-7

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Lanfear R, Calcott B, Ho SYW, Guidon S. 2012. PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol. Biol. Evol. 29(6): 1695–1701. https://doi.org/10.1093/molbev/mss020

Lang K. 1948. Monographe der Harpacticiden I, II. [Reprint]. Konigsten (Germany): Otto Koeltz Science Publ; p. 1682.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23(21): 2947–2948. https://doi.org/10.1093/bioinformatics/btm404

Lazzaretto I, Libertini A. 1986. Karyological comparison among different Mediterranean populations of the genus *Tigriopus* (Copepoda Harpacticoida). Bolletino di Zool. 53(2): 197–201. https://doi.org/10.1080/11250008609355503

Machida RJ, Miya MU, Nishida M, Nishida S. 2004. Large-scale gene rearrangements in the mitochondrial genomes of two calanoid copepods *Eucalanus bungii* and *Neocalanus cristatus* (Crustacea), with notes on new versatile primers for the srRNA and COI genes. Gene. 332: 71–78. https://doi.org/10.1016/j.gene.2004.01.019

Marrone F, Brutto SL, Arculeo M. 2010. Molecular evidence for the presence of cryptic evolutionary lineages in the freshwater copepod genus *Hemidiaptomus* G.O. Sars, 1903 (Calanoida, Diaptomidae). Hydrobiologia 644(1): 115–125. https://doi.org/10.1007/s10750-010-0101-6

Mastrantonio V, Porretta D, Bellini R, Nascetti G, Urbanelli S. 2015. Molecular systematics and origin of the Mediterranean Sea rock-pool mosquitoes of the *Aedes mariae* (Diptera: Culicidae) Complex. Ann. Entomol. Soc. Am. 108(4): 593–599. https://doi.org/10.1093/aesa/sav031

McAllen R. 1999. *Enteromorpha intestinalis*–A refuge for the supralittoral rock-pool harpacticoid copepod *Tigriopus brevicornis*. J. Mar. Biol. Assoc. United Kingdom. 79(6): 1125–1126. https://doi.org/10.1017/S0025315499001393

Monard A. 1935. Note préliminaire sur la faune des Harpacticoides marins d'Alger. Bull. des Trav la Stn d'aquiculture pêche Castiglione 1–85.

71

Park EO, Lee S, Cho M, Yoon SH, Lee Y, Lee W. 2014. A new species of the genus *Tigriopus* (Copepoda: Harpacticoida: Harpacticidae) from Antarctica. Proc. Biol. Soc. Washingt. 127(1): 138–154.
https://doi.org/10.2988/0006-324X-127.1.138

Powlik JJ. 1998. Seasonal abundance and population flux of *Tigriopus californicus* (Copepoda: Harpacticoida) in Barkley Sound, British Columbia. J. Mar. Biol. Assoc. United Kingdom. 78(2): 467–481.
https://doi.org/10.1017/S0025315400041564

Puillandre N, Lambert A, Brouillet S, Achaz G. 2012. ABGD, Automatic barcode gap discovery for primary species delimitation. Mol. Ecol. 21(8): 1864–1877.
https://doi.org/10.1111/j.1365-294X.2011.05239.x

Raisuddin S, Kwok KWH, Leung KMY, Schlenk D, Lee JS. 2007. The copepod *Tigriopus*: A promising marine model organism for ecotoxicology and environmental genomics. Aquat. Toxicol. 83(3): 161–173.
https://doi.org/10.1016/j.aquatox.2007.04.005

Richterich P. 1998. Estimation of errors in "raw" DNA sequences: A validation study error rate prediction accuracy for six projects. Genome Res. 8(1): 251–259.
https://doi.org/10.1101/gr.8.3.251

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61(3): 539–542.
https://doi.org/10.1093/sysbio/sys029

Schizas NV. 2012. Misconceptions regarding nuclear mitochondrial pseudogenes (Numts) may obscure detection of mitochondrial evolutionary novelties. Aquat. Biol. 17(1): 91–96.
https://doi.org/10.3354/ab00478

Song H, Buhay JE, Whiting MF, Crandall KA. 2008. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proc. Natl. Acad. Sci. 105(36): 13486–13491.
https://doi.org/10.1073/pnas.0803076105

Underwood AJ, Skilleter GA. 1996. Effects of patch-size on the structure of assemblages in rock pools. J. Exp. Mar. Bio. Ecol. 197(1): 63–90.
https://doi.org/10.1016/0022-0981(95)00145-X

Van Douwe C. 1913. *Tigriopus fulvus* Fisher, var. adriatica, ein typischer Rock pools-Copepode. Biol. Zentralblatt. 33: 256–258.

