Natural or human mediated – Biogeography of widespread Mediterranean invertebrates with poor dispersal capacities

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All that concerns the Mediterranean is of the deepest interest to civilized man, for the history of its progress is the history of the development of the world; the memory of the great men who have lived and died around its banks; the recollection of the undying works that have come thence to delight us for ever; the story of patient research and brilliant discoveries connected with every physical phenomenon presented by its waves and currents, and with every order of creatures dwelling in and around its waters. The science of the Mediterranean is the epitome of the science of the world.

Edward Forbes

On Early Explorations in the Mediterranean. In *Memoir of Edward Forbes F.R.S.* (1861)
by George Wilson and Archibald Geikie, p. 279.

Abbreviations

AIC	Akaike Information Criterion
AMOVA	Analysis of Molecular Variance
B.C.	Before Christ
BF	Bayes factor
bp	Base pairs
BP	Before present
COX1	Cytochrome oxidase subunit 1
CTAB	Cetrimonium bromide
ESS	Effective sampling size
F_{ST}	Fixation index
G/Γ	Gamma variation
GTR	General Time Reversible Model
H	Haplotype
H_0	Null hypothesis
HKY	Hasagawa-Kishino-Yano Model
I	Invariable sites
ICZN	International Commission on Zoological Nomenclature
ITS-1	Internal transcribed spacer region 1
IUCN	International Union for Conservation of Nature
ka	Kiloannum
LGM	Last glacial maximum
ln L	Logrithmic maximum likelihood
ln P	Logarithmic Bayes factor
log	Logarithm
Ma	Megaannum
MCMC	Monte Carlo Markov Chain
mrca	Most recent common ancestor
MSC	Messinian Salinity Crisis
Mya	Million years ago
ND1	NADH dehydrogenase subunit 1
PCR	Polymerase chain reaction
PI	Parsimony informative
PMS	Phylogenetic Model Selection
post. prob.	Posterior probability
rRNA	Ribosomal ribonucleic acid
s.d.	Standard deviation
S.E.	Standard error
SP	Statistical Parsimony
sp. nov	Species novum
tmrca	Time to the most recent common ancestor
tRNA	Transfer RNA
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

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General Introduction

1. Biogeography

Paradoxical biogeographical patterns as a challenge for research

In autumn 1911 Alfred Wegener (1880 – 1930) was studying in the library of Marburg University when he came across an article about fossils of terrestrial species which have been found on both sides of the Atlantic Ocean. Fascinated by this paradox, Wegener began to collect more examples of identical organisms that had, hundreds of million years ago, lived on two or more continents simultaneously. A strong evidence for his later theory was the biogeography of the prehistoric freshwater reptile *Mesosaurus*. Its fossilised remains were found in southern Africa and eastern South America. As *Mesosaurus* was living in freshwater habitats and could not have crossed the Atlantic Ocean, this distribution indicated that the two continents were connected in prehistoric times. At the beginning of the 20th century, such cases were explained by former land bridges between continents.

Besides the fragmented distribution of fossilised terrestrial and limnic species, Wegener noticed the close fit between the coastlines of South America and Africa. Could the paradoxical biogeography of prehistoric organisms be caused by the continents having once been joined together?

Wegener knew that for such a theory to be accepted a large number of supporting evidence would be required, which he collected in a pioneering interdisciplinary approach by studying prehistoric biogeography, rock strata and climate. In the year 1912, he proposed his theory of “continental drift” for the first time in a geological journal. In his later book “The Origin of Continents and Oceans” he claimed that approximately 300 million years ago (Mya) the continents had formed a super-continent, called Pangaea (Greek: "all the Earth"). Starting about 200 Mya, Pangaea split and its pieces had been drifting apart ever since. Wegener was not the first to suggest that the continents had once been connected, but he was the first to present extensive evidence from various fields. So the paradox of the freshwater reptile *Mesosaurus* living on continents separated by an ocean was a puzzle that finally led to the discovery of continental drift (further reading in “Alfred Wegener: Creator of the Continental Drift Theory” by Lisa Yount, 2009).

Species which are known as poor dispersers, but widely distributed in areas separated by geographical barriers for dispersal (e.g. oceans, mountain ranges, deserts), are still a major challenge to biogeography research.

Reconstructing the history of species

The reconstruction of a species' colonisation history often implicates difficulties like incomplete sampling throughout the range or taxonomic uncertainty in the study species. Species which are believed to occupy a wide and fragmented range, in reality often represent several cryptic lineages occurring in the distribution area (amongst others: Jackson and Austin 2010; Marko and Moran 2009; Piller and Bart Jr. 2009). As cryptic species, by definition, can not be identified through fossil and morphological comparisons and their occurrence is not predictable for a specific taxon or region (Pfenninger and Schwenk 2007), molecular analyses of specimens from a comprehensive sampling are necessary to resolve phylogenies.

The phylogeny of a species is a requirement for all other analyses reconstructing the mode and timing of its dispersal. Two extreme findings can occur in the phylogeny of a species with a widespread and disjunct range:

First, if the divergence in a species' phylogeny is shallow throughout its range, it can be assumed that the separated populations are in occasional contact. This maintained contact over barriers and great distances can be made possible through passive dispersal. Vectors for passive dispersal can be wind, water currents, animals and humans. For example, numerous freshwater invertebrates are known to hitchhike with insects, amphibians, reptiles, waterfowl, beavers or boats (Bailey et al. 2003; Bilton et al. 2001; Green and Figuerola 2005). Through passive transport by waterfowl, tiny freshwater crustaceans of the genus *Daphnia* are able to colonise lakes and ponds which are thousands of kilometers apart (Figuerola et al. 2005). This mode of dispersal also works for much bigger organisms than water fleas. In a study on 17 000 wading birds resting in the Odiel marshes of south-western Spain during autumn migration, four individuals with a live cockle (*Cerastoderma edule*) attached to one leg were found (Green and Figuerola 2005). Such studies demonstrate that the knowledge of a species' ecology, habitat and interactions with other taxa is a precondition to make qualified assumptions for possible mechanisms of dispersal.

In the second case, if there is a deep divergence in the phylogeny of a species, this biogeographical pattern could mirror the palaeogeographic history of the region. Geological as well as climatic events (e.g. plate tectonics, glacial cycles) can create barriers to migration, and genetic differences accumulate after populations were separated. A well dated geological event, that had strong influence on the American fauna and flora, is the closure of the Isthmus of Panama. When this land bridge, connecting North and South America, was completed 2.8 Mya, populations of marine species in the Atlantic and Pacific became separated (Coates et al. 2005). But the same geological event, that produced an insurmountable barrier for marine species, also opened a migration corridor for terrestrial species, resulting in the "Great American Interchange" (Marshall et al. 1982; Stehli and Webb 1985; Webb and Barnosky 1989). This is just one example illustrating that a profound knowledge of an area's geological and climatic history is essential for the reconstruction of a species demographic past.

Vicariance versus dispersal

In cases of poor dispersers with widespread and fragmented distributions, it is often disputed whether vicariance or dispersal were the dominant forces underlying the biogeographical patterns (Austin et al. 2003; Givnish et al. 2004; Keppel et al. 2009; Yoder and Nowak 2006). According to vicariance theory, ancestral taxa were widespread and diversification/speciation was caused by a fragmentation of their former range (Sparks and Smith 2005; Yoder and Nowak 2006). In contrast, the main principle of dispersal theory is that of "centres of origin" from which taxa spread, were subsequently isolated and evolved separately (Dobzhansky 1937; Mayr 1954).

It is the rough rule of thumb that if the phylogenetic history of a species is congruent with a known sequence of geological vicariance events in the history of its range, a vicariance scenario is supported (e.g. Sparks and Smith 2005). Therefore, the dating of splits in a species phylogeny plays a decisive role in distinguishing between vicariance and dispersal scenarios (Yoder and Nowak 2006). As vicariance events affect all species in a certain region, cooccuring taxa often show common biogeographical patterns (e.g. Hausdorf and Hennig 2005; Taberlet et al. 1998). Conversely, dispersal is typically inferred if phylogenies reveal lineages from one geographic region nested deeply within clades from another separated area (e.g. Austin et al. 2003; Givnish et al. 2004; Yoder and Nowak 2006). Dispersal leads to clade specific patterns, which differ from biogeographical patterns of other taxa in the same region.

In my thesis I focused on invertebrate species occurring in the Mediterranean region. The next paragraphs expose briefly which tectonic and climatic events, during the last 30 Ma, affected biogeographical patterns in fauna and flora of the Mediterranean area by offering favourable conditions for speciation by vicariance or dispersal.

2. The Mediterranean – a model region for biogeographical studies

The Mediterranean area is a model region for biogeographical studies because of its complex and well known geological history, as well as high levels of biodiversity and endemism, especially in plants and terrestrial invertebrates (Blondel and Aronson 1999; Comes 2004; Hausdorf and Hennig 2005; Sfenthourakis and Legakis 2001).

Figure 1: Satellite photograph of the Mediterranean Sea (http://commons.wikimedia.org/wiki/File:Mediterranian_Sea_16.61811E_38.99124N.jpg)

Momentous vicariance events in the Mediterranean were, for example, the splitting of the Tyrrhenian plate, the fragmentation of the Aegean islands or the formation of the Mid-Aegean trench.

The drift of continental micro-plates in the Western Mediterranean

In the Oligocene (34 – 23 Mya), the Western Mediterranean did not exist in the form we know it today. Micro-plates (so called terranes) located in the area of present-day's Betis (Southern Spain), Balearic Islands, Corsica, Sardinia, Calabria, Sicily, Kabylies (Algeria) and Rif range (Morocco), were part of the Tyrrhenian plate which was situated on the Southern European – Iberian continental margin (Rosenbaum et al. 2002a; see Figure 1, Chapter 4). Approximately 30 Mya, this plate split off the European continent and broke into smaller terranes. Around 22 Mya all these micro-plates, with the exception of the Betis- and Rif-terranes which were connected until 12 Mya, drifted separately in south or south-east direction through the Mediterranean. 15 Mya the Kabylies-terrane connected with the African continent in the region of Algeria, 5 Ma later the Rif-plate merged with Morocco. All other micro-plates reached their current positions approximately in the Pliocene between 5.3 and 2.6 Mya. The terranes, isolated from the continent and from each other for millions of years, offered ideal conditions for allopatric speciation and are an example for a strong vicariance event. The influences of the movement of micro-plates are visible especially in the biogeography of western Mediterranean plants, like the maritime pine and the cork oak (Magri et al. 2007), but it might have been as influential on poor faunal dispersers. This event is also a possible explanation for the paradox that the Northern African flora and fauna are more closely related to the south-western European than to the African ones, and why there is such a high endemism rate in this region (Jeannel 1952).

Fragmentation of the Aegean Islands and formation of the Mid-Aegean trench

Similar to the geological development of the western Mediterranean, the eastern Mediterranean was strongly influenced by the fragmentation of the landmass "Ägäis". During the early and middle Miocene (23 – 12 Mya), the Aegean region was a continuous landmass, until between 12 and 8 Mya the southern part broke off the continent and fragmented into the Aegean Islands (Creutzburg 1963; Dermitzakis and Papanikolaou 1981).

These southern plates have been stretched over 400 km southwards while the north remained locked in position close to the neighbouring mainland (Dermitzakis 1990; Meulenkamp 1985; Steininger and Rögl 1984). At the end of the middle Miocene (~12 Mya) the Mid-Aegean trench developed and was fully completed around 9 Mya, resulting in an additional separation of the western and eastern Aegean islands (Dermitzakis and Papanikolaou 1981). Nowadays, the Aegean archipelago consists of a large number of islands of various sizes; its southern limit is defined by the Hellenic arc running from the Peloponnesus through Kithira, Crete, Karpathos and Rhodes to south-west Turkey. The islands on the Aegean plate had been in contact with the surrounding coast and with each other for various periods and at different times, resulting in high levels of diversity and endemism caused by vicariance events. Biogeographical studies on poor dispersers like terrestrial invertebrates or legless reptiles showed that the present distribution of species in the Aegean often mirrors patterns of the palaeogeographical past. For example, Hausdorf and Hennig (2005) pointed out that the present composition of land snails, isopods, tenebroid beetles and butterflies on the Aegean islands was influenced to different degrees by the palaeogeography of the region, from the Miocene until recent events during the ice ages.

Besides the previously described vicariance events, the geological history of the Mediterranean additionally offered favourable conditions for terrestrial and limnic animal species to disperse across the marine basin, for example during the Messinian Salinity Crisis or the glacial cycles.

The Messinian Salinity Crisis

The Messinian Salinity Crisis (MSC), i.e. the desiccation of the Mediterranean Sea between 5.96 and 5.33 Mya, was an unprecedented event removing the marine barrier to dispersal in the Mediterranean area (Krijgsman et al. 1999). It resulted from a tectonic uplift (Duggen et al. 2003) which led to the closure of the Strait of Gibraltar and the subsequent isolation from the Atlantic Ocean. For approximately 700 000 years Mediterranean species had the possibility to reach otherwise isolated islands or coasts by migration through desiccated parts of the basin or freshwater drainages. It was proposed that some terrestrial and limnic species used these favourable conditions for migration, for example reptiles, crickets, freshwater fish and crabs (Allegrucci et al. 2009; Brandis et al. 2000; Ketmaier et al. 2008; Kornilios et al. 2010). Approximately 5.33 Mya, the barrier at the Strait of Gibraltar broke and the Mediterranean basin was re-filled in the Zanclean flood (Cita 1972), resulting in allopatric isolation of populations on both sides of the basin or on islands.

The Quaternary ice ages

At the beginning of the Quaternary, around 2.6 Mya, the arctic ice cap built up. Major climatic oscillations occurred during the last 700 000 years with a dominant 100 000 cycle, interrupted by relatively warm interglacials (Webb and Bartlein 1992); the last glacial period ended approximately 13 000 years ago (Debeaulieu et al. 1994). During the Quaternary glacial cycles many species went through repeated expansions/contractions of their range, characterised by extinctions of northern populations when the temperature decreased and a subsequent northward expansion from southern refugia during interglacials. Studies on the biogeography of European animal and plant species, amongst others the European brown bear (*Ursus arctos*), the meadow grasshopper (*Chorthippus parallelus*), the silver fir (*Abies alba*) and the white oak (*Quercus spp.*), showed that Northern Europe was mainly re-colonised from Iberian and Balkanic refugia and Italian lineages were frequently isolated because of the Alps barrier (Taberlet et al. 1998). Higher species richness and genetic diversity was often found in Southern Europe were species persisted during glacial periods, whereas successive bottlenecks led to a loss of genetic diversity in the north. The polar regions and mountain ranges like the Alps were entirely covered by ice during cold periods, so that the large volume of accumulated ice reduced sea levels by up to 125 m and precipitation sank (Bar-Matthews et al. 1999; Fleming et al. 1998;). This drop of sea-level during glacial maxima resulted in the connection of many Mediterranean islands (especially in the Aegean) and diminished the distance between coasts considerably, thereby opening potential migration corridors for terrestrial species (Beutler 1979; Dermitzakis 1990).

Anthropogenic influences

As mentioned beforehand, passive dispersal supported by vectors is a possibility for long distance colonisation independent from a species locomotive abilities and geological or climatic conditions. Unintended anthropogenic transport, as a by-product of human migration and trade, is presently the most common way of passive dispersal in the densely populated areas of Mediterranean coasts.

Additionally to its complex geological history, the biodiversity of the Mediterranean area was considerably shaped, at least since the Neolithic era (ca. 10 500 – 5 000 years BP), by human landscape management and species introductions, which strongly impacted the indigenous flora and fauna (Zeder 2008). Coming from the Near East (Levant), Neolithic farmers started around 10 500 years BP to migrate first into the Balkans and then continuously colonised the whole of Europe in expansion waves westwards during the following 4 500 years (Davison et al. 2006; Turney and Brown 2007). Domesticated crop and animal species were dispersed over Europe by settlers spreading the Neolithic culture. Numerous obsidian artefacts from around the Mediterranean basin document the efficiency of early Neolithic seafaring (Tykot 2002). Consequently, the Mediterranean has served as an exchange place of biodiversity associated to human use since the Neolithic (Zeder 2008). But also species not beneficial for human agriculture or consumption were spread unintendedly, for example as blind passengers in ship ballasts or weeds mixed with crop seeds (Wilson et al. 2009). In the course of globalisation, the number of accidental transports of non-indigenous species has increased rapidly. Estimations are that currently there are more than 500 introduced species in the Mediterranean Sea (Galil 2007), 6.2 new plant species, capable of naturalisation, arrive each year in Europe (Lambdonet et al. 2008) and the Global Invasive Species Database lists 33 alien species in the Mediterranean that are supposed to threaten native biodiversity (www.issg.org). These numbers illustrate the increasing effectiveness of humans as vectors for passive dispersal and their importance for present day species expansions.

3. Study organisms

In my thesis I focused on Mediterranean invertebrate species which are, as a consequence of their ecology, considered as poor dispersers. Nonetheless, freshwater crabs of the genus *Potamon* and land snails of the genus *Tudorella* occupy large areas that are discontinuous and separated by the Mediterranean Sea. For both groups the suspicion was raised that humans could have been involved as vectors in their dispersal history and presently both are threatened by human disturbance of their habitats.

Freshwater crabs of the genus Potamon

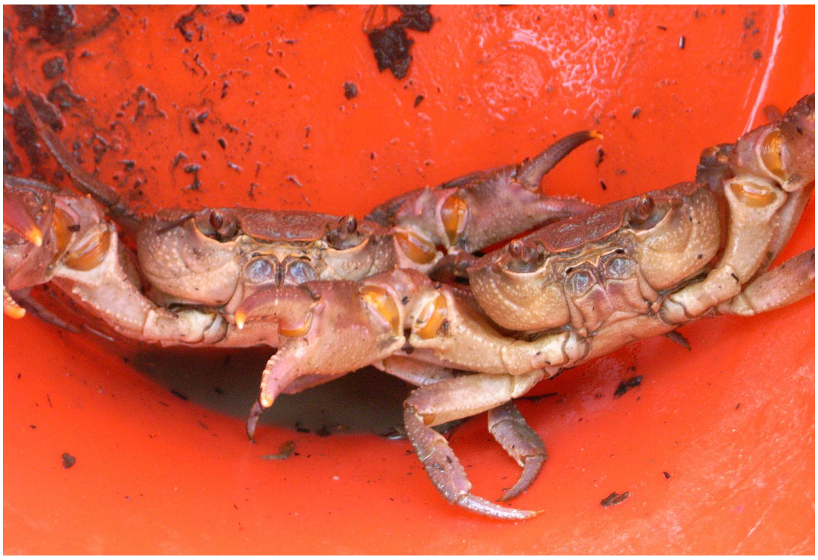

Figure 2:
Two male crabs of the species *Potamon pelops*, showing their bright orange coloured joints in an aggressive posture.
© R. Jesse

The genus *Potamon* has a highly fragmented geographic distribution in a number of Mediterranean countries, i.e. Algeria, Italy, Malta, Greece, the Aegean and Ionian Islands and Turkey (Brandis et al. 2000; see Figure 1, Chapter 1). Currently, the Eastern Mediterranean is the European biodiversity hotspot for potamid freshwater crabs (for example *Potamon pelops*; Figure 2), but the origin of the genus *Potamon* is still unknown.