Ventura M, Petrusek A, Miró A, Hamrová E, Buñay D, De Meester L, Mergeay J. 2014. Local and regional founder effects in lake zooplankton persist after thousands of years despite high dispersal potential. Mol. Ecol. 23(5): 1014–1027.
https://doi.org/10.1111/mec.12656

Waller CL, Worland MR, Convey P, Barnes DKA. 2006. Ecophysiological strategies of Antarctic intertidal invertebrates faced with freezing stress. Polar. Biol. 29(12): 1077–1083.
https://doi.org/10.1007/s00300-006-0152-3

Walter TC, Boxshall G. 2018. World of Copepods database. *Tigriopus fulvus* (Fischer, 1860). [Place of publication unknown]: World Register of Marine Species; [2018 Nov 15] http://www.marinespecies.org/aphia.php?p=taxdetails&id=116184.

Wells JBJ. 2007. An annotated checklist and keys to the species of Copepoda Harpacticoida (Crustacea). Auckland (New Zealand): Magnolia Press. (Zootaxa; vol. 1568); ISBN 978-1-86977-110-2.

Williams DD. 2007. The Biology of Temporary Waters. London: Oxford University Press; ISBN: 9780198528128.

Zhang J, Kapli P, Pavlidis P, Stamatakis A. 2013. A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29(22): 2869–2876.
https://doi.org/10.1093/bioinformatics/btt499
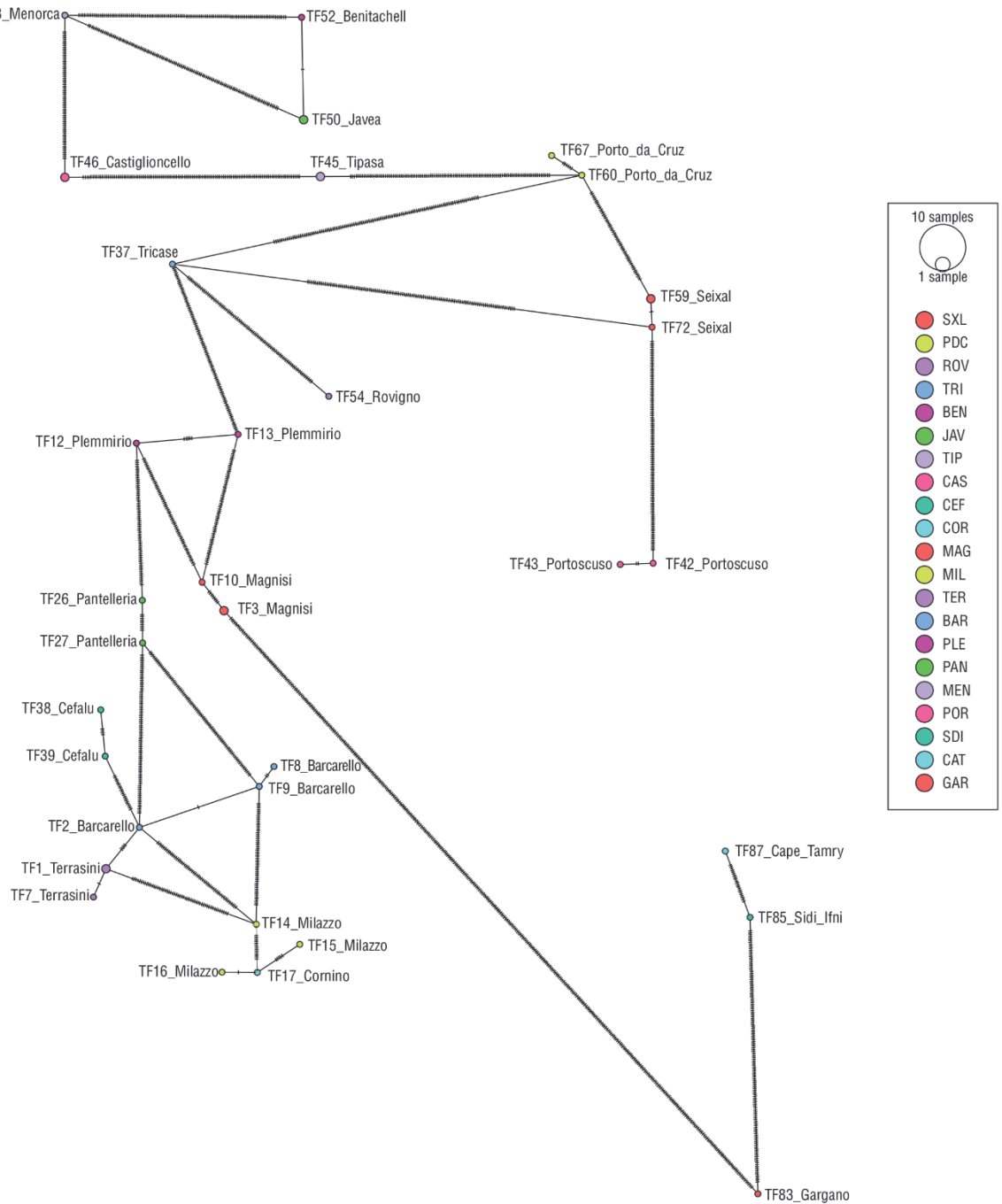
82

Supplementary material

Figure S1. Minimum-spanning haplotype network based on a 552-bp long fragment of the mtDNA COI of *Tigriopus fulvus*. Dashes indicate substitution steps. Each circle represents a haplotype and its size is proportional to its frequency. The analyzed specimens are reported using the codes listed in Table 1.

Figura S1. Red de haplotipos de expansión mínima basada en un fragmento de 552 pb de largo del ADN mitocondrial del gen COI de *Tigriopus fulvus*. Los guiones indican sustituciones. Cada círculo representa un haplotipo y su tamaño es proporcional a su frecuencia. Los especímenes analizados se identifican con los códigos que se enumeran en la Tabla 1.
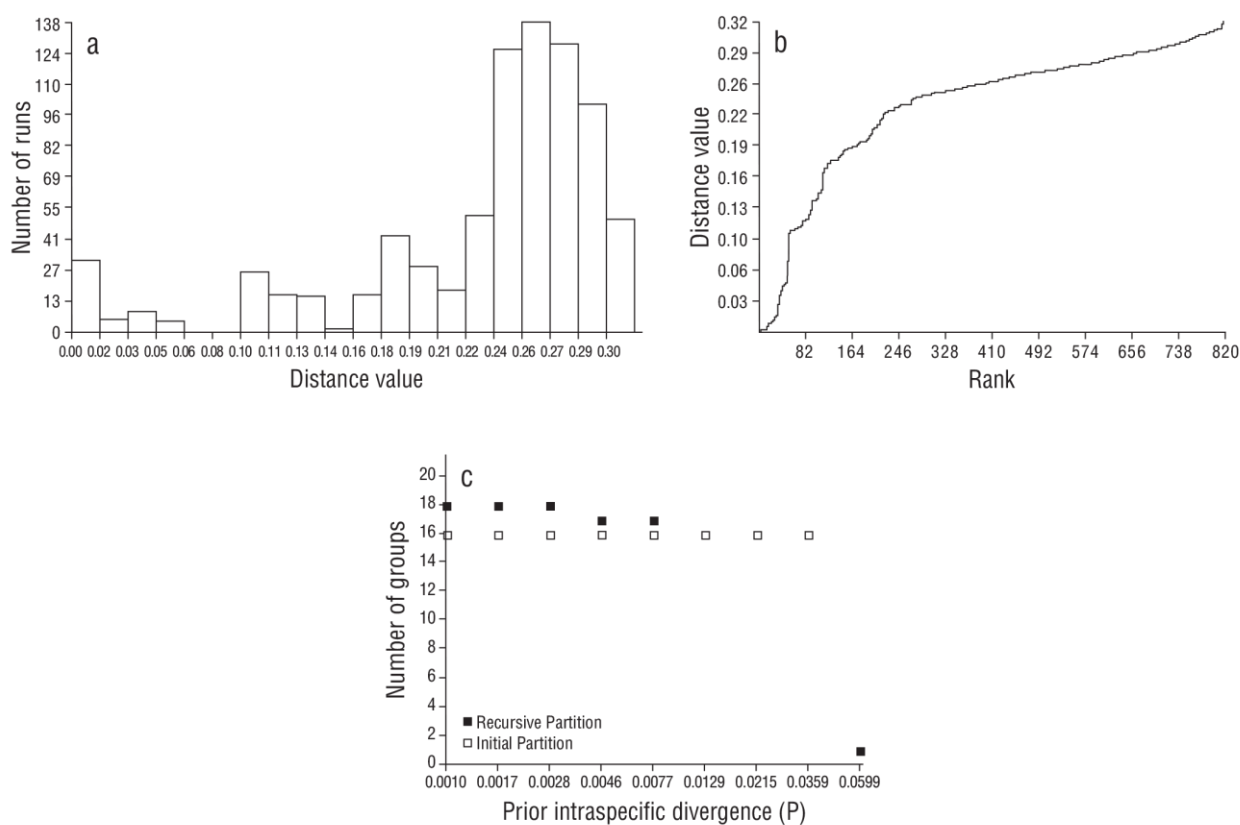
Figure S2. Results of the ABGD analysis using distances based on the K2p model, and the mtDNA dataset. (**a**) Hypothetical distribution of pairwise differences; (**b**) ranked pairwise differences; (**c**) automatic partition of the dataset. The number of groups inside the partitions (initial and recursive) are reported as a function of the prior limit between intra- and interspecies divergence.