The taxonomy of the European freshwater crabs was reviewed and discussed in the last decades and is so far not completely solved. Previous to this study, 16 species of four subgenera within *Potamon* were recognised in Europe. The taxonomy of the European freshwater crabs is based on the morphology of the male reproductive apparatus, especially the first gonopods (Brandis et al. 2000). The gonopods, covered by the abdomen, are assumed to be environmentally neutral and therefore qualified for species delimitation. Because of the high phenotypic plasticity in the genus *Potamon*, several variants, so-called "nationes", on subspecies-level have been described (Pretzmann 1978, 1983, 1986a, 1986b). A genetic revision of the Mediterranean freshwater crab species has previously not been conducted.

To resolve a species' taxonomy is especially important if it is threatened. To legitimate and plan conservation measurements, it is a prerequisite to find hotspots of genetic diversity and to assess whether the species is indigenous in an area or was introduced by humans. Four freshwater crab species of the Mediterranean area are currently classified as "near threatened" in the red list of the IUCN (Cumberlidge et al. 2009). The crabs have few predators, but they are vulnerable to pollution, regulation and desiccation of streams and to introductions of non-indigenous crayfish species (Gherardi and Holdich 1999). The oldest fossils of potamid crabs (with an approximate age of 17 Ma) were found in Central Europe as far north as Engelswies in Southern Germany (Klaus and Gross 2010). 130 years ago, Lago di Garda in Italy was described as their northern limit of distribution (Bettoni 1884; Garbini 1894; Kingsley 1880). Due to human consumption and habitat degradation, the crabs declined observably during the last century and their current range is defined by the river Po as northern limitation (Jesse et al. 2009).

Figure 3:
Representative habitat of *Potamon* in Central Italy.
© R. Jesse

Considering the ecology of these crustaceans, their present range, comprising areas separated by an ocean, raised questions about their past mode and routes of dispersal. European freshwater crabs live in streams and lakes where they shelter under stones and among vegetation. Especially during arid conditions and while moulting, they stay in their burrows dug deeply into river banks. Figure 3 shows a natural, unaltered stream such as European freshwater crabs prefer as habitat. The crabs feed mostly at night, consuming a wide variety of food items, like algae and moss, tadpoles, insects, small frogs and fish (Gherardi et al. 1987). Freshwater crabs are bound to their aquatic habitat and as gill breathers prefer to disperse during humid and temperate weather conditions (Gherardi et al. 1988). They avoid saltwater and are sedentary because of their brood care behaviour which causes the juveniles to mostly stay in the parental drainage basin (Gherardi et al. 1988; Micheli et al. 1990). Due to these ecological characteristics, freshwater crabs probably have a low dispersal capacity, especially across marine barriers, and speciation was more likely caused by vicariance events. However, Brandis et al. (2000) proposed that *Potamon algeriense* coming from the Balkans crossed the Mediterranean basin actively during the MSC and was isolated in Northern Africa after the Zanclean flood 5.33 Mya. It remains thus unclear to what extent the speciation of *Potamon* was influenced by vicariance and/or dispersal.

Terrestrial snails of the genus Tudorella

In contrast to freshwater crabs which have a long tradition in the Mediterranean cuisine and were portrayed on coins and other archaeological remains during Greek and Roman periods (Brandis 1997), land snails of the genus *Tudorella* (Figure 4) did not attract so much attention and less studies on their ecology have been conducted.

Figure 4:
Tudorella sulcata sensu stricto from Martigues, at the coast of Southern France
© D. Pavon

The origin of the land snail genus *Tudorella* P. Fischer, 1885 in the Mediterranean can be traced back to the beginning of the Miocene (Véla et al. 2008). The present range of *Tudorella* comprises coastal areas in the western Mediterranean, from the Iberian Peninsula to Malta and from Tunisia to Morocco. The time of residence in each area was not possible to estimate by fossils, as shells can not be matched to extant species (Véla et al. 2008).

The high level of phenotypic plasticity and sexual dimorphism in these snails (Martínez-Ortí and Robles 2005), in combination with their fragmented range, has long since encouraged authors to describe new species within the genus *Tudorella*, mostly on the subspecific level (Pallary 1898; Potiez and Michaud 1838; Sowerby 1847). Until now, two species (*T. ferruginea* and *T. sulcata*) and one to five subspecies of *Tudorella sulcata* were recognised (Giusti et al. 1995; Sacchi 1958). A DNA taxonomy approach, taking into account populations of all areas in the range, has not been undertaken yet, but would be a necessity for possible future conservation measures.

The terrestrial gastropods have a covert life style, spending most of the day borrowed in the soil under shrubs and limestone rocks, and feed on detritus (habitat shown in Figure 5). Like freshwater crabs, they migrate and feed mostly at night and during moist weather periods (Véla et al. 2008). Because of their low ability to disperse and their need for coastal vicinity and areas with shrubs and limestone, the snails are threatened by coastal urbanisation especially in Southern France and Spain.

Figure 5:
Typical habitat of *Tudorella* on Sardinia.
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4. Thesis outline

Aims of the study

In my thesis, I aimed to analyse the biogeographical patterns of the two genera *Potamon* and *Tudorella*, which both occupy a wide and disjunct range in the Mediterranean area, despite their supposedly limited dispersal abilities. To evaluate if their dispersal was human mediated or achieved independently was one of the main tasks throughout this study.

I investigated each organism group in three contexts:

1) As a prerequisite for all further analyses, I resolved the **taxonomy** with molecular markers to delimit evolutionary lineages. The main questions in the taxonomic context were:

- Are the morphologically described species congruent to genetic lineages?
- Can cryptic diversification be detected in the studied genus/species?

2) Biogeographic analyses on **genus** level included all species occurring in the Mediterranean area. My aim was to answer the following questions:

- Does the diversification and temporal splitting pattern of the genus mirror the palaeogeographical and climatic history of the distribution area?
- Where was the origin of the lineages in this region?
- Was vicariance or dispersal the main factor shaping their present biogeography?

3) Finally, I reconstructed the phylogeography of one **species** in each genus by analysing the genetic population structure in a range wide assessment, to find out:

- Where did the species originate and what was its route of colonisation?
- What was the time frame of its range expansion(s)?
- Could humans have been the vector for long distance dispersal?

General overview

The first three chapters give attention to freshwater crabs of the genus *Potamon*.

In **Chapter 1**, I studied the biogeography of the genus *Potamon* in the Mediterranean area. All species occurring in that region were analysed with two mitochondrial and one nuclear marker in a DNA taxonomy approach. After species delimitation, I reconstructed the phylogeny and the temporal splitting pattern of the genetic lineages as well as test different hypotheses for the cause of major splits in the phylogeny. Phylogenetic areal analysis was applied to evaluate if the Aegean was the origin of the extant European freshwater crabs.

In **Chapter 2**, I described the cryptic species *Potamon pelops* sp. nov., which was found in the course of my study on *Potamon fluviatile* (see Chapter 3). The taxonomic position of the species was determined by analyses of two mitochondrial and one nuclear marker as well as a morphological investigation of male first gonopods and spermatophores.

Chapter 3 highlights the phylogeography of *Potamon fluviatile*. This particular species in the genus *Potamon* is distributed on the Italian Peninsula and Malta, as well as Serbia, Albania and Greece on the Balkans. By analysing specimens from across their range with two mitochondrial markers, I resolved the phylogeny of the species. With this phylogenetic information used in a model selection approach, I could infer the most likely route of dispersal between the disjunct areas in Italy and the Balkans. In a Bayesian demographic analysis I estimated the population growth in *P. fluviatile* and reconstructed the approximate time of expansion to the new area.

The last two chapters present studies on terrestrial snails of the genus *Tudorella*.

In **Chapter 4**, I conducted a revision of the genus *Tudorella* based on molecular analyses of two mitochondrial and one nuclear marker. Species delimitation was the basis to reconstruct the biogeography of the genus. In a model selection approach, I tested four different biogeographical hypotheses to estimate whether the snails crossed the Mediterranean basin actively during the Messinian Salinity Crisis or passively through the fragmentation and drift of the Tyrrhenian micro-plates.

Chapter 5 presents a study on the phylogeography of *Tudorella sulcata* sensu stricto which has a current range comprising coastlines of France, Sardinia and Algeria. The phylogeny of the species was resolved by analysing populations from all three regions with the mitochondrial marker COX1. I reconstructed the origin and sequence of expansion by testing nine different dispersal hypotheses in a model selection approach. The time frame of their migration and the resulting population growth were inferred by demographic analysis.

Chapter 1

Evolution of freshwater crab diversity in the Aegean region (Crustacea: Brachyura: Potamidae)

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Abstract

The aim of this study was to estimate the influence of the palaeogeographic and climatic history of the Aegean region on the diversity of freshwater crabs of the genus *Potamon* and to test whether this area served as source or reservoir in species diversity. Necessary species delimitation was accomplished by phylogenetic analyses of the mitochondrial markers COX1 and ND1, partial 16S rRNA gene and the tRNALeu gene. We found 14 genetic lineages of which nine could be assigned to previously recognized species. Temporal estimates of the splitting pattern in the phylogeny of *Potamon* indicated that a combination of geological and climatic events influenced their diversification. Within *Potamon*, the lineages separated into a western and an eastern group. This first split in the genus occurred approximately 8.3 – 5.5 Mya, thus possibly correlated with the Messinian Salinity Crisis. A likelihood approach to geographic range evolution suggested for most species, occurring in the Aegean area, an origin in the Middle East. Moreover, there were no insular endemics in the central Aegean archipelago, therefore low sea-levels during the Pleistocene glacial periods possibly enabled dispersal to these islands, but subsequent rise in sea-level did not cause speciation. Nevertheless, the diversification of most lineages occurred during the Pleistocene epoch thus coinciding with Quaternary fluctuations of the climate.

Molecular Phylogenetics and Evolution, in press.

1.1. Introduction

The Aegean Archipelago was in its relatively short history subject of extreme geological and geomorphological alterations (Dermitzakis 1990). During the Early and Middle Miocene (23 – 12 million years ago; Mya), the Aegean region was part of a continuous landmass. Fragmentation started with the collision of the African / Arabian tectonic plate with the Eurasian plate in the Middle Miocene (~16 Mya; Krijgsman 2002; Steininger and Rögl 1984) and the formation of the Mid-Aegean trench (12 – 9 Mya; Dermitzakis and Papanikolaou 1981). Subsequently, the former landmass was divided into a large number of islands of various sizes. These were connected and isolated repeatedly during their geological history, e.g. during the Messinian Salinity Crisis (5.96 – 5.33 Mya) when the Mediterranean basin fell partly dry (Krijgsman et al. 1999) or during the Pleistocene (2.58 Mya – 11.70 kya) due to eustatic sea-level changes (Perissoratis and Conispoliatis 2003).

Because of this complex and well-known paleogeographical history and its high levels of diversity and endemism, the Aegean region has become a key area for biogeographical studies (Hausdorf and Hennig 2005). Studies on invertebrate (Fattorini 2002; Heller 1976; Sfenthourakis 1996; Subai 1996; Welter-Schultes and Williams 1999) and vertebrate (Poulakakis et al. 2005) terrestrial taxa, as well as plants (Bittkau and Comes 2005) showed that the distribution of Aegean species often mirrors palaeogeographical patterns and processes. For land-snails (Welter-Schultes and Williams 1999) and tenebroid beetles (Fattorini 2002), it was tested whether their present distributions can be explained by active colonisation, or rather by vicariant events caused by the geological history. In both studies, the second hypothesis was better supported by the data, consistent with the suggested limited dispersal ability of these organisms.

Freshwater crabs are also considered to have a rather low dispersal capacity and a high potential for drainage specific endemism (Daniels et al. 2002): (1) supposedly, they are only capable to surmount longer terrestrial distances outside their aquatic habitat under humid conditions (Gherardi et al. 1988a, 1988b); (2) their philopatry is increased by the absence of marine planctonic larvae and their brood care behaviour which causes the juveniles to stay in the parental habitat (Gherardi et al. 1988b; Micheli et al. 1990); (3) freshwater crabs are expected to have a low capacity to disperse over marine barriers (Ng and Rodriguez 1995). Although freshwater crabs are to some extent capable to endure higher salinity, most investigated species do not withstand actual sea water conditions, and are obligate freshwater species (Morris and van Ardt 1998). Previous phylogeographic studies in the Aegean area focused on terrestrial invertebrates. Here, we consider the genus *Potamon* as a suitable group to approach Aegean freshwater biogeography, and to investigate the correlation between speciation, geological history and complex geography within this area.

The genus *Potamon* is situated at the western periphery of the distribution area of the family Potamidae, the latter having its main species diversity in East and Southeast Asia with more than 500 currently described species (Ng et al. 2008). The present distribution of the genus ranges from Northern Africa to the Middle East. The last revision of *Potamon* recognised 16 species, assigned to four subgenera that were distinguished by characters of the male reproductive organs and discrete distribution areas (Brandis et al. 2000): (1) the subgenus *Euthelphusa* occurring in the western Mediterranean area (*P. fluviatile,*: Italian Peninsula, Sicily and Malta; *P. algeriense:* Maghreb) and on the Balkan (*P. fluviatile*) and Peloponnesus peninsulas (*P. pelops*), (2) the subgenus *Potamon* ranging from the Aegean islands to the Levant (*P. potamios*, *P. rhodium*, *P. setiger* and *P. bileki*) , (3) the subgenus *Pontipotamon* that demarcates the northern distribution limit from the Balkan peninsula to the tributaries of the Caspian Sea (*P. ibericum*, *P. bilobatum*), and (4) the subgenus *Orientopotamon*, that ranges from Asia Minor to the Hindu Kush (eight species).

A population genetic study of *Potamon fluviatile* showed that freshwater crabs can colonise new areas in a relatively short time intervals if favoured by geographic conditions (Jesse et al. 2009). This species expanded its range from the Southern Balkans to the Italian Peninsula and Sicily approximately 15 000 years BP, when most of the Adriatic Sea was dry as a consequence of the low sea-levels during the last glacial maximum. In contrast, the split between *P. fluviatile* on the Balkans and *P. pelops* on the Peloponnesus peninsula was probably caused by the formation of the Gulf of Corinth followed by allopatric speciation (Jesse et al. 2010). So it remains contentious to what extent the diversification of this freshwater crab genus was influenced by vicariance or dispersal events.

Also the centre of origin for the genus *Potamon*, and of the family Potamidae in general, still remains unknown. While the by far earliest record of potamids are Early Miocene fossils of the genus *Potamon* from Central Europe (see Klaus and Gross 2010), species diversity argues for an origin of the Potamidae in East Asia, with subsequent dispersal to the Near East and the Mediterranean area (thus assuming an incomplete fossil record of Neogene Asian freshwater crabs, see Klaus et al. in press). Yet, the Aegean area is at least the Mediterranean biodiversity hotspot for the genus *Potamon*. The distribution area of five currently recognised species (see Brandis et al. 2000) and of all four subgenera meet in this small and highly structured geographical region.

In the present study, we aimed to clarify the phylogeny and phylogeography of the freshwater crab genus *Potamon* in the Aegean region by analysing specimens of eleven described species with both mitochondrial and nuclear genetic markers. In detail, we addressed the following questions:

1. Are the morphologically described species congruent to evolutionary lineages?
We assessed the distribution of evolutionary lineages in the Aegean area in a DNA taxonomy approach to obtain monophyletic taxonomic units necessary for biogeographic analysis.

2. How did the palaeogeographical and climatic history of the Aegean region influence the diversification in *Potamon*? Did the region serve as centre of origin, or as an area where evolutionary lineages accumulated that originated outside the Aegean area?

1.2. Material and Methods

Sampling

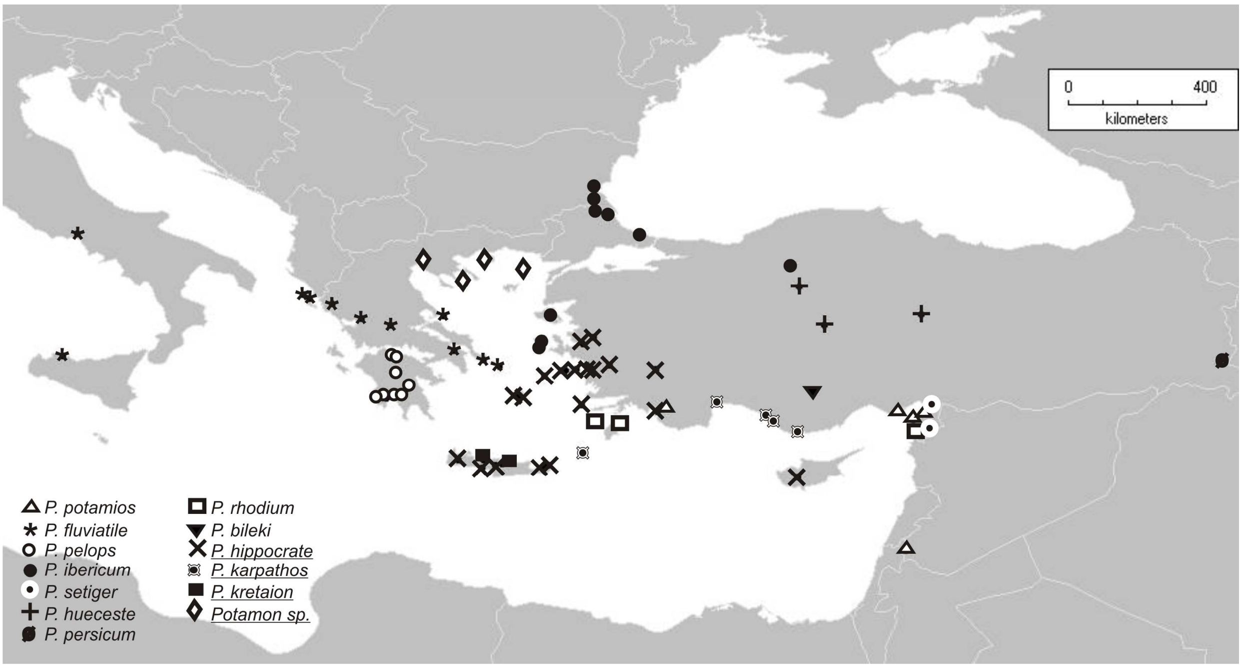

Figure 1: Sampling sites of the analysed species in the genus *Potamon*. *P. algeriense* from Algeria not shown

Museum tissue samples, stored in ethanol, were taken from 110 specimens originally assigned to eleven species in the revision of Brandis et al. (2000), and covering all four subgenera of the freshwater crab genus *Potamon* (see Table 1 for museum voucher numbers and provenance). 26 genetic sequences originated from two previous studies (see Table 1) on *Potamon fluviatile* (Jesse et al. 2009) and *Potamon pelops* (Jesse et al. 2010). *Paratelphusula martensi* and *Himalayapotamon emphysetum*, both genera being closely related to the genus *Potamon* (Shih et al. 2009), served as outgroups in phylogenetic reconstruction. Maps of distribution (Figure 1) were designed with the program DIVA-GIS vers. 5.2.0.2.