Figura S2. Resultados del análisis ABGD utilizando distancias basadas en el modelo K2p, con base en el conjunto de datos de ADN mitocondrial. (**a**) Distribución hipotética de las diferencias por pares; (**b**) las diferencias por pares ordenadas; (**c**) partición automática del conjunto de datos. El número de grupos dentro de las particiones (inicial y recursivo) se identifican en función del límite anterior entre la divergencia intraespecífica e interespecífica.
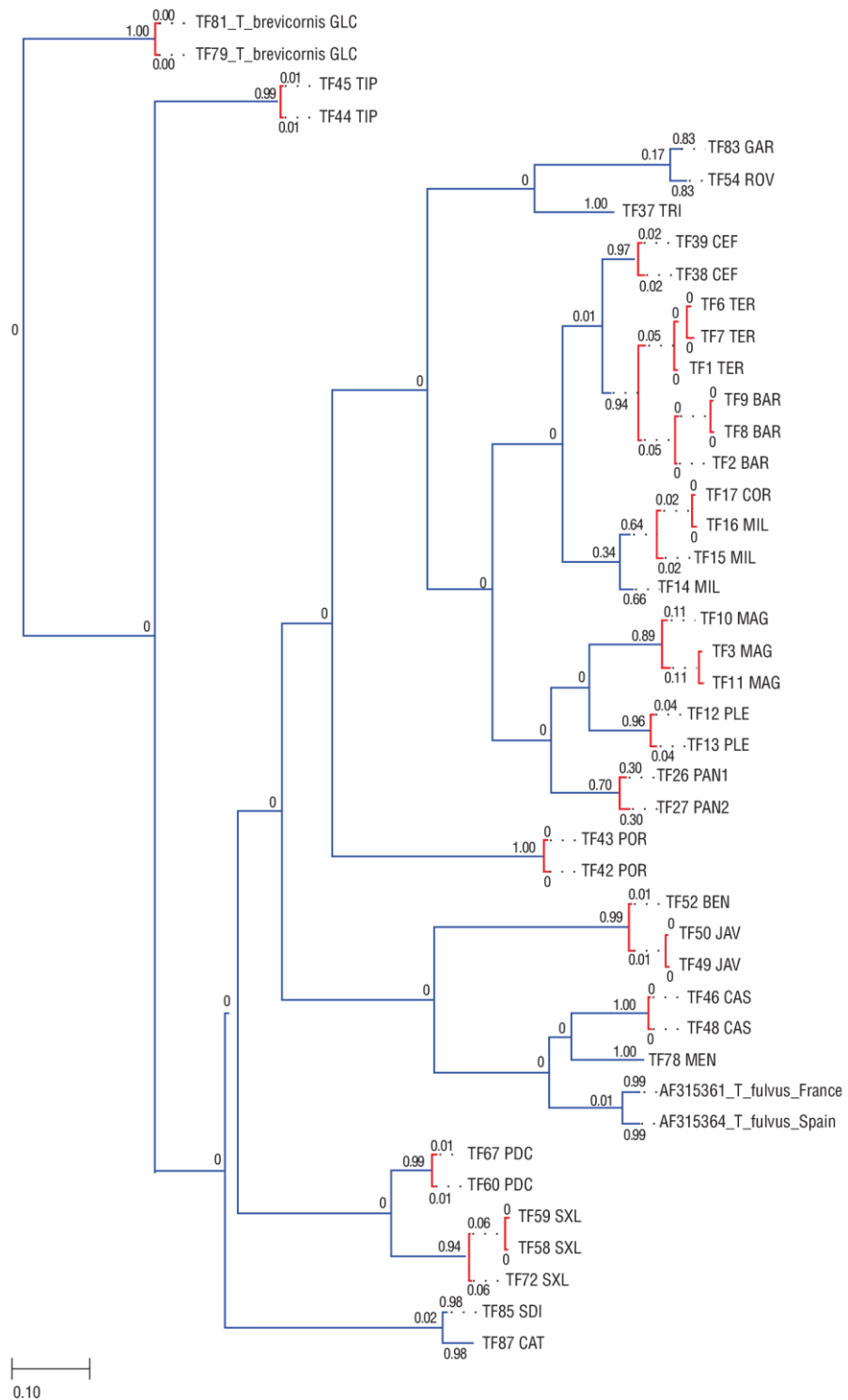
Figure S3. Putative species singled out by the bPTP model. The analyzed specimens are reported using the codes listed in Table 1.

Figura S3. Especies putativas señaladas por el modelo bPTP. Los especímenes analizados se identifican con los códigos que se enumeran en la Tabla 1.

LUCA VECCHIONI[1], FEDERICO MARRONE[1], MIGUEL RODILLA[2], EDUARDO J. BELDA[2], MARCO ARCULEO[1]

1 Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Università degli Studi di Palermo, via Archirafi,18, I-90123 Palermo, Italy.
2 Research Institute for Integrated Management of Coastal Areas, Universitat Politècnica de València, C/Paranimf 8nº 1, E-46730, Gandía (Valencia), Spain.