Table 1: Study specimens with origin, obtained sequences and museum vouchers; Names underlined are the three lineages elevated from subspecies rank and one newly identified cryptic clade in this study, individuals printed in bold were included from previous studies; *= originally described as *P. potamios*; **= originally *P. ibericum*; ***= originally *P. rhodium*; ****= originally *P. setiger*; NHMW= Naturhistorisches Museum, Wien; MZUF= Museo Zoologico dell' Università, Firenze; SMF= Senckenberg Museum, Frankfurt; ULLZ= University of Louisiana, Lafayette, Zoological Collection;

Abbreviation	Sampling site and year	COX1	ND1	28S	Voucher number
Potamon algeriense					
***PA*-Alg**	Morocco, Gorges du Zegzel; 2005	X	X	X	SMF 31799
Potamon bileki					
PB-Kara	Turkey, Province Karaman, Karaman; 1970	--	X	--	NHMW 3785
Potamon fluviatile					
PF-And	Greece, Island of Andros; 1978	X	X	--	NHMW 4326/1
PF-Eub1	Greece, Island of Euböa; 1980	X	X	--	NHMW 4784
PF-Eub2	Greece, Island of Euböa; 1980	X	X	--	NHMW 4794
***PF*-H 1**	Italy, Ausonia; 2005	X	X	--	MZUF2947
***PF*-H 9**	Greece, Island of Euböa; 1989	X	X	--	NHMW 17041
***PF*-H 10**	Greece, Stephanos; 1978	X	X	--	NHMW 4327
***PF*-H 11**	Greece, Paramythía; 2003	X	X	X	SMF 30166
***PF*-H 12**	Greece, Vítoli; 2003	X	X	--	ULLZ 3792
***PF*-H 13**	Greece, Island of Corfu; 2002	X	X	--	SMF 29345
***PF*-H 14**	Greece, Patíopulon; 2003	X	X	--	NHMW 21248
***PF*-H 15**	Greece, Sporades; 1980	X	X	--	SMF 15122
***PF*-H 16**	Greece, Island of Corfu; 2002	X	X	--	SMF 29345
***PF*-H 17**	Italy, Palermiti; 2006	X	X	--	SMF pending
***PF*-H 18**	Greece, Island of Corfu; 2002	X	X	--	SMF 29345
Potamon hippocrate					
PR-Ayd1***	Turkey, Province Aydin, Aydin; 1969	X	X	--	NHMW 4088
PP-Ayd2*	Turkey, Province Aydin, Kusadasi; 1968	X	--	--	SMF 5443
PP-Ayd3*	Turkey, Province Aydin, Söke; 1969	X	--	--	NHMW 4806
PP-Cos1*	Greece, Island of Cos; 1979	X	X	--	NHMW 4507
PP-Cos2*	Greece, Island of Cos; 1979	X	X	--	NHMW 4507
PP-Cos3*	Greece, Island of Cos; 1979	X	X	--	NHMW 4507
PP-Cyp1*	Island of Cyprus; 1980	X	--	--	NHMW 4505
PP-Cre1*	Greece, Island of Crete; 1980	X	X	--	NHMW 4798
PP-Cre2*	Greece, Island of Crete; 1980	X	X	--	NHMW 4798
PP-Cre3*	Greece, Island of Crete; 1965	X	X	--	NHMW 4296
PP-Cre4*	Greece, Island of Crete; 1971	X	--	--	NHMW 3839
PP-Cre7*	Greece, Island of Crete; 1972	X	--	--	NHMW 4351
PP-Cre8*	Greece, Island of Crete; 1942	X	X	--	NHMW 1699
PP-Cre9*	Greece, Island of Crete; 1942	X	X	--	NHMW 1697
PP-Cre10*	Greece, Island of Crete; 1942	X	X	--	NHMW 1697
PP-Cre11*	Greece, Island of Crete; 1942	X	--	--	NHMW 1697
PP-Cre12*	Greece, Island of Crete; 1942	X	X	--	NHMW 1697

Table 1 (continued)

Abbreviation	Sampling site and year	COX1	ND1	28S	Voucher number
PR-Den***	Turkey, Province Denizli, Denizli; 1969	X	X	--	NHMW 4091
PR-Ika***	Greece, Island of Ikaria; 1976	X	X	--	NHMW 4295
PR-Izm1***	Turkey, Province Izmir, Izmir; 1969	X	X	X	NHMW 4083
PR-Man***	Turkey, Province Manisa, Manisa; 1969	X	--	--	NHMW 4807
PP-Mug1*	Turkey, Province Mugla, Fethiye; 1977	X	X	--	SMF 24118
PP-Nax1*	Greece, Island of Naxos; 1981	X	X	--	NHMW 17045
PP-Nax2*	Greece, Island of Naxos; 1980	X	--	--	NHMW 4790
PP-Sam1*	Greece, Island of Samos; 1932	X	X	--	NHMW 3214
PP-Sam2*	Greece, Island of Samos; 1936	X	X	--	NHMW 3216
PR-Sam3***	Greece, Island of Samos; 1979	X	X	--	NHMW 4509
PR-Sam4***	Greece, Island of Samos; 1979	X	X	--	NHMW 4509
PR-Sam5***	Greece, Island of Samos; 1987	X	X	--	NHMW 17054
Potamon hueceste					
PH-Ank1	Turkey, Province Ankara, Ankara; 1885	--	X	--	NHMW 3220
PH-Ank5	Turkey, Province Ankara, Sereflikochisar; 1972	--	X	--	NHMW 4133
PH-Kay	Turkey, Province Kayseri, Kayseri-Sivas; 1975	X	X	X	NHMW 4134
Potamon ibericum					
PI-Ank2	Turkey, Province Ankara, Kizilcahaman; 1969	X	X	--	NHMW 3983
PI-Ank3	Turkey, Province Ankara, Kizilcahaman; 1966	X	--	--	NHMW 3981
PI-Ank4	Turkey, Province Ankara, Kizilcahaman; 1967	--	X	--	NHMW 3982
PI-Chi1	Greece, Island of Chios; 1986	X	X	X	NHMW 5694
PI-Chi2	Greece, Island of Chios; 1986	X	X	--	NHMW 5694
PI-Chi3	Greece, Island of Chios; 1975	X	X	--	NHMW 4365
PI-Fra1	France, Saint-Guilhem-le-Désert; 1994	X	X	--	NHMW 21934
PI-Fra2	France, Saint-Guilhem-le-Désert; 1994	X	X	--	NHMW 21934
PI-Izm2	Turkey, Province Izmir, Izmir; 1969	X	X	--	NHMW 3956
PI-Ist	Turkey, Province Istanbul, Istanbul; 1967	X	--	--	NHMW 21931
PI-Kir	Turkey, Province Kirkladi, Demirkoy; 1967	X	X	--	NHMW 4311
PI-Les	Greece, Island of Lesbos; 1975	X	X	--	NHMW 4364
PI-Bul1	Bulgaria, Malko Tarnovo; 1980	X	--	--	NHMW 4816/48
PI-Bul2	Bulgarien, SO, Krushevets; 1980	X	--	--	NHMW 4811
PI-Bul3	Bulgaria, Mladezhko; 1980	X	--	--	NHMW 4813/48
Potamon karpathos					
PP-Ant1*	Turkey, Province Antalya, Alanya; 1987	X	X	X	NHMW 17050
PP-Ant2*	Turkey, Province Antalya, Antalya; 2001	X	X	--	SMF 28771
PP-Ant3*	Turkey, Province Antalya, Demirtas; 1969	--	X	--	NHMW 4108
PP-Kar1*	Greece, Island of Kárpathos; 1977	X	X	--	NHMW 4293
PP-Kar2*	Greece, Island of Kárpathos; 1963	X	X	--	SMF 24091
PP-Kar3*	Greece, Island of Kárpathos; 1960	X	X	--	SMF 2635
PP-Kar4*	Greece, Island of Kárpathos; 1960	X	X	--	SMF 2607
PP-Kar5*	Greece, Island of Kárpathos; 1977	X	X	--	NHMW 4294
PN-Mer1	Turkey, Province Mersin, Anamur; 1969	X	X	--	NHMW 17044
PN-Mer2	Turkey, Province Mersin, Anamur; 1969	X	X	--	NHMW 17044

Table 1 (continued)

Abbreviation	Sampling site and year	COX1	ND1	28S	Voucher number
Potamon kretaion					
PP-Cre5	Greece, Island of Crete; 1980	X	X	X	NHMW 4796
PP-Cre6	Greece, Island of Crete; 1982	X	X	--	NHMW 4801
Potamon pelops					
PE-Pel1	Peloponnesus; 1984	X	X	--	NHMW 21905
PE-Pel2	Peloponnesus; 2007	X	X	--	SMF pending
PE-Pel3	Peloponnesus; 2007	X	X	--	SMF pending
PE-Pel4	Peloponnesus; 2007	X	X	--	SMF pending
PE-Pel5	Peloponnesus; 2007	X	X	--	SMF pending
PE-Pel6	Peloponnesus; 2007	X	X	--	SMF pending
PE-Pel7	Peloponnesus; 2007	X	X	--	SMF pending
PE-Pel8	Peloponnesus; 2007	--	--	X	SMF pending
PE-Pel9	Peloponnesus; 2007	X	X	--	SMF pending
PE-Pel10	Peloponnesus; 1984	--	--	X	NHMW 21905
Potamon persicum					
Pp-Van	Turkey, Province Van, Baskale; 1972	X	X	X	SMF 5882
Potamon potamios					
PP-Ada	Turkey, Province Adana, Bebeli; 1982	X	X	X	SMF 24103
PP-Isr	Israel, Golan; 1980	X	X	--	NHMW 4501
PP-Mug2	Turkey, Province Mugla, Üzümlü; 1964	X	X	--	NHMW 3263
PP-Mug3	Turkey, Province Mugla, Üzümlü; 1964	X	X	--	NHMW 3263
PS-Hat4****	Turkey, Province Hatay, Arsuz; 1966	X	--	--	NHMW 4127
PS-Hat7****	Turkey, Province Hatay, Iskenderum; 1977	X	X	--	SMF 24155
Potamon rhodium					
PS-Hat2****	Turkey, Province Hatay, Antakya; 1982	--	X	--	SMF 24168
PS-Hat3****	Turkey, Province Hatay, Antakya; 1988	X	X	--	SMF 24173
PR-Rho1	Greece, Island of Rhodes; 1971	X	X	--	NHMW 17028
PR-Rho2	Greece, Island of Rhodes; 1971	X	X	--	NHMW 17028
PR-Rho3	Greece, Island of Rhodes; 1971	X	X	--	NHMW 17028
PR-Rho4	Greece, Island of Rhodes; 1977	--	--	X	NHMW 4292
PP-Til*	Greece, Island of Tilos; 1983	--	X	--	SMF 24093
Potamon setiger					
PS-Hat1	Turkey, Province Hatay, Amanus; 1982	X	X	X	SMF 24167
PS-Hat5	Turkey, Province Hatay, Antakya; 1979	X	X	--	SMF 24160
PS-Hat6	Turkey, Province Hatay, Antakya; 1979	X	X	--	SMF 24160
***Potamon* sp.**					
PI-Cha1**	Greece, Peninsula of Chalkidike; 1976	X	X	--	NHMW 4281
PI-Cha2**	Greece, Peninsula of Chalkidike; 1927	--	X	--	NHMW 3208
PI-Sth1**	Greece, Island of Samothraki; 1981	--	X	--	NHMW 4512
PI-Sth2**	Greece, Island of Samothraki; 1934	--	X	--	NHMW 3200
PI-Sth3**	Greece, Island of Samothraki; 1934	X	--	--	NHMW 3200
PI-Tha1**	Greece, Island of Thassos; 1970	--	X	X	NHMW 4283
PI-Tha2**	Greece, Island of Thassos; 1975	--	X	--	NHMW 4288

DNA extraction, fragment amplification and sequencing

Genomic DNA was extracted from muscle tissue of a walking leg using the Puregene Kit (Gentra Systems). The selective amplification of a 574 bp fragment of cytochrome oxidase subunit I (COX1), a 612 bp fragment of subunit 1 of the nicotinamide adenine dinucleotide dehydrogenase (ND1) with neighbouring regions (part of the 16S rRNA gene and the tRNA of leucine) and 605 bp of the nuclear marker 28S rRNA was carried out by polymerase chain reaction (PCR). PCR (40 cycles; denaturing 94°C 45s/ annealing 48°C 1min/ extension 72°C 1min) was conducted with the primers COL6b and COH6 for the COX1 gene, 16L11, NDL5, NDH5 and NDH2 for the partial 16S rRNA, tRNA Leu and ND1 genes (see Jesse et al. 2009) and 28Sa and 28Sb for the 28S rRNA gene (see Jesse et al. 2010). The PCR products were purified with the Sure Clean Plus Kit (Bioline) and precipitated with ethanol. The products were sequenced with the ABI Big Dye terminator mix (Big Dye Terminator® v 3.1; Applied Biosystems) on an ABI Prism ™ 310 Genetic Analyser. Sequences were analysed with the program ABI Sequencing Analysis®3.4 (Applied Biosystems), manually proofread and aligned with ClustalW (Thompson 1994) in BioEdit (Hall 1999). All sequences aligned unambiguously and protein coding sequences translated without stop codons. Sequences are available in GenBank, accession numbers are HQ223109 – HQ223282.

DNA taxonomy, phylogenetic inference and divergence time estimation

To reveal evolutionary lineages within the genus *Potamon*, and to confirm the identity of museum specimens, we calculated two unrooted trees for the ND1 (including 5′ neigbouring regions; 92 specimens) and COX1 (94 specimens) partitions (MrBayes, 10 M generations, four chains, burn-in 10 %, default prior settings for the substitution model). As we did not succeed to amplify both genes for the whole material (see Table 1), the independent analyses of the two partitions were neccessery for unambiguous assignment of all specimens to one of the lineages.

For phylogenetic inference, divergence time estimation and temporal hypothesis testing, Bayesian analyses were performed in BEAST vers. 1.5.3 (Drummond and Rambaut 2007). We included one representative from each of the 13 lineages retrieved in the previous two analyses, and used five separate alignment partitions (COX1, ND1, the 5′ flanking regions of

ND1, 16S rRNA and tRNALeu, and the nuclear encoded 28S rRNA gene). Molecular clock and substitution models for the five gene partitions were unlinked. We let the analysis run for 50 million generations. To ensure stationarity, the analysis was repeated three times from different random starting points and convergence of parameters was checked in Tracer vers. 1.5 (Rambaut and Drummond 2007). After a burn-in phase of 10 %, trees were sampled every 1000th generation. Models of sequence evolution were set for all partitions individually (28S rRNA: GTR+G; COX1: GTR+G; 16S rRNA: HKY; tRNALeu: HKY+G; ND1: HKY+G). We chose the models of evolution as suggested by jModeltest 0.1 (Posada and Crandall 1998), a Yule tree prior and an uncorrelated relaxed molecular clock.. Normal prior distribution with 10 % standard deviation was set for COX1 and 16S rRNA mean rates (COX1 2.33% and 16S rRNA 0.88% per Ma; based on Schubart et al. 1998). The earliest split within *Euthelphusa* (C2), separating north African *Potamon algeriense* from the other two species, was calibrated with the end of the Messinian period (5.3 Mya, Krijgsmann et al. 1999) as lower cut-off value (gamma-shaped, alpha=1.9, scale parameter=1.0) . Dispersal of *Potamon* to Northern Africa was likely to have occurred during the latest Messinian, after the Mediterranean basin had desiccated during the Messinian Salinity Crisis (see Brandis et al. 2000) and brackish to freshwater conditions prevailed thereafter (Orszag-Sperber 2006). A maximum clade credibility tree was computed with TreeAnnotator 1.5.3 (see Figure 4).

We applied a Bayesian model selection approach to decide which biogeographic scenario best fits the data (Klaus et al. 2010; Pfenninger et al. 2010). To test if the first split (C1, Figure 4) within the genus *Potamon* coincides with the formation of the Mid-Aegean trench we conducted three additional analyses with the same parameter settings, but calibrated in addition the node (C1) with three different time intervals. We assumed an age of 10.5 ± 1.75 Mya for divergence during the trench formation (mean of the normal prior distribution and standard deviation). This value covers the supposed period of this geological event (12.0 – 9.0 Mya; Dermitzakis and Papanikolaou 1981). In the other two analyses C1 was set before the trench formation (14.5 ± 1.75 Mya) and after (6.5 ± 1.75 Mya), such that the prior distributions slightly overlap.

Biogeographic analysis

To evaluate if the Aegean region served as a centre of origin for freshwater crab species or if the extant species dispersed into the Aegean region from surrounding areas, we applied a parametric biogeographic method as implemented in Lagrange vers. 20091004 (Ree and Smith 2008). Lagrange reconstructs the range inheritance at a node with a likelihood approach, based on dispersal and extinction rates that are estimated via a simulation process (Ree et al. 2005). We assigned the clades to the following areas: A = Africa; B = Balkan Peninsula, the Aegean islands and the eastern tributaries flowing into the Aegean Sea; C = Anatolia, tributaries to the Black Sea, South- and Southwest Turkey, Cyprus and the Levant. We calculated three models, one unconstrained model (H_0) with the above parameter settings, one enabling only dispersal from the West (areas A, B) to the East (area C; $H_{West-->East}$), and one model which allowed only dispersal from the East into the Aegean region ($H_{East-->West}$). The global maximum likelihood at the root of the three models was compared and the models ranked according to the Akaike Information criterion (AIC).

1.3. Results

Mitochondrial lineages and phylogeny

By analysing COX1 sequences of 94 individuals, we found 14 genetic lineages of which nine could be assigned to existing species (Figure 2). Bayesian analysis of 92 specimens with the marker ND1 showed a congruent pattern (Figure 3). ND1 analysis additionally included one individual of *Potamon bileki* (only present in Figure 3), from which solely this fragment could be obtained. The phylogenetic inference validated the classification of the genus *Potamon* into four subgenera based on male gonopod morphology (Brandis et al. 2000), with the reservation that some Middle East *Potamon* species not be evaluated in this regard here. The 13 lineages separated into a western group (all three species of the subgenus *Euthelphusa*) and an eastern group (ten lineages in the subgenera *Potamon, Pontipotamon* and *Orientopotamon*) (Figure 4). Uncorrected pairwise distances within lineages ranged from 0.0 to 2.7 %, between lineages from 3.1 to 11.2 %.