SYSTEMATICS AND PHYLOGEOGRAPHY OF *TIGRIOPUS FULVUS* (COPEPODA, HARPACTICOIDA, HARPACTICIDAE) IN THE MEDITERRANEAN SEA AND EASTERN ATLANTIC OCEAN

The copepod genus *Tigriopus* Norman, 1869 is worldwide distributed in coastal rock-pools and presently includes 12 valid species. In the Mediterranean and eastern Atlantic Ocean, the species *Tigriopus fulvus* (Fischer, 1860), its subspecies *T. f. adriaticus* Van Douwe, 1913 and *T. f. algiricus* Monard, 1935, and possibly *Tigriopus minutus* Božić 1960 are reported to occur, but a sound revision of the group based on modern morphological and molecular standards is to date lacking.

We used phylogenetic and coalescence-based approaches to assess the diversity, distribution and phylogeography of *Tigriopus* populations throughout the study area, and to investigate the relationships among the aforementioned taxa, including in the analyses also a population of the closely-related North Atlantic *T. brevicornis* (Müller, 1776).

Our results indicated that all the studied Mediterranean and eastern Atlantic populations studied belong to *T. fulvus* s.s, and no support was found for the taxa of subspecific rank described for the species. Moreover, the high level of inter-populations mitochondrial DNA differentiation and the absence of shared haplotypes among different populations of *T. fulvus* revealed a pronounced molecular structuring on geographical basis even for small geographical distances.

6.2 Publications

Marrone, F., **Vecchioni, L**., Deidun, A., Mabrouki, Y., Abdeslam, A., Arculeo M. (2020). DNA taxonomy of the potamid freshwater crabs from Northern Africa (Decapoda, Potamidae). Zoologica Scripta. (*In press*)

Vecchioni, L., Sala, J., Arculeo, M., Marrone, F. (2020). On the occurrence of *Eudiaptomus gracilis* (G.O. Sars, 1863) (Copepoda: Diaptomidae) in Madeira (Portugal). Limnetica. (*In press*)

Giovos, I., Arculeo, M., Doumpas, N., Katsada, D., Maximiadi, M., Mitsou, E., Paravas, V., Naasan Aga-Spyridopoulou, R., Stoilas, V.O., Tiralongo, F., Tsamadias, I., **Vecchioni, L**., Moutopoulos, D.K. (2019). Assessing multiple sources of data to detect illegal fishing, trade and mislabelling of elasmobranchs in Greek markets. Marine Policy. doi:10.1016/j.marpol.2019.103730.

Faraone, F.P., Barraco, L., Giacalone, G., Muscarella, C., Schifani, E., **Vecchioni, L**. (2019). First records of the Brahminy blindsnake, *Indotyphlops braminus* (Daudin , 1803), in Italy (Squamata : Typhlopidae). Herpetology Notes, 12: 1225–1229.

Arizza, V., **Vecchioni, L**., Caracappa, S., Sciurba, G., Berlinghieri, F., Gentile, A., Persichetti, M.F., Arculeo, M., Alduina, R. (2019). New insights into the gut microbiome in loggerhead sea turtles *Caretta caretta* stranded on the Mediterranean coast. PLoS ONE, 14(8): 1–14. doi:10.1371/journal.pone.0220329.

Vecchioni, L., Marrone, F., Naselli-Flores, L. (2019). On the occurrence and distribution of *Calanipeda aquaedulcis* Kritschagin, 1873 (Copepoda, Calanoida, Pseudodiaptomidae) in Sicily, Italy, with some notes on coexistence and species replacement in calanoid copepods. Advances in Oceanography and Limnology, 10(1): 18–23. doi:10.4081/aiol.2019.8177.

Vecchioni, L., Marrone, F., Rodilla, M., Belda, E.J., Arculeo, M. (2019). An account on the taxonomy and molecular diversity of a marine rock-pool dweller, *Tigriopus fulvus* (Copepoda, Harpacticoida). Ciencias Marinas, 45(2): 59–75. doi:10.7773/cm.v45i2.2946.

Vecchioni, L., Marrone, F., Deidun, A., Adepo-Gourene, B., Froglia, C., Sciberras, A., Bariche, M., Burak, A.Ç., Foka-Corsini, M., Arculeo, M. (2019). DNA Taxonomy Confirms the Identity of the Widely-Disjunct Mediterranean and Atlantic Populations of the Tufted Ghost Crab *Ocypode cursor* (Crustacea: Decapoda: Ocypodidae). Zoological Science, 36(4): 322. doi:10.2108/zs180191.

Belaiba, E., Marrone, F., **Vecchioni, L**., Bahri-Sfar, L., Arculeo, M. (2019). An exhaustive phylogeny of the combtooth blenny genus *Salaria* (Pisces, Blenniidae) shows introgressive hybridization and lack of reciprocal mtDNA monophyly between the marine species *Salaria basilisca* and *Salaria pavo*. Molecular Phylogenetics and Evolution, 135: 210–221. doi:10.1016/j.ympev.2019.02.026.

Vecchioni, L., Marrone, F., Belaiba, E., Tiralongo, F., Bahri-Sfar, L., Arculeo, M. (2019). The DNA barcoding of Mediterranean combtooth blennies suggests the paraphyly of some taxa (Perciformes, Blenniidae). Journal of Fish Biology, 94(2): 339–344. doi:10.1111/jfb.13897.

Vecchioni, L., Marrone, F., Arculeo, M., Meyer-Rochow, V.B. (2018). On the Occurrence of the Invasive Freshwater Limpet *Ferrissia californica* (Mollusca: Gastropoda: Planorbidae) in Japan. Zoological Science, 35(2): 149–152. doi:10.2108/zs170149.

Vecchioni, L., Deidun, A., Sciberras, J., Sciberras, A., Marrone, F., Arculeo, M. (2017). The late Pleistocene origin of the Italian and Maltese populations of *Potamon fluviatile* (Malacostraca: Decapoda): insights from an expanded sampling of molecular data. European Zoological Journal, 84(1): 575–582. doi:10.1080/24750263.2017.1405084.

Vecchioni, L., Marrone, F., Arculeo, M., Arizza, V. (2017). Are there autochthonous *Ferrissia* (Mollusca: Planorbidae) in the Palaearctic? Molecular evidence of a widespread North American invasion of the Old World. European Zoological Journal, 84(1): 411–419. doi:10.1080/24750263.2017.1350759.

6.3 Proceedings

Vecchioni, L., Arculeo, M., Stoch, F., Ketmaier, V., Marrone, F. (2019). Diversity and phylogeography of the fairy shrimp *Chirocephalus kerkyrensis* (Branchiopoda: Anostraca) from the central Mediterranean area. 80° Congresso Unione Zoologica Italiana, Roma, Italy.

Vecchioni, L., Marrone, F., Rodilla, M., Belda, E.J., Arculeo, M. (2018). Systematics and phylogeography of *Tigriopus fulvus* (Copepoda, Harpacticoida, Harpacticidae) in the Mediterranean Sea and eastern Atlantic Ocean. 79° Congresso Unione Zoologica Italiana, Lecce, Italy.

Vicari, F., **Vecchioni, L**., Arculeo, M., Marrone, F. (2018). The legacy of quaternary colonization waves on the current mtDNA diversity of two Sicilian diaptomids. 79° Congresso Unione Zoologica Italiana, Lecce, Italy. Awarded with the Price "UZI-CSFI 2018" by the UZI "Giovani Ricercatori" Committee.

Vecchioni, L., Deidun, A., Sciberras, J., Sciberras, A., Marrone, F., Arculeo, M. (2017). mtDNA sequences reveals an overlooked diversity hotspot for the freshwater crab genus *Potamon* in North Africa (Decapoda, Brachyura, Potamidae). Second Joint Meeting of Société Zoologique de France and Unione Zoologica Italiana, Torino, Italy.

Vecchioni, L., Deidun, A., Marrone, F., Arculeo, M. (2017). Molecular evidences for a late Pleistocene origin of the Italian and Maltese population of the species *Potamon fluviatile* (Malacostraca, Decapoda). XIII incontro dei dottorandi in ecologia e scienze dei sistemi acquatici" organizzato dalla Società Italiana di Ecologia (SItE), dall'Associazione Italiana di

Oceanologia e Limnologia (AIOL) e dall'Italian Network for Lagoon Research (LaguNet), presso l'Università degli Studi di Palermo.

6.4 28S rRNA Alignment

TAG	TAXON	ALIGNED 28S SEQUENCE
TF9	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGTGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF10	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF13	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF17	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGTCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF27	*T. fulvus*	GGGCAGAGCCCAACACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGTGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF38	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGTGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA

TAG	TAXON	ALIGNED 28S SEQUENCE
TF45	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCACTGGCGGCACCGTCACGTGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGGGCTGTCCCTGTGCGTGGTTCTTGATCAGCAATGACTTGGCCGCGTGCTTGGGGCCCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGTGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGGACGGCTCCCCTCTCTGTCGTGAGTATGTCTGCGGCGTCTCCGACCCGTCTTGAAACACGGACCACGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCACTGAGAGCACA
TF14	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF23	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGTGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF28	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--CGTTCCTGTGCGTGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTGCGGGGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CTTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTTGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTACAATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF43	*T. fulvus*	GGGCAGAGCCCAACACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTTCCTGTGCGTGGTTCTCGATCAACAATGACTTGGCCGCGTGCTCGGGGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CTTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTTGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF46	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTCCTCTCGTGCGGATGGCCAGTCCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--CGTTCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTCGGGGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCTTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATATCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA