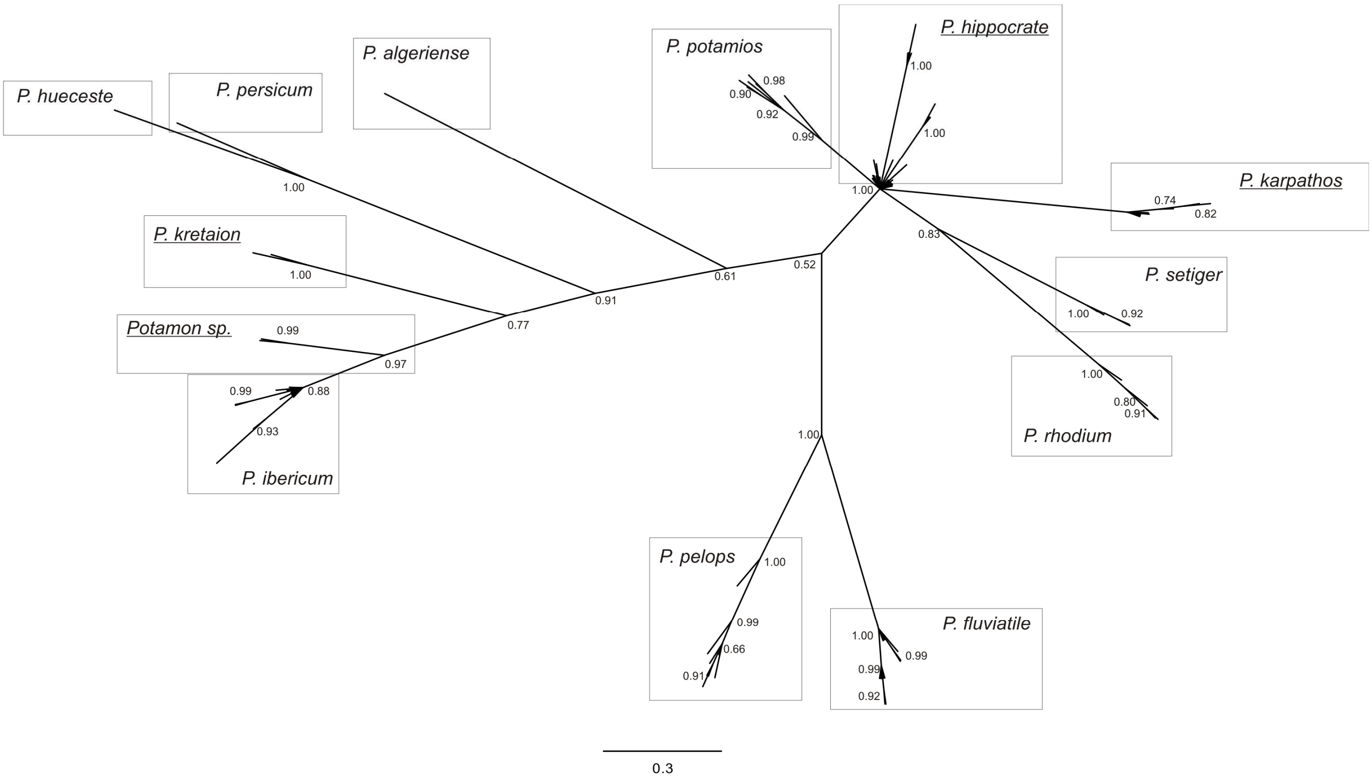

Figure 2: Unrooted majority rule consensus tree calculated by Bayesian analysis for the COX1-fragment. Numbers on nodes indicate their Bayesian posterior probability, values lower than 0.5 are not shown.

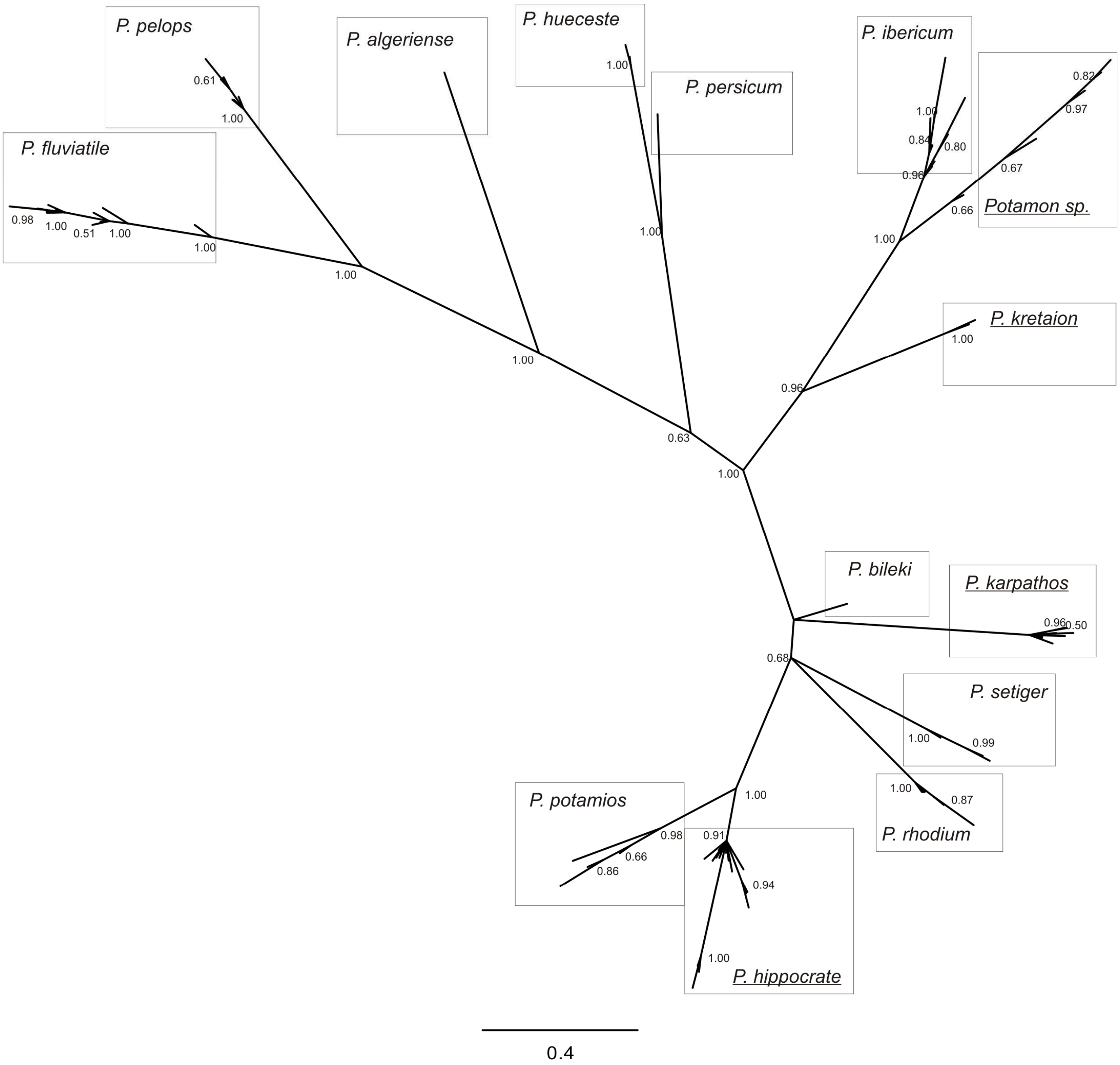

Figure 3: Unrooted majority rule consensus tree calculated by Bayesian analysis for the ND1-fragment. Numbers on nodes indicate their Bayesian posterior probability, values lower than 0.5 are not shown.

Taxonomically unrecognised, but highly divergent lineages occurred in the subgenera *Pontipotamon* and *Potamon* (underlined in all figures). Individuals assigned as *Potamon ibericum* clustered into two lineages (*P. ibericum* and *Potamon* sp.*)* with an uncorrected genetic distance of 3.2 ± 0.1 %. The other three unrecognised lineages occurred at the type localities from previously described subspecies of *Potamon potamios*: *Potamon potamios karpathos* from Karpathos; *Potamon potamios hippocrate* from Kos (both clustering within the subgenus *Potamon*) and *Potamon potamios kretaion* from Crete clustering within the subgenus

Pontipotamon. The uncorrected pairwise genetic distances between these three clades and their sister clades were: *P. potamios kretaion* / *P. ibericum*: 8.3 ± 2.1 %; *P. potamios hippocrate* / *P. potamios*: 4.2 ± 1.0 %; *P. potamios karpathos* / *P. potamios*: 10.9 ± 2.6 %. The mean distances within the three lineages are: *P. potamios kretaion* 1.1 ± 0.6 %, *P. potamios hippocrate* 2.4 ± 0.7 % and *P. potamios karpathos* 0.2 ± 0.1 %.

Model testing and temporal splitting pattern

The constraint of node C1 to 6.5 ± 1.75 Mya, after formation of the Mid-Aegean trench, received the best support by the data (Table 2). Bayes factor analysis provided substantial to strong support for this model in comparison to the scenarios constraining node C1 before or during formation of the Mid-Aegean trench (applying the interpretation guidelines for Bayes factor comparisons by Kass and Raftery 1993).

Table 2: Probability of different temporal models, their standard error (S.E.) and logarithmic bayes factors (ln P) between the models as calculated in Tracer. Tested were the unconstrained phylogenetic analysis, calibrated with the latest Messinian for node C2 (see Figure 4), and the three models constraining node C1 before, during and after the formation of the Mid-Aegean trench.

			unconstrained	before	during	after
Model	**ln P (model | data)**	**S.E.**		**formation of the Mid-Aegean trench**		
unconstrained	-5832.40	± 0.14	_	1.34	0.40	-0.13
before trench formation (node C1: 14.5 ± 1.75 Mya)	-5835.48	± 0.14	-1.34	_	-0.94	-1.47
during trench formation (node C1: 10.5 ± 1.75 Mya)	-5833.33	± 0.13	-0.40	0.94	_	-0.54
after trench formation (node C1. 6.5 ± 1.75 Mya)	-5832.09	± 0.13	0.13	1.47	0.54	_

The first split within *Potamon*, dividing a western and an eastern group (node C1), occurred 8.3 – 5.5 Mya (95% credibility interval, see Figure 4). The split of *Orientopotamon* from *Potamon* and *Pontipotamon* (6.6 – 3.1 Mya) covered the period of the Messinian Salinity Crisis. The subgenera *Pontipotamon* and *Potamon* separated 5.4 – 2.3 Mya. The speciation events within the subgenera (apart from *P. algeriense*) were estimated to have happened during the Pleistocene (Figure 4).

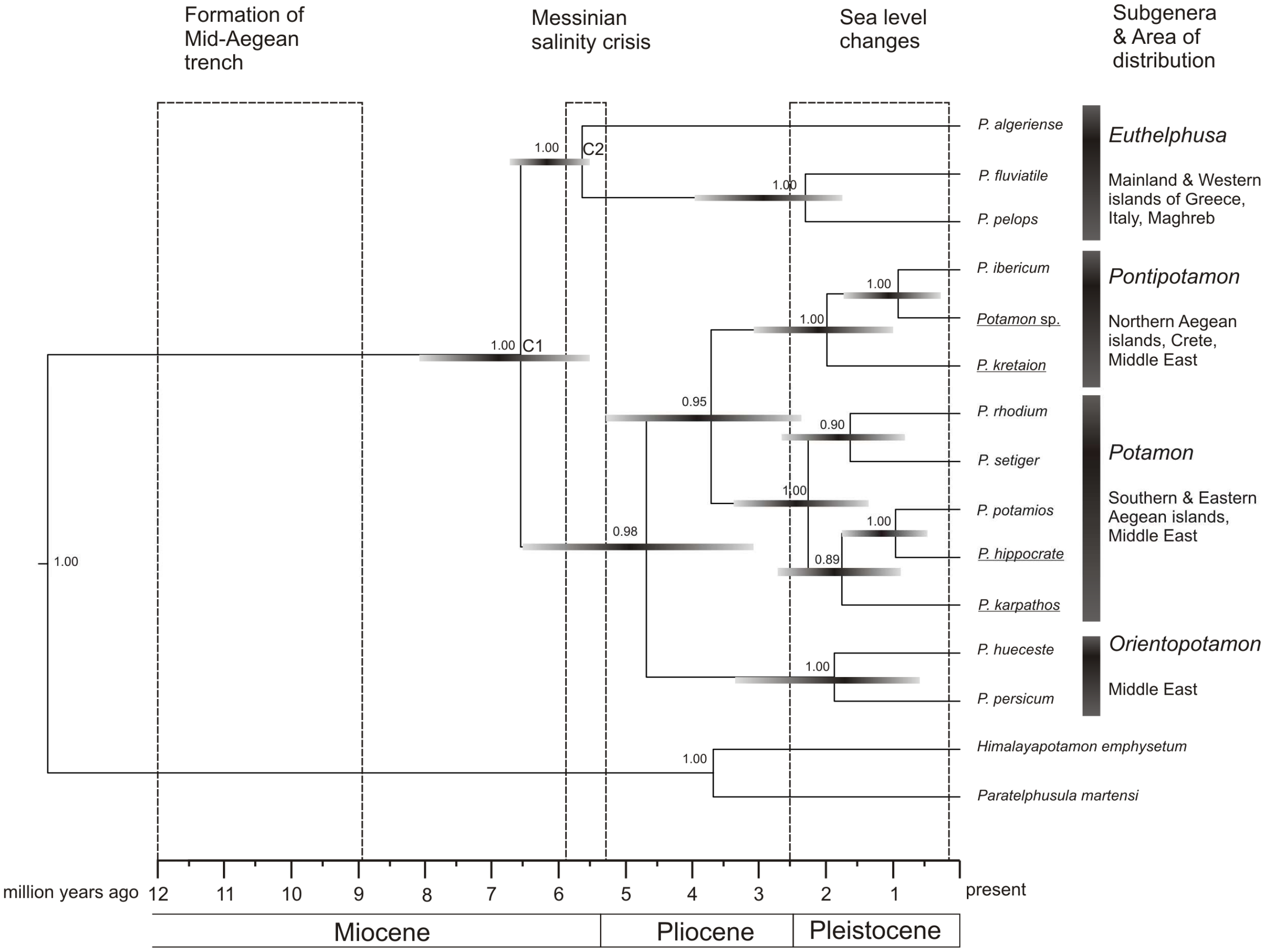

Figure 4: Maximum clade credibility tree of the concatenated data set (COX1, 16S rRNA, ND1, tRNALeu & 28S rRNA) and inferred divergence times among circum-Mediterranean species of the genus *Potamon* (Bayesian analysis with BEAST). Bars at the nodes represent 95 % posterior density credibility intervals, numbers represent posterior probabilities. Node C1 was subject to temporal constraints for the model testing approach, node C2 was calibrated with the MSC.

Aegean region as centre of origin

Among the three dispersal models calculated in Lagrange, the scenario enabling only dispersal from Asia Minor (area C) into the Aegean region (area B) and to Africa (area A) had the best global maximum likelihood at the root (-ln L = 21.0; AIC = 50), in contrast to the $H_{W\text{-->}E}$ (-ln L = 23.52; AIC = 55.04) and the H_0 model (-ln L = 21.69; AIC = 55.38). Thus, most probably the origin of the genus *Potamon* was located in the east of the Aegean region (Asia Minor or Middle East), with subsequent dispersal into western Asia Minor, to the Aegean islands and the Balkan Peninsula.

1.4. Discussion

Morphological versus molecular taxonomy

The last revision of the genus *Potamon* recognised 16 species based on the morphology of the male reproductive apparatus, especially the first gonopods (Brandis et al. 2000). This morphological character was supposed to be environmentally neutral and also served to delimitate the four subgenera within *Potamon*. On subgenus level there was no discrepancy in our study between morphological and DNA taxonomy within the circum-Mediterranean species. On species level, however, it had been previously shown that cryptic speciation occurred in the genus (*Potamon pelops*, Jesse et al. 2010). Applying the unified species concept (De Queiroz 2007), which suggests that separately evolving metapopulation lineages should be treated as single species, we propose that the three former subspecies of *Potamon fluviatile* (later transferred to *Potamon potamios*, see Pesta 1937) indeed present biological species: *Potamon hippocrate* Ghigi, 1929 (type locality: Island of Cos), *Potamon karpathos* Giavarini, 1934 (type locality: Island of Karpathos) and *Potamon kretaion* Giavarini 1934 (type locality: Crete). These had been initially delimited by carapace morphometrics (Ghigi 1929; Giavarini 1934), but their subspecies status was rejected in Brandis et al. (2000), as they showed no differences in gonopod morphology. We strongly recommend to exclude these three subspecies from *Potamon potamios* and elevate them to species rank within the subgenera *Potamon* and *Pontipotamon*. Our genetic results support the identification as species by previous morphometric analysis, thus stressing the value of this method in brachyuran taxonomy.

The species *Potamon ibericum* (Bieberstein 1809) has its type locality in Georgia at the coast of the Black Sea. Therefore, the specimens sampled from localities in the coastal areas of the Black Sea, as well as the Turkish west coast could be identified as *Potamon ibericum*. A reciprocally monophyletic sister lineage that was morphologically assigned to *P. ibericum* was identified in northern Greece (Figure 1). The two genetic lineages have an average uncorrected pairwise distance of 3.2 ± 1.0 %, within the lineages ranging from 0.6 to 0.2 %. Additionally, no geographic overlap of the clade ranges could be detected. We suggest therefore that these individuals may belong to a separately evolving, cryptic lineage, requiring further taxonomic attention. For clarity, we referred to the lineage from the Northern Aegean area hereafter as *Potamon* sp. (Figures 2-4).

In consideration of the above arguments, we suggest that the total species number within the genus *Potamon* is at least 21. The taxonomy based on the male copulatory system is well established and congruent with the present phylogenetic data down to subgenus level. Also on species level, the use of gonopod characters did not lead to polyphyletic assemblages (although we identified frequent misidentifications, see Table 1). However, a more accurate resolution on species-level can obviously be achieved by additional analyses with molecular markers and morphometrics.

Origin of the Mediterranean freshwater crab fauna

Based on Lagrange analyses, the genus *Potamon* in the Aegean area most likely had its origin in Asia Minor and/or the Middle East, as dispersal from these eastern regions fitted the present distribution patterns best. This result is congruent with the distribution of potamid freshwater crab diversity. Highest species numbers can be found in East and South East Asia where the potamid subfamily Potamiscinae dominates with more than 460 species while the western subfamily Potaminae only comprises 47 species. Considering this diversity pattern, Pretzmann (1987) already suggested a dispersal hypothesis for the colonisation of Europe by potamid crabs originating from the Middle East.

Interestingly, the distribution pattern of the Eastern Aegean and Anatolian species shows strong affinities to the distribution pattern of haplotype groups of water frogs that diversified approximately at the same time as the freshwater crabs (Çiğdem et al. 2010). With *P. ibericum* a northern group reaches into the Aegean area, while the more southern Aegean islands were colonized by Pamphylian and Cilician lineages that also reached Cyprus. Assuming similar dispersal pathways for amphibians and freshwater crabs, this corroborates an eastern origin for the genus *Potamon*.

Divergence time estimates and fossil record

Lagrange analyses, in combination with divergence time estimates (Figure 4), raised questions on the identity and relationship of Central European fossils of *Potamon*. Currently five fossil species are recognised from geological basins around the Alps. *Potamon quenstedti* from Southern Germany (Klaus and Gross 2010), the first occurrence dating back 16.5 Ma, predates the most recent common ancestor of the extant Mediterranean representatives of the genus *Potamon* at least by eight million years. As the assignment of these fossils to the genus *Potamon* is morphologically indisputable, they can therefore be seen as evidence for an earlier dispersal of potamids into Europe. The cooling of the climate at the end of the Miocene was a possible cause for the subsequent extinction in Western Europe. Replacement of the Miocene/Pliocene *Potamon castelliense* by extant *Potamon fluviatile* in Italy after the last glacial period had already been demonstrated (Jesse et al. 2009; Klaus and Gross 2010). In contrast, Pliocene and Pleistocene fossils of *Potamon antiquum* from Hungary fit well to our present time estimates and are thus potentially extinct members of the Pleistocene radiation. It was already proposed that this species in fact represents evidence for the northern distribution limit of *Potamon ibericum* during phases of warmer climate (Klaus and Gross 2010). Conclusively, the speciation of lineages in the Mediterranean area during the Miocene to Pleistocene most likely represents a secondary colonisation of the region.

The Mid-Aegean trench and the Messinian salinity crisis: possible vicariant events

The circum-Mediterranean *Potamon* species are divided into a western (subgenus *Euthelphusa*) and an eastern clade (subgenera *Potamon, Pontipotamon* and *Orientopotamon*) (see Figure 4). It was tempting to attribute this separation to the formation of the Mid-Aegean Trench (12 – 9 Mya; Dermitzakis and Papanikolaou 1981), as this geological event had been proven in various studies to be a major force in shaping biogeographical patterns in Aegean species (Parmakelis et al. 2005; Poulakakis et al. 2008; Simaiakis and Mylonas 2008). Yet, the time estimate (95% credibility interval: 8.3 – 5.5 Mya) and the model selection approach suggested that this divergence event had occurred only after formation of the trench, implying dispersal across this barrier or bypassing at the northern fringe of the Aegean Sea. Additionally, the Lagrange analyses implied an eastern origin of the most recent common ancestor of *Potamon* (see above). Maybe moderately wide marine barriers could have been surmounted by rafting as proposed for other terrestrial species like rodents (Rowe et al. 2010), worm lizards (Vidal et al. 2008) and frogs (Measey et al. 2007).

Dispersal of freshwater organisms across the Mid-Aegean trench with subsequent vicariant isolation could have been a likely event during the latest Messinian (5.96 – 5.33 Mya). The

200 ka following the evaporitic phase of the Messinian salinity crisis were dominated by brackish to freshwater conditions in much of the Mediterranean basin until the re-flooding at the end of the Messinian period (the so-called "Lago-Mare" facies, see Orszag-Sperber 2006). As the time estimate of the initial east-west divergence in *Potamon* overlaps with the latest Messinian, the respective palaeogeographic conditions can at least not be excluded as cause for the initial diversification.

Diversification of Potamon *during Pliocene and Pleistocene*

A time period of increased diversification of *Potamon* was the Pleistocene (Figure 4). Between 3.96 and 0.3 Mya (according to the 95% credibility interval of divergence times), all lineages currently situated in the Aegean region speciated. This coincides with the establishment of a Mediterranean type of climate with marked seasonality and stable summer draughts 3.2 – 2.8 Mya (Suc 1984; Thompson 2005). The trend of gradual cooling and drying culminated in the onset of the Pleistocene climatic oscillations after 2.8 Mya (Klotz et al. 2006). During the subsequent glacial periods the sea-level fluctuated up to 125 m (Fleming et al. 1998) thus opening and closing repeatedly potential dispersal corridors for terrestrial and limnic species. In previous studies, it had been assumed that biogeographic patterns in some species of the Aegean islands were caused by these Pleistocene sea-level changes (Allegrucci et al. 2009; Fattorini 2002). The Pleistocene terrestrial connections to the Aegean islands could explain the present insular occurrence of *Potamon* in the Aegean region. However, as the Aegean islands do not host endemic freshwater crab species and the respective lineages originated, according to the Lagrange analysis, east of the Aegean area, the Pleistocene sea-level changes have most probably not been involved in allopatric speciation. Only with *P. kretaion* on Crete we identified one island endemic.

As an alternative explanation, we propose that the Pliocene/Pleistocene climatic change itself might have contributed to the speciation of freshwater crabs. As aquatic organisms, the range of freshwater crabs is dependent on freshwater systems which are affected by the precipitation regime. Moreover, potamid freshwater crabs (as gill breathers) need humid conditions for prolonged survival outside water bodies and successful migration into neighbouring drainage systems (Gherardi et al. 1988a; 1988b). Thus, recurrent gene-flow between populations could have been diminished during phases of seasonal climate or even aridity. Especially local aridification of southwest Anatolia 2.6 – 1.8 Mya (Alçiçek 2010; Fortelius et al. 2006), a region where five of the extant *Potamon* species occur, could have favoured allopatric speciation. The Pliocene transformation to seasonal Mediterranean climate with winter precipitation and summer draught was already shown to influence the diversification of Mediterranean plants (Bittkau and Comes 2009; Presti and Oberprieler 2009) and Anatolian water frogs (Çiğdem et al. 2010). However, this hypothesis requires that reduced gene flow in the freshwater crabs not necessarily excludes rare colonisation events, e.g. to the Aegean islands.

The occurrence of *Potamon hippocrate* on Crete and Cyprus can not be explained straightforward by palaeogeographic data. This species managed, despite the likely absence of terrestrial connections during the Pleistocene, to colonise Crete as well as Cyprus (both isolated since the end of the Messinian; see Çiğdem et al. 2010). Anthropogenic involvement in the spread of *Potamon* species with present disjunct distribution patterns, like *P. hippocrate*, *P. rhodium*, *P. potamios* is another thinkable possibility, especially as *Potamon* was used for human consumption and played a role in the ancient Greek culture (Brandis 1997). As there are no fossils to date the time of residence in most areas, and as we do not have extensive population sampling of each species, we can not exclude this hypothesis. Unlike anthropogenic translocation, the possibility of human involvement in the speciation of lineages within *Potamon* can be clearly neglected. The lowest estimated 95% credibility interval for the speciation of lineages in *Potamon* predates the arrival of first modern humans in Europe by around 200 ka (Harvati et al. 2008).

Conclusions

This study is one of few attempts to reveal the biogeographic history of a freshwater taxon in the Aegean area based on molecular markers. Although freshwater crabs are considered to be moderately mobile and sensitive to salt water, major geological events, like the formation of the Mid-Aegean trench or the Pleistocene sea-level changes, did not result in vicariant speciation. Only the re-flooding of the Mediterranean basin at the end of the Miocene probably correlates chronologically with the separation of the Mediterranean species of

Potamon into an eastern and a western group. In comparison, the speciation patterns of reptiles (Poulakakis et al. 2005), beetles (Fattorini 2002), scorpions (Parmakelis et al. 2006a, b) and land snails (Parmakelis et al. 2005) are supposed to be affected by diverse tectonic events in the Aegean region.

Within the genus *Potamon* we found no insular endemics in the Aegean archipelago (with exception of *P. kretaion* on Crete), indicating that Pleistocene terrestrial connections might have enabled dispersal into the Aegean area. Yet, the subsequent isolation by marine transgression did not cause speciation. In fact, the diversification of *Potamon* most probably occurred east of the Aegean area during the Late Pliocene and Pleistocene. This coincides with climatic fluctuations and increased aridity in Anatolia, thus we hypothesise a correlation between these climatic oscillations and speciation events.

The present phylogeography of freshwater crabs in the Eastern Mediterranean area is highly similar to the distribution pattern of water frog haplotype groups that diversified approximately at the same time (Çiğdem et al. 2010).This indicates that there are either (1) profound differences between biogeographic patterns of terrestrial and freshwater organisms, with terrestrial groups being more affected by recent vicariant events than species with an amphibian lifestyle; or (2) that the congruent biogeographic pattern of water frogs and freshwater crabs is simply due to their diversification in the same time period and thus affected by the same geological setting, independent from the freshwater habitat. The phylogeographic investigation of terrestrial organisms with similar distribution areas and simultaneous diversification could test the validity of these assumptions.

Chapter 2

Identification of a cryptic lineage within *Potamon fluviatile* (Herbst) (Crustacea: Brachyura: Potamidae)

Ruth Jesse, Christoph D. Schubart, Sebastian Klaus

Abstract

Based on mitochondrial and nuclear sequence data we identify and describe an evolutionary separate lineage (*Potamon pelops* sp. nov.) within the potamid freshwater crab species *Potamon fluviatile* (Herbst 1785).

So far, *Potamon pelops* sp. nov. is only reported from the Peloponnesus Peninsula (Greece), probably allopatric to *P. fluviatile*. This cryptic lineage is not identical with the infrasubspecies *Potamon fluviatile fluviatile* nation *laconis* Pretzmann 1983, the latter being within the morphological variation of *Potamon fluviatile* and *Potamon pelops*. Morphologically, *Potamon pelops* sp. nov. differs from *Potamon fluviatile* probably in spermatophore packing (cleistospermia in *P. fluviatile* versus coeno- and cleistospermia in *P. pelops*).

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2.1. Introduction

Freshwater crabs are an important part of aquatic ecosystems (Dobson et al. 2002; Marijnissen et al. 2009), and can strongly dominate benthic invertebrate biomass (Abdallah et al. 2004; Dobson et al. 2007). With currently about 1300 described species, freshwater crabs also contribute substantially to aquatic biodiversity of the tropics and subtropics (Ng et al. 2008; Yeo et al. 2008). A recent evaluation of the conservational status of the global freshwater crab fauna classified four of the five currently recognised species of the Mediterranean area in the category "near threatened" (Cumberlidge et al. 2009). Although these species have large ranges, many populations already vanished because of human impact on the natural water balance, especially on the Ionian Islands (Dirk Brandis, personal communication), the southern and eastern Peloponnesus (authors' personal observation) and the Aegean Islands (Pretzmann 1995). Species delimitation and taxonomy are crucial for spatial classification of biodiversity and definition of conservational units – especially respective to cryptic speciation (Cook et al. 2008; Crandall et al. 2009). Cryptic species can be regarded as separately evolving metapopulation lineages (De Queiroz 2007) that were treated as a single species due to morphological similarity, until revealed by molecular techniques (Pfenninger and Schwenk 2007).

Freshwater crabs – independent from the marine habitat and lacking planctonic larvae – are claimed to have limited dispersal capabilities with lineages being drainage basin specific (Daniels et al. 2002; Daniels 2003; Daniels et al. 2006; Ng and Rodríguez 1995). Thus, one should expect reduced gene flow between hydrographic systems which potentially leads to speciation by allopatry and, in absence of morphological evolution, to cryptic species.

Potamon fluviatile (Herbst 1785) was the first scientifically described freshwater crab species. It comprises the westernmost natural freshwater crab populations in Europe and occurs on the Italian Peninsula (its type locality, Pretzmann 1983), on Sicily, the western Balkan Peninsula and the Peloponnesus (Brandis et al. 2000). In the course of a previous study on the phylogeography of *Potamon fluviatile* (Jesse et al. 2009), a specimen from the Peloponnesus (LAN, see Table 1) showed a cytochrome oxidase subunit 1 sequence strongly deviant from previously detected haplotypes of *Potamon fluviatile*. To elucidate the identity of this specimen and investigate the possibility that an evolutionary separate lineage exists on the Peloponnesus, we conducted a broader sampling and additionally included specimens from other Mediterranean freshwater crab species of the genus *Potamon*. Morphologically, several variants ("infrasubspecies" or "nationes") of *Potamon fluviatile* were already described (Pretzmann 1967, 1978, 1983, 1986a, 1986b). Among them is *Potamon fluviatile* nation *laconis* Pretzmann 1978, described from the Taygetos and Parnon mountains on the southern Peloponnesus.

Does this variant have a morphological basis?
And if so, does it represent a monophyletic and genetically divergent lineage, as possibly indicated by preliminary sequence data?

2.2. Material and Methods

Material

Specimens from three sampling sites were collected on the Peloponnesus in 2007 and additional individuals from two populations were obtained from museum collections (Table 1). These locations are distributed among drainage basins of three provinces (Laconia in the south, Arcadia in the center and Achaia in the north of the Peloponnesus) and include the two main hydrographic basins, the Evrótas and the Alfeiós systems. In the molecular phylogenetic analyses we included specimens of all Mediterranean species of *Potamon* (*P. fluviatile, P. ibericum, P. rhodium, P. potamios* and *P. algeriense*) for comparison. In part, sequences originated from a previous study (Jesse et al. 2009; see Table 1 for accession numbers). Specimens of *Potamon fluviatile* from the southern Greek mainland cover the whole range adjacent to the Peloponnesus from the Adriatic to the Aegean Sea (Figure 1). Two species from the potamid subfamily Potaminae that are adjacent to the range of the genus *Potamon* in the East *Himalayapotamon emphysetum* (Alcock 1909) and *Paratelphusula martensi* (Wood-Mason 1875; following the taxonomy of Brandis and Sharma 2005) served as outgroup in the phylogenetic analyses. As these two genera are the closest relatives of the genus *Potamon*, they are the most appropriate outgroups to investigate phylogenetic relationships within the genus *Potamon* (see Shih et al. 2009 for phylogenetic relationships within the Potamidae).

Morphological analyses

First gonopods were investigated by light microscopy and photographed. The male second gonopod was dehydrated and embedded with Spurr's resin. Cross-sections of 350 nm thickness were cut with an ultra-microtome and stained with Richardson's blue.

Table 1: Studied specimens with GenBank accession numbers and museum voucher identification. Sequences with the letter code "EU" are derived from a previous study (see Jesse et al. 2009). Abbreviations: NHMW= Naturhistorisches Museum, Wien; MZUF= Museo Zoologico dell' Università, Firenze; SMF= Senckenberg Museum, Frankfurt am Main; ULLZ= University of Louisiana, Lafayette, Zoological Collection, *(paratype natio *laconis*)

Specimen	Sampling site	COX1	ND1	28S rRNA	Voucher number
***Potamon pelops* sp. nov.**					
AKR	Greece, Peloponnesus, Achaia, Akrata, 38°9.47'N–22°19.94'E, 2007 leg. Jesse & Klaus	HM244844	HM244855	HM244829	SMF 36931
GOR	Greece, Peloponnesus, Arcadia, ruins of Gortys, 37°32.41'N–22°2.79'E, 2007 leg. Jesse & Klaus	HM244845	HM244856	HM244830	SMF 36932
LAN	Greece, Peloponnesus, Arcadia, 24 km SE of Lancadia, 1978 leg. Pretzmann	HM244846	HM244857	HM244831	NHMW 4323
VAS–1 (holotype)	Greece, Peloponnesus, Laconia, Vassarás, Xagdariás river, 37°11.55'N–22°26.69'E, 2007 leg. Jesse & Klaus	HM244847	HM244858	HM244832	SMF 36933
VAS – 2	Greece, Peloponnesus, Laconia, Vassarás, Xagdariás river, 37°11.55'N–22°26.69'E, 2007 leg. Jesse & Klaus	HM244848	HM244859	HM244833	SMF 36934
TAY*	Greece, Peloponnesus, Laconia, Taygetos mountains, 1969 leg. Rausch	HM244849	HM244860	—	NHMW 4305
Potamon fluviatile					
UMB; H1	Central Italy, Umbertide	EU908226	EU908249	HM244834	MZUF 2948
EUB; H9	Greece, Euboea	EU908234	EU908257	HM244835	NHMW 17041
STP; H10	Greece, Stephanos	EU908235	EU908258	—	NHMW 4327
PAR; H11	Greece, Paramythiá	EU908236	EU908259	HM244836	SMF 30166
VIT; H12	Greece, Vítoli	EU908239	EU908260	—	ULLZ 3792
PAT; H14	Greece, Patíopulon	EU908240	EU908262	—	NHMW 21248
SLU; H17	Southern Italy, San Luca	EU908243	EU908265	HM244837	MZUF 2957
Potamon algeriense					
	Morocco, Gorges du Zegzel	EU908247	EU908268	HM244838	SMF 31799
Potamon ibericum					
	Greece, Island of Chios	HM244850	—	HM244839	NHMW 5694
Potamon rhodium					
	Greece, Island of Rhodos	HM244851	HM244861	HM244840	NHMW 17028
Potamon potamios					
	Turkey, Köprü Irman	EU908248	EU908269	—	uncatalogued
Paratelphusula martensi					
1	India, West Bengal	HM24444852	HM244862	HM244841	SMF 29343
2	India, West Bengal	HM244853	—	HM244842	SMF 29343
Himalayapotamon emphysetum					
	Nepal, Lumbini Province, Bhut Khola at Bhut Pul, 27° 44.74' N 83° 28.51' E, 2000 leg. Brandis	HM244854	—	HM244843	SMF 26076

Figure 1: Map of southern Greece showing the sampling sites of *Potamon pelops* sp. nov. on the Peloponnesus Peninsula and of *Potamon fluviatile* from the Greek mainland (see also Table 1).