TAG	TAXON	ALIGNED 28S SEQUENCE
TF89	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGTGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF83	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGTGGTGCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCCCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCTTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGTAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF7	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGTGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF49	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGCGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGAG--TGTCCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTCGGGGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGAGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF52	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGCGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGAG--TGTCCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTCGGGGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGAGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF54	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGTGGTGCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCCCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCTTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA

TAG	TAXON	ALIGNED 28S SEQUENCE
TF58	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATCGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACAACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGAG--TGTTCCTGTACGTGGTTCTTGATCAGCAATGACTTGGCCGCGTGCTCGGGGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CTTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATATCTGTCGTGAGTAGGTCGGCGGCGTCTCCTACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCAAAACCTAAAGGCGCAGTGAAAGCAAA
TF67	*T. fulvus*	GGGCAGAGCCCAACACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGGCCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGAG--TGTTCCTGTGCGTGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTCGGGGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATATCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF85	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCACTGGCGGCACCGTCACGTGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTTCCTGTGCGTGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGGGCCCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCTCTGGGTGAAGTTTTCGGACGTCCTCGGACGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCCTATCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF87	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCACTGGCGGCACCGTCACGTGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTTCCTGTGCGTGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGGGCCCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCTCTGGGTGAAGTTTTCGGACGTCCTCGGACGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCTCTTCACTGGCACGGCCCCCCTATCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF92	*T. fulvus*	GGGCAGAGCCCAACACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTCCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--CGTCCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTCGGGGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCCTTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATATCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF97	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGTGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA

TAG	TAXON	ALIGNED 28S SEQUENCE
TF101	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGTGGTCCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTCGTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF105	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGTGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCTTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF104	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGTGTGCTTGGTGCGCCCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCTTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF84	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGTGGTGCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCCCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCGCTCGTGCTGGACCGAGCCCTTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGTAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF109	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF110	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA

TAG	TAXON	ALIGNED 28S SEQUENCE
TF111	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTCGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCAACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF112	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTCGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGCGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCAACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF113	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTCGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGTGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF114	*T. fulvus*	GGGCAGAGCCCAGCACCGAACCGCTGGCGGCACCGTCACGCGGCATGTGGTGTTCGGGAGAGTCTTCTCTCGTCGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCCGTCCGTGCGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTCGCGGG--TGTGCCTGTGCGAGGTTCTCGATCAGCAATGACTTGGCCGCGTGCTTGGTGCGCTCGTTGGCTGCTCTTCTCCCG-TCCTTCACACGACGAACCACTCGTGCTGGACCGAGCCCCTGGGTGAAGTTTTCGGACG-CCTCGG-CGGACTGGGAGCCCCGGGTGGCTGGCTTCGGCGCGGGCGGGTAGTTAAGGGAGCTCGTATAGCGAGTGTCT--TCACTGGCACGGCCCCCATCTCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
EU370444	*T.cf. fulvus*	GGGCAGAGCCCAGCACCGAACCACTGGCGGCTCCGTCACGTGGCATGTGGTGTTCGGGAGAGTCTTCTCTCATGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCTGTCCGTGTGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCATGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAATAGTACGTGAAACTGTGGAGTGGTAAACGGAGACGACCTCGAAGTCTGGGTGGGAGGTTCAGGCTTGCGGC--GGTCCCTGTGCTCGGTTCTTGATCAGCAATGACTTGGCCGGGTATTGTGGGCCTCTGTTGGCTGCTCTTCTCCTG-CCCTTCACACGACGAACCACTCGTGCTGGACCGTGCCGCTGGGTGAAGTTTTCGACCG-CTTCGG--CGGTTGGCAGCCCCGGCTGGTTGGCTTCGGCGCGGGCGGGTAGTTAAGGGGGCTCGTATAGCGAGTGTCT---CACTGGCACGGCCCCCATATCTGTCGTGAGTAGGTCGGCGGCGTCTCCGWCCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGTTCATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF106	*T. californicus*	GGGCAGAGCCCAGCACCGAACCCCTGGCAGTTCTGTCACGTGGCATGTGGTGTTTGGGAGAGTCTTCTCGCGAAGGAATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTACGGCGGTCGTTCTTTCGCGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCTCAAAGTGCGTGGTAAACTCCACGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGCAAACGGAGACGACCTCGAAGTCGGAGTGGGAGGTTCAGGTCTGAGCG--TGGCCGTCATCGGGGTTCTTGATCAGCAATGACTTGGCCTTGGTGTCTGGTTACGTTTTGGCTGCTCTTCTCTCGCTCTTTCACACGACGAACCACTCGTGCTGGACCGAGACCTCGGGTGAAGTTGGTGGCGAGTTTCGA-CTTGCTGCCAGCCCCGGGTGTTTGGCTTCGGCGCGGGCGGGTAGTTAAGGAGGCTCGTATAGCGAGTGTCT--TTGCTGACACGGCCTTCATATCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT-CAATGGGTTGTCTAAACCCATAGGCAGAGTGAAAGCAAA