Molecular phylogenetic analyses

Genomic DNA was extracted from muscle tissue of a walking leg from individuals conserved in 70% ethanol using the CTAB method (Doyle and Doyle 1987). The selective amplification of sequence fragments from the cytochrome oxidase subunit 1 (COX1), from the subunit 1 of the nicotinamide adenine dinucleotide dehydrogenase (ND1) with neighbouring regions (3'-end of the 16S rRNA and the leucine tRNA gene) and a fragment of the nuclear 28S rRNA was carried out by polymerase chain reaction (PCR). The standard program (40 cycles; denaturing 94 °C 45 s annealing 48 °C 1 min extension 72 °C 1 min) was conducted with the primers COL6b (5'-ACA AAT CAT AAA GAT ATY GG-3') and COH6 (5'-TAD ACT TCD GGR TGD CCA AAR AAY CA-3') (Schubart and Huber 2006) for the COX1 gene, 28Sa-modified (5'-GAC CCG TCT TGA ARC ACG GA-3') and 28Sb (5'-TCG GAA GGA ACC AGC TAC-3') (Whiting et al. 1997) for the 28S rRNA and 16L11 (5'-AGC

CAG GTY GGT TTC TAT CT-3'), NDL5 (5'-TTG CTG GWT GRT CTT CWA ATT G-3'), NDH5 (5'-GCY AAY CTW ACT TCA TAW GAA AT -3') and NDH2 (5'-GCT AAA TAT ATW AGC TTA TCA TA -3') (Schubart 2009) for the ND1 gene. The PCR products were purified with the Sure Clean Plus Kit (Bioline). The PCR products were sequenced with the ABI Big Dye terminator mix (Applied Biosystems) in an ABI Prism automated sequencer (ABI Prism ™ 310 Genetic Analyzer). Sequences were analysed with the program ABI Sequencing Analysis®3.4 (Applied Biosystems) and manually proofread with the DNA sequencing software Chromas 1.55 (Technelysium, Pty. Ltd. 1998, Queensland, Australia). Sequences were aligned with Clustal W (Thompson et al. 1994) as implemented in the software BioEdit (Hall 1999) and adjusted manually. This resulted in a total alignment length of 1850 bp (COX1: 529 bp; 16S rRNA: 29 bp; tRNALeu: 97 bp; ND1: 820 bp; 28S rRNA: 375 bp). GenBank accession numbers are given in Table 1.

The phylogeny was inferred by maximum likelihood and Bayesian phylogenetic analyses. For both approaches, the molecular data was partitioned into the respective gene fragments. The Bayesian inference was performed in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). For each partition the model of sequence evolution was applied as suggested by jModelTest 0.1.1 (Posada 2008; COX1: GTR + gamma; 16S rRNA: HKY; tRNALeu: HKY + gamma; ND1: GTR + gamma; 28S rRNA: GTR + gamma). We ran two parallel analyses for 10×10^6 generations with four chains each. We sampled 10^4 trees in each run and discarded the first 10^3 as burn-in. Convergence of the two runs was analysed in Tracer 1.5 (Rambaut and Drummond 2007). The maximum likelihood analysis was conducted with RaxML 7.0.3 (Stamatakis 2006a). The protein coding ND1 and COX1 genes were additionally partitioned into codon positions. A GTR + gamma model of sequence evolution was used for the final maximum likelihood search, as suggested by the Akaike information criterion in jModelTest. For rapid bootstrapping (10^3 replicates) CAT approximation of rate heterogeneity is implemented in RaxML (Stamatakis 2006b). A second maximum likelihood analysis was done with the 28S rRNA partition alone (GTR + gamma, partitioned codon positions, 10^3 bootstrap replicates). Uncorrected p-distances were calculated in MEGA4 (Tamura et al. 2007) for the protein-coding mitochondrial data of *P. fluviatile* and the specimens from the Peloponnesus (COX1 and ND1 genes, 1349 bp).

2.3. Results

Morphological analyses

The morphological comparison of the first gonopod, part of the male reproductive apparatus, yielded no evidence that individuals from the Peloponnesus differ from other specimens of *Potamon fluviatile*. We examined the type series of the infrasubspecies "*laconis*" Pretzmann 1983, housed in the Natural History Museum Vienna (NHMW 4305, 4306, 3119). The criteria that define this infrasubspecies (Pretzmann 1983; 1986) appear to be within the morphological range of *Potamon fluviatile*. In detail, these are:

(1) a first gonopod with a shallow mesial bulge at its distal part (see Figure 2A for comparison);

(2) a straight subterminal segment of the first gonopod (also curved in specimens from the Peloponnesus, see Figure 3A,B);

(3) a second gonopod with only sparse and short setae – however, specimen SMF 36931 (AKR) from the northern Peloponnesus shows an ostentatiously long and dense setation on the first gonopod (Figure 2A);

(4) less projecting epigastric lobes (but see Figure 5A, displaying well-developed epigastric lobes).

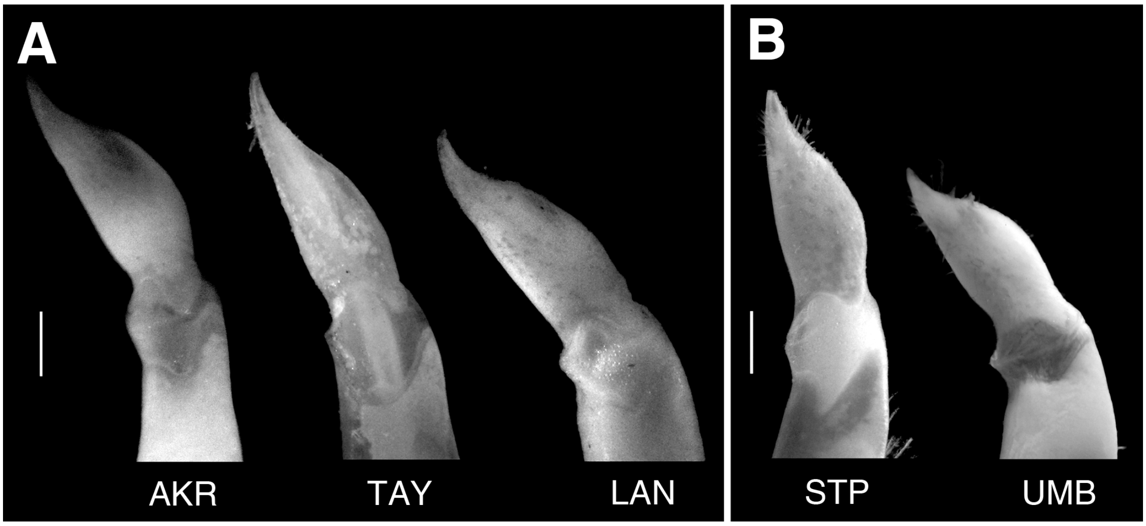


Figure 2: Comparison of distal parts of the first gonopod (dorsal view). (A) *Potamon pelops* sp. nov., (B) *Potamon fluviatile* (Herbst 1785). Abbreviations: see Table 1; Scale bars = 0.1 cm.

The flexible zone between the terminal and subterminal part of the first gonopod can not discern between individuals from the Peloponnesus versus individuals from mainland Greece and Italy, although there are subtle differences between specimens (Figure 2A,B). Nevertheless, their first gonopods differ profoundly from other species of the genus *Potamon* (Brandis et al. 2000). Cross-section of the second gonopod revealed a tube-like morphology that is characteristic for the genus *Potamon* (Brandis et al. 1999).

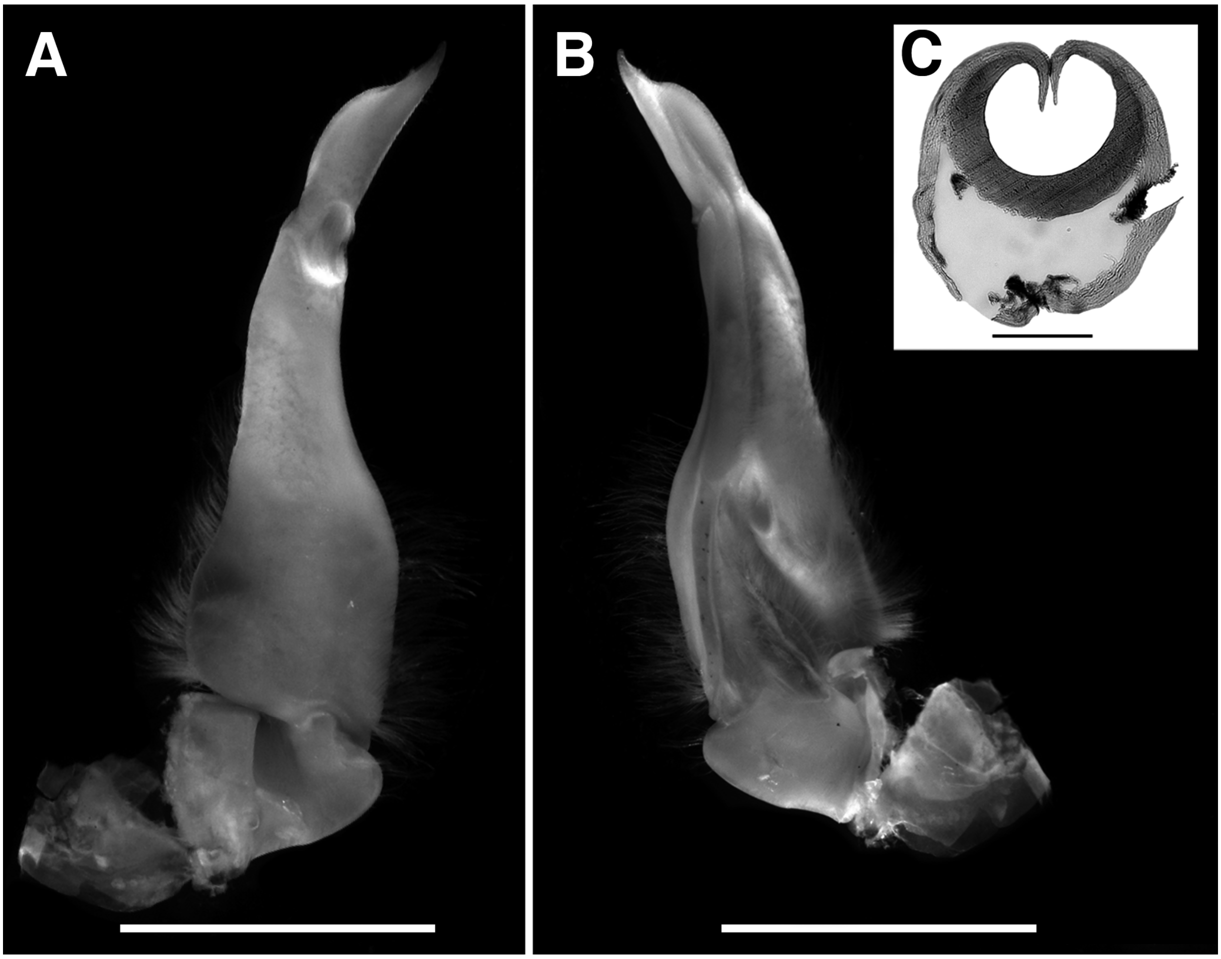


Figure 3: (A,B) First gonopod of the holotype specimen of *Potamon pelops* sp. nov. (SMF 36933; VAS–1); (A) dorsal view, (B) ventral view. (C) Cross-section of the second gonopod (distal part) of SMF 36932 (GOR). Scale bars in A and B = 0.5 cm; in C = 50 µm.

Molecular analyses

Bayesian inference and maximum likelihood analyses resulted in identical phylogenetic topologies (Figure 4). All specimens that are classified as *Potamon fluviatile* by morphology clustered together with high support. However, this group diverged into two reciprocal monophyletic clades. One clade comprised all specimens from the Peloponnesus, including one specimen from the type series of *Potamon fluviatile* natio *laconis* (TAY; see Pretzmann 1983), and the other clade specimens originating from Italy and mainland Greece. This dichotomy also appeared with high support (bootstrap value = 100) in the maximum likelihood analysis of the nuclear encoded 28S rRNA partition only (data not shown), thereby excluding putative hybridisation phenomena. The difference in 28S rRNA sequences between both clades consisted of two transversions that change the corresponding amino acid coding (tryptophan and glycine vs. proline and leucine). The uncorrected p-distances of the protein-coding mitochondrial sequences ranged between the specimens from the Peloponnesus from 0.1 – 0.5 %, and within *Potamon fluviatile* of mainland Greece and Italy from 0.1 – 0.9 %. Between both lineages, p-distances were ranging from 6.8 – 7.4 %.

There is strong evidence that this genetic pattern was generated by two independent coalescent processes, as indicated by: (1) reciprocal monophyly of both clades in the phylogenetic analyses; (2) deep genetic differentiation between both clades while differentiation within each clade is only shallow; (3) allopatry, as both clades are separated by the Gulf of Corinth.

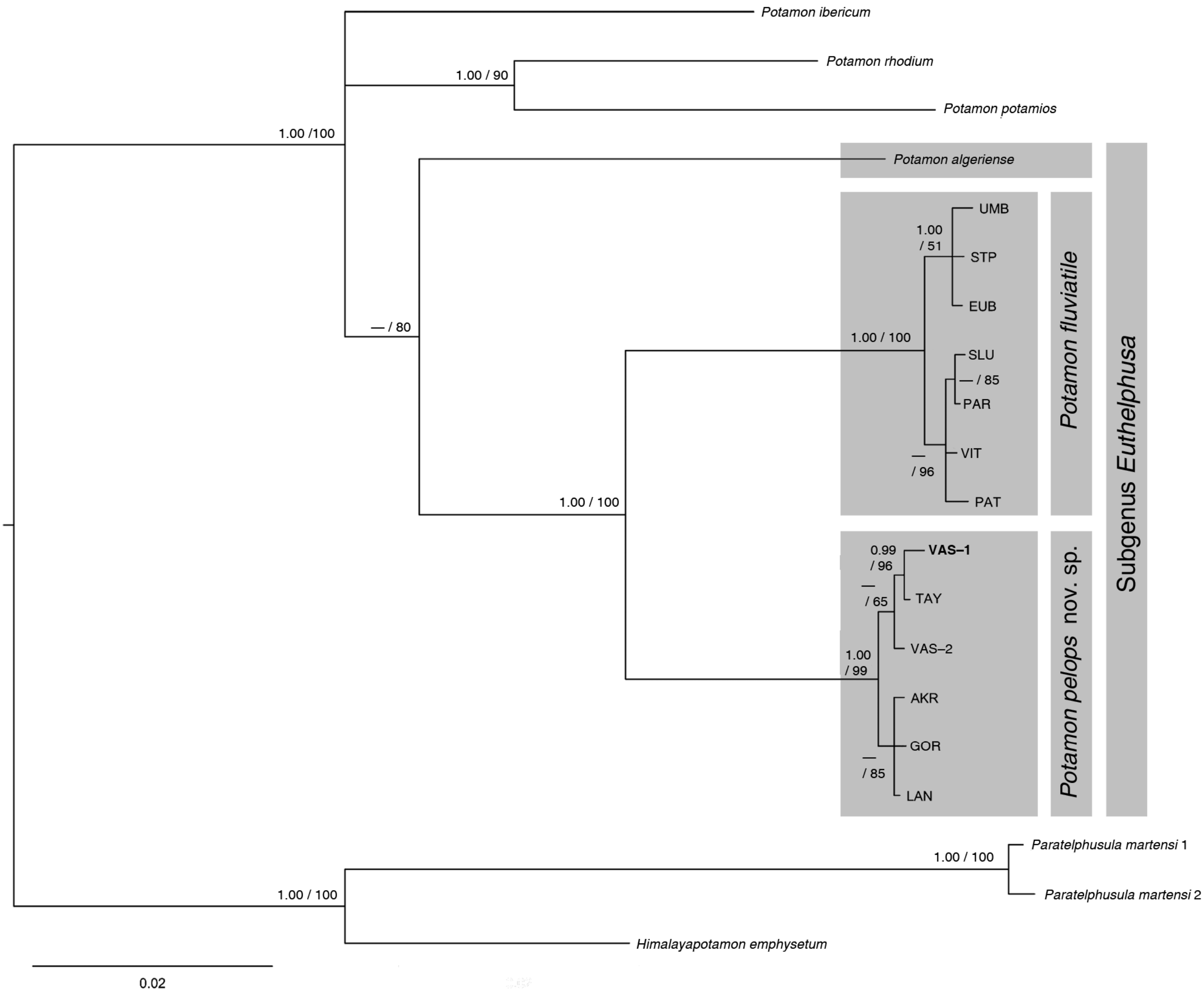
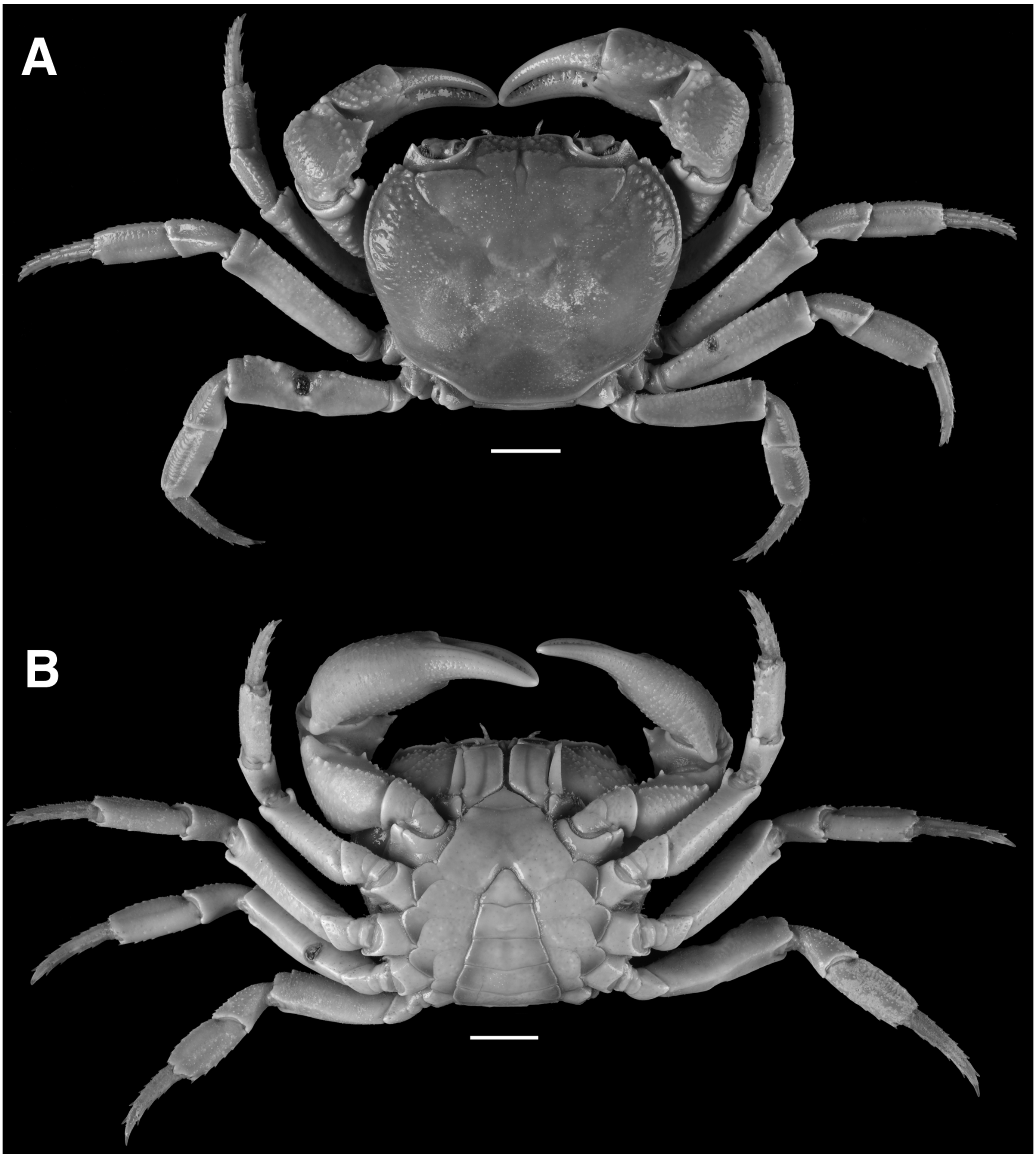


Figure 4: Consensus tree (Bayesian phylogenetic inference) of the genus *Potamon* in the Mediterranean area. At nodes the respective posterior probabilities (>0.90) and the bootstrap values of the maximum likelihood analysis (>50) are given. For abbreviations see Table 1. The holotype of *Potamon pelops* sp. nov. (VAS–1) is indicated in bold letters.

Although we could not detect any discerning differences in the outer morphology of specimens from both clades (including the male reproductive apparatus), a previous study identified ultrastructural differences in spermatophore morphology: in one specimen from the Peloponnesus (GOR; SMF 36932) both coenospermia (several spermatozoa packed into a single spermatophore) and cleistospermia (only a single spermatozoon per spermatophore) occurred (Klaus and Brandis 2010), while for *Potamon fluviatile* so far only cleistospermia has been described (Guinot et al. 1997). This profound difference of the spermatophores was preliminarily attributed to intraspecific variation or to putative seasonal changes in sperm packing (Klaus and Brandis 2010). However, in combination with the deep genetic differentiation we detected here, we conclude that this morphological difference reflects divergent evolutionary histories. Therefore, we will treat both clades as separate species.



Figur 5: Male holotype of *Potamon pelops* sp. nov. (VAS–1, SMF 36933). (A) dorsal, (B) ventral view. Scale bars = 1 cm.

Taxonomy

Suborder **Brachyura** Latreille, 1802

Superfamily **Potamoidea** Ortmann, 1896

Family **Potamidae** Ortmann, 1896

Subfamily **Potaminae** Ortmann, 1896

Genus ***Potamon*** Savigny, 1816

Subgenus ***Euthelphusa*** Pretzmann 1962

***Potamon pelops*, **sp. nov.

(Figure 5)

1978 *Potamon* (*Euthelphusa*) *fluviatilis fluviatilis* natio *laconis* – Pretzmann: 339.

1983 *Potamon* (*Euthelphusa*) *fluviatilis fluviatilis* natio *laconis* – Pretzmann: 382.

1986a *Potamon fluviatilis* natio *laconis* – Pretzmann: 338.

1986b *Potamon fluviatilis* natio *laconis* – Pretzmann: 259.

partim 2000 *Potamon fluviatile* (Herbst 1785) – Brandis, Türkay and Storch: 28.

partim 2000 *Potamon potamios* (Olivier 1804) – Brandis, Türkay and Storch: 9.

2009 *Potamon* sp. – Jesse et al.: 2211.

Material examined

Holotype: Male specimen, SMF 36933, Peloponnesus, Laconia, Vassarás, Xagdariás river, a tributary of the Evrótas, 37°11.55'N–22°26.69'E, 2007 leg. Jesse and Klaus. Carapace width 45 mm, carapace length 38 mm.

Additional material examined: In addition to the material used for genetic analyses (Table 1), we investigated the morphology of the following specimens that originated from the Peloponnesus. However, without genotyping or ultrastructural investigation, assignment to *Potamon pelops* sp. nov. has to remain tentative and is based on historical biogeographic consideration (see Discussion). 1 ♀, NHMW 4303, Corinthia, 1976 leg. Malicky; 3 ♂, SMF 2642, Corinthia, Lake Stymphalos, 1965 leg. Kinzelbach; 5 ♂, NHMW 4323, Arcadia, 24 km E of Langadia 1978 leg. Pretzmann; 4 ♂, NHMW 3120, Arcadia, 1926 leg. Beier; 2 ♂ 1 ♀, NHMW 4306, Laconia, Parnon mountains, 1976 leg. Aspöck (paratype natio *laconis*); 1 ♀, NHMW 4780, Laconia, Agios Ellisseos, 1980 leg Malicky; 2 ♂ 2 ♀, NHMW 3119, Laconia, Taygetos mountains, 1937 leg. Werner (paratype natio *laconis*); 1 ♂ 1 ♀, NHMW 4305, Laconia, Taygetos mountains, 1969 leg. Rausch (paratype natio *laconis*).

Abbreviations:

NHMW = Naturhistorisches Museum Wien; SMF = Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt am Main.

Description

The morphology is very similar to *Potamon fluviatile* (see Brandis et al. 2000). The carapace is smooth, the anterolateral margins serrated, the postorbital cristae are straight, the epigastric lobes are projecting. The right chela is slightly larger than the left one, the pleon is triangular with straight lateral margins. The terminal part of the first gonopod is mesially bulging, the subterminal part is curved. The flexible zone is V-shaped. Males have both coeno- and cleistospermic spermatophores.

Distribution

This species is so far only documented from the Peloponnesus, Greece. It occurs in clear water streams and runlets at elevations from near sea-level up to 1000 m altitude in the mountain ranges. In the whole eastern part of the Peloponnesus (Argolis and eastern Laconia) freshwater crabs could not be found during April 2007 (see Figure 6); although reported from that region in earlier studies (e.g. Pretzmann 1983). Although this study is based on a limited number of specimens, we postulate that *Potamon pelops* sp. nov. occurs exclusively on the Peloponnesus and in allopatry to *Potamon fluviatile*. This assumption is based on (1) the sampling in all major drainage basins of the Peloponnesus, and (2) on the postulated historical biogeography of *Potamon pelops* sp. nov. (see Discussion).

Etymology

In Greek mythology Pelops is the son of the Phrygian king Tantalus, and eponym for the Peloponnesus Peninsula. The name is used as a noun in apposition.

Figure 6:
Type locality of *Potamon fluviatile fluviatile* natio *laconis* Pretzmann 1983 in April 2007
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Remarks

Names of infrasubspecies category are nomenclaturally not available (ICZN 1999, article 45.5), and as such is *Potamon fluviatile fluviatile* nation *laconis* Pretzmann 1983. In any case, this unavailable name is here regarded as a synonym of *Potamon pelops* sp. nov.

Brandis et al. (2000) listed in their material of *Potamon potamios* three specimens from the Peloponnesus. However, these specimens comprised a juvenile (SMF 24095), disarticulated fragments lacking the reproductive apparatus (SMF 24094), and a specimen with first gonopods as described for *Potamon fluviatile* (SMF 24096). Therefore, an isolated occurrence of *Potamon potamios* on the Peloponnesus is not supported.

2.4. Discussion

The male gonopods of freshwater crabs, consisting of two pairs of strongly modified pleopods which serve to transmit spermatophores during copulation (see Brandis et al. 1999 for a detailed description), are frequently used for species delimitation (e.g. Balss 1914; Bott 1970; Ng 1988). Brandis et al. (2000) revised the genus *Potamon* by means of first gonopod morphology and considered especially the shape of the first gonopods terminal part and of the flexible zone between terminal and subterminal part for species delimitation. The shape of this flexible zone is thought to influence the angle of the terminal joint of the first gonopod during copulation (Brandis et al. 1999). Thereby, the taxonomic system of Brandis et al. (2000) is directly based on structures necessary for successful sperm transfer. However, the present study gives evidence that morphological differentiation of the external male reproductive structures of brachyuran crabs represents only a sufficient, not a necessary modification regarding species delimitation. Although we expected these copulatory structures to be under strong selection (Arnquist 1998; Eberhard 2010), the evolution of gonopodal structures is likely to be a slower process.

One possible scenario that could explain the separation of the two sister species *Potamon fluviatile* and *Potamon pelops* sp. nov. is the post-glacial expansion of *Potamon*. Pliocene and Pleistocene climate cooling possibly lead to the extinction of all Miocene potamids in Italy (Klaus and Gross 2010). Moreover, molecular data argue for a very recent dispersal of *Potamon fluviatile* from the Balkans to Italy after the last glacial period, approximately 15 000 years before present (Jesse et al. 2009). Under the assumption, that also the Greek mainland was *Potamon*-free during Pleistocene glacial maxima, the Peloponnesus Peninsula could have served as a glacial refuge. In consequence, the split of *Potamon fluviatile* and *Potamon pelops* sp. nov. could be the result of a single dispersal event out of the Peloponnesus, maybe during an interglacial. The southern Balkan and the Peloponnesus were shown to serve as Pleistocene glacial refuge for many species (Hewitt 1996; Taberlet et al. 1998), e.g. arvicoline rodents on the southern Peloponnesus (Mitsainas et al. 2009) or flightless acridid grasshoppers on the southern Balkan (Lunt et al. 1998).

Regarding the large genetic distances between *Potamon pelops* sp. nov. and *Potamon fluviatile*, an earlier, Pliocene age of this split seems to be more probable (see Taberlet et al. 1998), especially as uncorrected p-distances are a conservative estimation. A Pliocene split of lineages would coincide with the formation of the Gulf of Corinth (Collier and Dart 1991; Doutsos et al. 1988) and complete palaeogeographic isolation of the Peloponnesus between 3.4 and 1.8 million years ago (Khondkarian et al. 2004). We consider this geographic isolation followed by allopatric speciation as a very likely explanation for divergence of the two clades, and, in consequence, as further evidence that the ranges of both species may not overlap. Endemism on the Peloponnesus Peninsula occurs in numerous taxa, e.g. cyprinid fishes (Doadrio and Carmona 1998), slowworms (Gvoždík et al. 2010) or staphylinid beetles (Sabella et al. 1998).

Under the scenario that *Potamon fluviatile* and *Potamon pelops* sp. nov. are allopatric, the delimitation of *Potamon pelops* sp. nov. reduces the range of *Potamon fluviatile*. Would this change the conservational status of *Potamon fluviatile*? As the range of *Potamon fluviatile* is without the Peloponnesus still very widespread, ranging from mainland Greece to Italy, it should not be considered as threatened. Nevertheless, *Potamon fluviatile* accounts for the Red List criterion of declining population levels (Jesse et al. 2009) and therefore, we are convinced that its current status as "near threatened" is justified (see Cumberlidge et al. 2009). Compared to *Potamon fluviatile*, *Potamon pelops* sp. nov. has a more restricted range. Especially the populations at lower altitude seem to be perilled by human impact due to withdrawal of water for agriculture and rural/urban construction, with many populations already lost in the last decades (authors' observation). For this species both the Red List criteria of declining population levels and geographic range applies. Although local populations might disappear in the future, populations at higher altitudes, especially in the province of Arcadia, do not seem to be under threat. Thus we propose this species to be classified as "near threatened".

Chapter 3

Disjunct distribution of the Mediterranean freshwater crab *Potamon fluviatile* – natural expansion or human introduction?

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Christoph D. Schubart

Abstract

Human mediated biological invasions are seen as an increasing danger for biodiversity. On the other hand, range expansions are natural processes. It is often practically not possible to tell these processes apart, like in the case of the freshwater crab *Potamon fluviatile*. This species has a disjunct distribution on the Balkan Peninsula, Italy, Sicily and Malta. An innovative analysis framework involving phylogeographic model selection and temporal coalescent analyses on a mitochondrial dataset (COX1 and ND1) could clarify that the origin of the species was on the Balkans and the colonisation of Italy proceeded from the northern Balkans via southern Italy in the Otranto Strait region. The population expansion associated with this invasion was estimated to have taken place 15 000 years before present (95% confidence interval 10 000 – 24 000 years). An anthropogenic introduction is therefore implausible and a natural expansion likely. We argue that the species should thus be included in the national conservation management in Italy.

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3.1. Introduction

The increase of worldwide trade as a consequence of the globalisation process leads currently to a dramatic rise in biological invasions (Perrings et al. 2005). Throughout human history, however, deliberate or inadverted introductions of alien species are a recurrent theme (Johnson and Wroe 2003; Lee et al. 2007; Matisoo-Smith and Robins 2004). Not all introduced species are harmful for their new environs, but some have the potential to significantly interfere with indigenous biota. Examples like the zebra mussel *Dreissena polymorpha* overgrowing native unionid bivalves in the Great Lakes of North America (Ricciardi et al. 1998) or the introduction of mammalian predators on islands, causing the extinction of native birds (Blackburn et al. 2005), clarify the dangers.

Besides all current debates about biological invasions caused by humans, their risks for the native biodiversity, human health and economy (Facon et al. 2006; Strayer et al. 2007), species ranges are dynamic and thus range expansions are also natural processes. For instance, the closure of the Isthmus of Panama connecting North and South America about 3 million years ago resulted in the Great American Interchange, greatly affecting biodiversity on both continents (Marshall et al. 1982). Other well-studied examples for natural range expansions were caused by the Pleistocene climatic and sea-level fluctuations. Between and after glacial periods in Europe, species expanded from their mostly southern refugia over Europe (Hewitt 1999) and species numbers, distributions and compositions in Europe changed dramatically within short periods (Gates 1993).

In certain cases, however, it remains unclear whether a species colonised its current range by natural expansion or anthropogenic introduction. A prominent case of such a debate, lasting 150 years, is the marine snail *Littorina littorea* occurring at the coasts of Northern America and Europe (Chapman et al. 2007; Chapman et al. 2008; Cunningham 2005; Ganong 1886). Especially for conservation management decisions, the mode of introduction is important information. The decision whether a species obtains legal protection or not depends often on its origin and time of residence in an area. An anthropogenically introduced species will less likely get a legal conservation status compared to a species which expanded naturally or is even endemic to the region (Grammont and Cuarón 2006).

The distribution of the European freshwater crab *Potamon fluviatile* (Herbst 1785) is such a disputed case. The present range of this species comprises large parts of Italy, Sicily and Malta, as well as the Balkans. As these areas are separated by the Mediterranean Sea, such a disjunct distribution requires an explanation in a moderately mobile, freshwater dependent organism. In the Aegean region, four species of the genus *Potamon* are described and it was therefore discussed whether *P. fluviatile* originates from this area and is invasive in Italy/Malta (Pretzmann 1987a). However, as the genus is also distributed in northern Africa, an Italian origin of the species can not be *a priori* excluded. Because *P. fluviatile* was extensively used for human consumption (the former species' name was *Potamon edule*, which derives from the Latin word "*edulis*" = edible), an anthropogenic introduction was advocated (Pretzmann 1987a). Moreover, since the presence of the species is documented for the Greek and Roman period on the Italian Peninsula by coins and other archaeological remains (Brandis 1997), the introduction was attributed to ancient Greek settlers, which colonised southern Italy and Sicily from 750 B.C. onwards (Cerchiai et al. 2004).

Due to human impact, *P. fluviatile* declined observably during the past 20 years. The crab used to be present as far north as Lago di Garda in Italy (Bettoni 1884; Garbini 1894; Kingsley 1880). Nowadays, the river Po marks its approximate northern distribution limit. A number of factors are made responsible for the decline of the species: pollution, regulation and desiccation of streams, human consumption and the introduction of exotic crayfish species (Gherardi and Holdich 1999). The species is not yet included in the IUCN red list, but this was strongly suggested already by Pretzmann (1984) and in a recent ecological study (Barbaresi et al. 2007). To make an informed conservation decision, it is therefore important to assess whether the presence of the species in Italy is indigenous, caused by natural expansion or due to human introduction.

On the basis of a species range wide assessment of the genetic population structure, we located the most likely origin of the species and determined which processes governed the expansion with advanced statistical and phylogeographic methods. Furthermore, we modelled with coalescent methods if and when an expansion of population size, logically associated with a spatial range expansion, took place.

3.2. Material and Methods

Species range and sampling sites

Potamon fluviatile (Herbst 1785) is distributed in Italy, Malta, southern Dalmatia, Albania and Greece (mainland up to Chalkidiki, western Ionian and Aegean Islands) (Brandis et al. 2000). The species does not occur on the Ionian Islands of Kefalonia and Zakynthos, as well as the Peloponnesus. Here, a cryptic species of the genus *Potamon* is distributed (Jesse et al. 2010). In total 111 specimens (1 – 5 per site) were analysed: 93 from 29 sampling sites collected in 2003-2006, and 18 of the remnant 13 sampling sites obtained from museum collections (Figure 1). Thus, the sampling covered the entire range of the species. Further information about sampling sites and sequenced individuals are given in Table 1. The congeners *Potamon algeriense* from Morocco and *Potamon potamios* from Turkey served as outgroups in phylogenetic reconstruction.

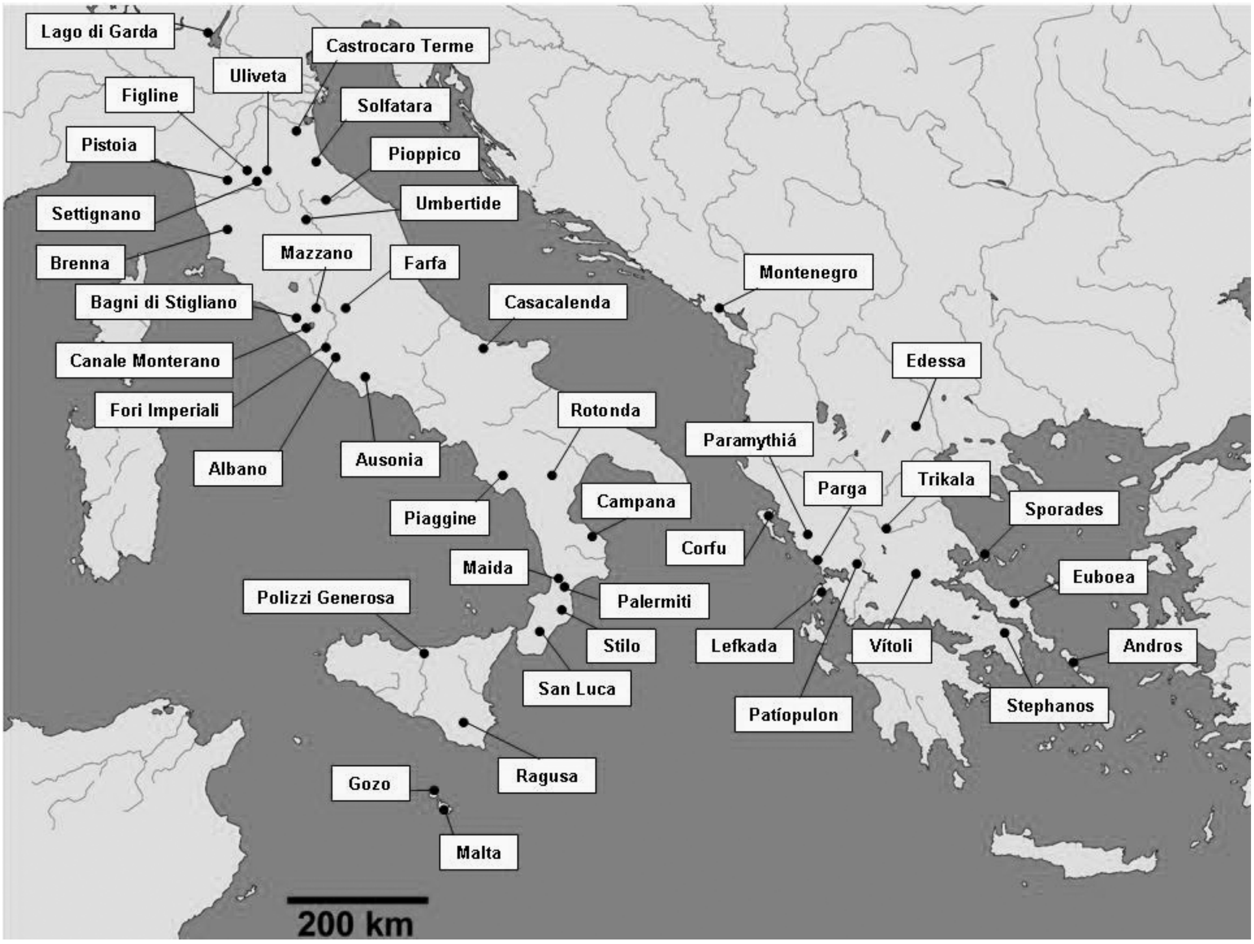

Figure 1: Sampling sites of *Potamon fluviatile* (own sampling and museum collections).