TAG	TAXON	ALIGNED 28S SEQUENCE
TF81	*T. brevicornis*	GGGCAGAGCCCAGCACCGAACCACTGGCGGCTCCGTCACGTGGCATGTGGTGTTCGGGAGAGTCTTCTCTCATGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCTGTCCGTGTGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCT CAAAGTGCGTGGTAAACTCCATGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGTAAACGGAGACGACCTCGAAGTCTGGGTGGGAGGTTCAGGCTTGCGGC-- GGTCCCTGTGCTCGGTTCTTGATCAGCAATGACTTGGCCGGGTATTGTGGGGCCTCTGTTGGCTGCTCTTCTCCTG-CCCTTCACACGACGAACCACTCGTGCTGGACCGTGCCGCTGGGTGAAGTTTTCGACCG-CTTCGG-- CGGTTGGCAGCCCTGGCTGGTTGGCTTCGGCGCGGGCGGGTAGTTAAGGGGGCTCGTATAGCGAGTGTCT---CACTGGCACGGCCCCCATATCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT- CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF79	*T. brevicornis*	GGGCAGAGCCCAGCACCGAACCACTGGCGGCTCCGTCACGTGGCATGTGGTGTTCGGGAGAGTCTTCTCTCATGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCTGTCCGTGTGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCT CAAAGTGCGTGGTAAACTCCATGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGTAAACGGAGACGACCTCGAAGTCTGGGTGGGAGGTTCAGGCTTGCGGC-- GGTCCCTGTGCTCGGTTCTTGATCAGCAATGACTTGGCCGGGTATTGTGGGGCCTCTGTTGGCTGCTCTTCTCCTG-CCCTTCACACGACGAACCACTCGTGCTGGACCGTGCCGCTGGGTGAAGTTTTCGACCG-CTTCGG-- CGGTTGGCAGCCCCGGCTGGTTGGCTTCGGCGCGGGCGGGTAGTTAAGGGGGCTCGTATAGCGAGTGTCT---CACTGGCACGGCCCCCATATCTGTCGTGAGTAGGTCGGCGGCGTCTCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT- CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF108	*T. brevicornis*	GGGCAGAGCCCAGCACCGAACCACTGGCGGCTCCGTCACGTGGCATGTGGTGTTCGGGAGAGTCTTCTCTCATGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCTGTCCGTGTGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCT CAAAGTGCGTGGTAAACTCCATGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGTAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTTGCGGC-- GGTCCCTGTGCTCGGTTCTTGATCAGCAATGACTTGGCCGGGTATTGTGGGGCCTCTGTTGGCTGCTCTTCTCCTG-CCCTTCACACGACGAACCACTCGTGCTGGACCGTGCCGCTGGGTGAAGTTTTCGACCG-CTTCGG-- CGGTTGGCAGCCCCGGCTGGTTGGCTTCGGCGCGGGCGGGTAGTTAAGGGGGCTCGTATAGCGAGTGTCT---CACTGGCACGGCCCCCATATCTGTCGTGAGTAGGTCGGCGGCGTCTCCAACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT- CATTGGGTTGTCTAAACCTAAAGGCGCAGTGAAAGCAAA
TF107	*T. brevicornis*	GGGCAGAGCCCAGCACCGAACCACTGGCGGCTCCGTCACGTGGCATGTGGTGTTCGGGAGAGTCTTCTCTCATGCGGATGGCCAGTCCAAGTCCACCTTGACTGGGGCCACGGCCCATAGAGGGTGATAGGCCCGTAAGGCGGCTGTCCGTGTGAGCTGACTTTTCCCTAGAGTCGAGTTGCTTGGGAGTGCAGCT CAAAGTGCGTGGTAAACTCCATGTAAGGCTAAATATGACCCCGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACTGTGGAGTGGTAAACGGAGACGACCTCGAAGTCTGGGCGGGAGGTTCAGGCTTGCGGC-- GGTCCCTGTGCTCGGTTCTTGATCAGCAATGACTTGGCCGGGTATTGTGGGGCCTCTGTTGGCTGCTCTTCTCCTG-CCCTTCACACGACGAACCACTCGTGCTGGACCGTGCCGCTGGGTGAAGTTTTCGACCG-CTTCGG-- CGGTTGGCAGCCCCGGCTGGTTGGCTTCGGCGCGGGCGGGTAGTTAAGGGGGCTCGTATAGCGAGTGTCT---CACTGGCACGGCCCCCATATCTGTCGTGAGTAGGTCGGCGGCGTCTCCAACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGTGCGAGT- CATTGGGTTGTCTAAACCTAAAGGCGCATTGAAAGCAAA