DNA extraction, fragment amplification and sequencing

Genomic DNA was extracted from muscle tissue of a walking leg from individuals conserved in 70 % ethanol using the Puregene Kit (Gentra Systems). The selective amplification of a 537 bp fragment from the cytochrome oxidase subunit I (COX1) and a 981 bp fragment from the subunit 1 of the nicotinamide adenine dinucleotide dehydrogenase (ND1) with neighbouring regions (end part of 16S rRNA and tRNA of leucine) was carried out by polymerase chain reaction (PCR). The standard program (40 cycles; denaturing 94 °C 45 s/ annealing 48 °C 1 min/ extension 72 °C 1 min) was conducted with the primers COL6b (5′-ACA AAT CAT AAA GAT ATY GG-3′) and COH6 (5′-TAD ACT TCD GGR TGD CCA AAR AAY CA-3′) (Schubart and Huber 2006) for the COX1 gene and the new primers 16L11 (5′-AGC CAG GTY GGT TTC TAT CT-3′), NDL5 (5′-TTG CTG GWT GRT CTT CWA ATT G-3′), NDH5 (5′-GCY AAY CTW ACT TCA TAW GAA AT -3′) and NDH2 (5′-GCT AAA TAT ATW AGC TTA TCA TA -3′) for the ND1 gene. The PCR products were purified with the Sure Clean Plus Kit (Bioline) and precipitated with ethanol. The products were sequenced with the ABI Big Dye terminator mix (Big Dye Terminator® v 1.1 Cycle Sequencing Kit; Applied Biosystems) in an ABI Prism automated sequencer (ABI Prism ™ 310 Genetic Analyzer). Sequences were analysed with the program ABI Sequencing Analysis®3.4 (Applied Biosystems) and manually proofread with the DNA sequencing software Chromas 1.55 (Technelysium, Pty. Ltd. 1998, Queensland, Australia) and aligned with the BioEdit Sequence Alignment Editor (Hall 1999).

Estimation of the mitochondrial haplotype phylogeny

The phylogeny of both genes in combination (COX1 and ND1) was inferred by Bayesian analyses performed by Mr Bayes vers. 3.1.2 (Ronquist and Huelsenbeck 2003). We chose a GTR + I + G model, assuming a gamma-shaped rate variation. We ran four Metropolis coupled Monte Carlo Markov chains (MC^3) for 5 million generations. After a burn-in phase of 10 000 generations, trees were sampled every 100th generation. A majority consensus tree, rooted with *P. potamios* and *P. algeriense,* was computed from the sampled trees. For all further analyses, ND1 was used exclusively. The statistical parsimony (SP) cladogram was constructed using TCS vers. 1.21 (Clement et al. 2000). The connection limit was set at 95 %

and gaps were treated as fifth character state. The few loops that occurred in the constructed networks were resolved according to the rules detailed in Pfenninger and Posada (2002).

Dispersal hypotheses testing by model selection

To compare the relevance of six explicit dispersal hypotheses, we applied a model selection approach (Johnson and Omland 2004; Stephens et al. 2007), introduced into phylogeography by Pfenninger and Posada (2002) and further developed in Depraz et al. (2008). To keep computations feasible, the 42 sampling sites were pooled into five geographic groups (Northern Balkans, Southern Balkans, Central Italy, Southern Italy and Sicily including Malta and Gozo, Figure 4A). These groups were chosen on the basis of a series of AMOVAs, aiming to minimise the number of groups and simultaneously maximising the variance between groups (Table 2). We tested two major hypotheses: origin of *P. fluviatile* in Italy and subsequent colonisation of the Balkans (*Out of Italy*) and *vice versa* (*Out of Balkans*). For each major hypothesis, we took three different dispersal scenarios into account: 1) a route via the northern Adriatic Sea along the coastline, thus connecting the northern part of the Balkan Peninsula with Central Italy, called *Adriatic route*, 2) a connection of Southern Italy with the Northern Balkan region via the *Strait of Otranto* and 3) *passive dispersal* in the course of the antique Greek colonisation of Sicily and southern Italy in the first millennium B.C., linking the southern part of the Balkan Peninsula with both Sicily and Southern Italy. This resulted in six distinct hypotheses. We chose the land-based (*Adriatic route*) and passive scenarios (*passive dispersal*) because the freshwater crab *P. fluviatile* cannot support sea water well and is not able to swim. Consequently, for overseas dispersal we only considered the Strait of Otranto hypothesis, as it was not deemed impossible that this distance could be crossed during low sea level periods by rafting.

The six hypotheses were translated into migration matrices (Figure 4B). These matrices were intended to contrast the different hypotheses, but they are unlikely to capture the subtleties of the actual gene-flow patterns. The maximum likelihood migration rate matrix of each model given the data was then estimated using MIGRATE-N version 2.3 (Beerli and Felsenstein 2001). The first genealogy was started with a random tree. Initial theta and migrant values were generated from an F_{ST} calculation. A static heating scheme with four different temperatures was applied. We ran ten short chains with 4×10^4generations each, from which 1 000 trees were recorded in regular intervals after a burn-in phase of 20 000 generations. These were followed by three long chains of 10^5 generations, from which 10^3 trees were sampled after a burn-in period of 2×10^3 generations. Parameter estimates were gained from the combination of the last chains. Log likelihood estimates cannot be directly compared over different runs with MIGRATE-N. We therefore ran a final analysis with an unconstrained migration model using the likelihood-ratio-test option to gain likelihood estimates that were comparable between the different hypotheses and their parameter sets. We used these estimates and the number of free parameters in each model to calculate the Akaike Information Criterion (AIC; Akaike 1974) and resulting Akaike weights to obtain measures of relative hypothesis support.

Past demography of Potamon fluviatile

To test whether our presumption of a population expansion is valid, we tested the data set of ND1 using mismatch distribution by Arlequin vers. 3.11 (Excoffier et al. 2005). We tested both demographic and spatial expansion models for pairwise differences with 1 000 bootstrap replicates. Past population demography for *P. fluviatile* was inferred using a Bayesian skyline plot model (Drummond et al. 2005) as implemented in BEAST 1.4 (Drummond and Rambaut 2003) and visualised in TRACER 1.3. This individual-based coalescent approach uses standard Monte Carlo Markov Chain (MCMC) sampling procedures to estimate a posterior distribution of gene genealogies and population parameters under a $\mathrm{GTR} + \mathrm{I} + \Gamma$ substitution model. A lognormal model that relaxes the molecular clock hypothesis was used (Drummond et al. 2006). These distributions were then used to generate credibility intervals that represent sequence evolution model, phylogenetic and coalescent uncertainty (Drummond et al. 2005). The hyperparameter m (number of grouped intervals) was set to 10. Four independent MCMC analyses of 10^7 steps were performed sampling every 500th generation for each marker/clade combination, with the burn-in set at 10^6 generations. We used the split between the haplotype lineages of *P. fluviatile* and *P. algeriense* (11.6 % sequence divergence in ND1), which probably took place during the Messinian Salinity Crisis (Brandis et al. 2000) to calibrate the molecular clock. Assuming that the split of haplotype lineages occurred during this event between 6.0 and

5.3 Mya (Krijgsman et al. 1999), we placed a normally distributed prior distribution with upper and lower two standard deviations falling on these temporal limits. This assumes that the most recent common ancestor of the haplotypes was present before the actual population split, in contrast to the species split which occurred after the populations were separated. The resulting value of 0.02 ± 0.006 (mean ± s.d.) sequence divergence per 1 million years agrees well with the accepted range for arthropods (Brower 1994; Schubart et al.1998).

Table 1: Studied specimens with origin and museum vouchers (*=for the gene ND1; Variability given as nucleotide diversity; abbreviations: N= number of specimens; NHMW= Naturhistorisches Museum, Wien; MZUF= Museo Zoologico dell' Università, Firenze; SMF= Senckenberg Museum, Frankfurt am Main; ULLZ= University of Louisiana, Lafayette, Zoological Collection)

Sampling sites	N	Haplotypes found*	Nucleotide diversity*	Voucher number
Central Italy				
Lago di Garda; 1929	1	H1	0.0000	NHMW 3097
Castrocaro Terme; 2005	5	H1	0.0000	MZUF 2647
Figline; 2004	2	H1	0.0000	MZUF 2717, 2718
Pistoia; 2005	3	H1	0.0000	MZUF 2751
Uliveta; 2005	3	H1, H4	0.0007	MZUF 2961
Settignano; 2003	5	H1	0.0000	SMF 30156
Solfatara; 2005	3	H1	0.0000	MZUF 2959
Brenna; 2005	3	H1	0.0000	MZUF 2747, 2750
Pioppico ; 2005	1	H1	0.0000	MZUF2952
Umbertide; 2005	5	H1, H5	0.0006	MZUF 2948
Bagni di Stigliano; 2005	4	H1	0.0000	MZUF 2951
Mazzano Romano; 2005	3	H1	0.0000	MZUF 2954
Canale Monterano; 2004	1	H1	0.0000	MZUF 2953
Farfa; 2005	2	H1	0.0000	MZUF 2949
Fori Imperiali/Roma; 2005	3	H1	0.0000	MZUF 2950
Albano; 2005	3	H1	0.0000	MZUF 2946
Ausonia; 2005	3	H1	0.0000	MZUF 2947
Casacalenda ; 2004	5	H1	0.0000	MZUF 2691
Southern Italy				
Piaggine; 2005	3	H1	0.0000	MZUF 2960
Rotonda; 2005	3	H1, H6	0.0014	MZUF 2956
Campania; 2006	2	H1, H3	0.0021	SMF pending
Maida; 2006	2	H1, H2	0.0055	SMF pending
Palermiti; 2006	2	H1, H2	0.0019	SMF pending
Stilo; 2006	2	H1, H7	0.0020	SMF pending
San Luca; 2005	4	H17	0.0000	MZUF 2957
Sicily & Malta				
Polizzi Generosa; 2004	5	H17	0.0000	MZUF 2955
Ragusa; 2005	5	H17	0.0000	MZUF 2958
Malta; 1985	1	H1	0.0000	NHMW 6342
Gozo; 1991	1	H17	0.0000	NHMW 17043

Table 1 (continued)

Sampling sites	**N**	**Haplotypes found***	**Nucleotide diversity***	**Voucher number**
Northern Balkans				
Virpazar; Montenegro; 2004	1	H19	0.0000	SMF pending
Edessa; Greece; 1978	1	H14	0.0000	NHMW 4328
Corfu; Greece; 2002	3	H13, H16, H18	0.0021	SMF 29345
Trikala; Greece; 1978	1	H11	0.0000	NHMW 4322
Paramythiá; Greece; 2003	5	H11	0.0000	SMF 30166
Parga; Greece; 1991	1	H11	0.0000	NHMW 23014
Patíopulon; Greece; 2003	3	H14	0.0000	NHMW 21248
Skiathos/Sporades; Greece; 1980	2	H14, H15	0.0032	SMF 15122
Southern Balkans				
Vítoli; Greece; 2003	1	H12	0.0000	ULLZ 3792
Lefkada; Greece; 1986	2	H11	0.0000	NHMW 23013
Euboea; Greece; 1989	4	H8, H9, H10	0.0013	NHMW 17041, 17042
Stephanos; Greece; 1978	1	H10	0.0000	NHMW 4327
Andros; Greece; 1978	1	H8	0.0000	NHMW 4326/1
Outgroups				
Potamon algeriense; Gorges du Zegzel; Morocco; 2005	1		0.0000	SMF 31799
Potamon potamios; Köprü Irman; Turkey; 2005	1		0.0000	

3.3. Results

Sequence variation

A total of 19 ND1 haplotypes, defined by 17 singleton mutations and ten parsimony informative (PI) sites, which resulted in an alignment of 981 positions were obtained. For the COX1 fragment, 13 haplotypes with an aligned length of 537 bp and nine singletons and five PI sites were observed. The combination of both genes resulted in 22 unique haplotypes with pairwise differences of 0.002 % up to 0.015 %. Analysis of ND1 haplotypes alone resulted in pairwise differences of 0.001 % up to 0.021 %. Nomenclature was based on ND1 haplotypes (H1-19; Table 1). In cases where COX1 increased the resolution of otherwise identical ND1 haplotypes, these additional haplotypes were designated with small letters in Figure 2 (e.g. 1a). Haplotypes that only occur in Figure 3 had no corresponding COX1 sequence and could thus not be used in the combined analysis. Haplotype sequences are registered in GenBank, accession numbers are EU908226 – EU908269. All sequences aligned unambiguously and translated without stop codons.

Phylogenetic relationships

Bayesian analyses of the combination of both genes, rooted with *P. potamios* (Figure 2), showed that ancestral haplotypes of *P. fluviatile* were found on the islands of Euboea (numbered H8a, H9, H10), Andros (H8b) and Stephanos (H10). Specimens sampled from Central and Southern Italy, as well as Malta (1, 1a/b, 2, 3, 6, 7) formed a group. A second cluster consisted of specimens collected from Sicily, Gozo and all other Balkan sampling sites (H11-19).

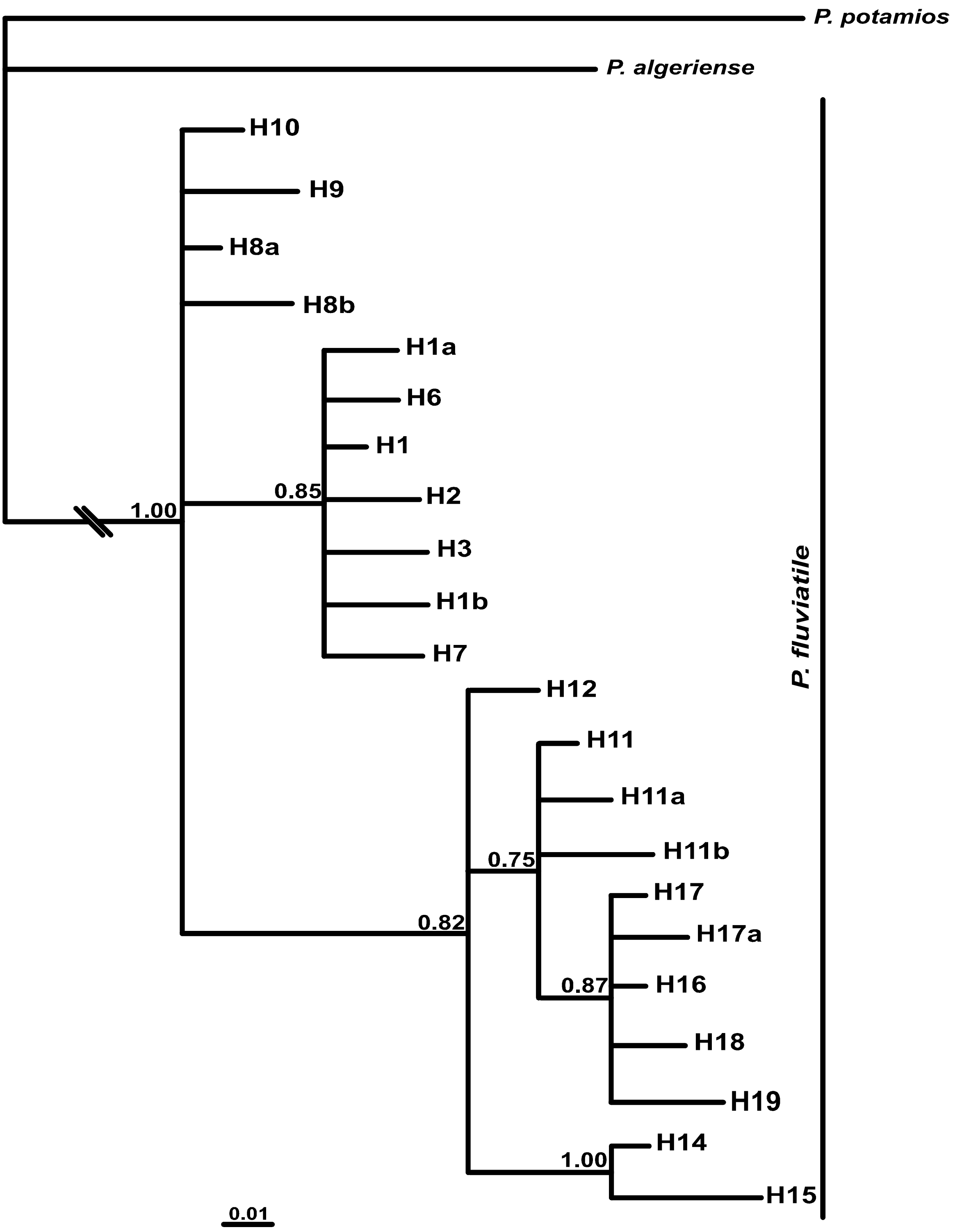

Figure 2: Phylogram of COX1 & ND1, Bayesian analyses of 1518 bp (981 bp of ND1 plus flanking region, 537 bp of COX1), 69 sequences, GTR + I + Γ model, posterior probability values major than 0.5 are reported on the tree branches

As ND1 sequences were more variable and available for more individuals than their COX1 counterparts, all further analyses were conducted with the ND1 dataset alone. The statistical parsimony analyses of ND1 alone resulted in a network compatible with the Bayesian consensus tree. The three clades were separated by three, six and nine mutational steps (Figure 3).

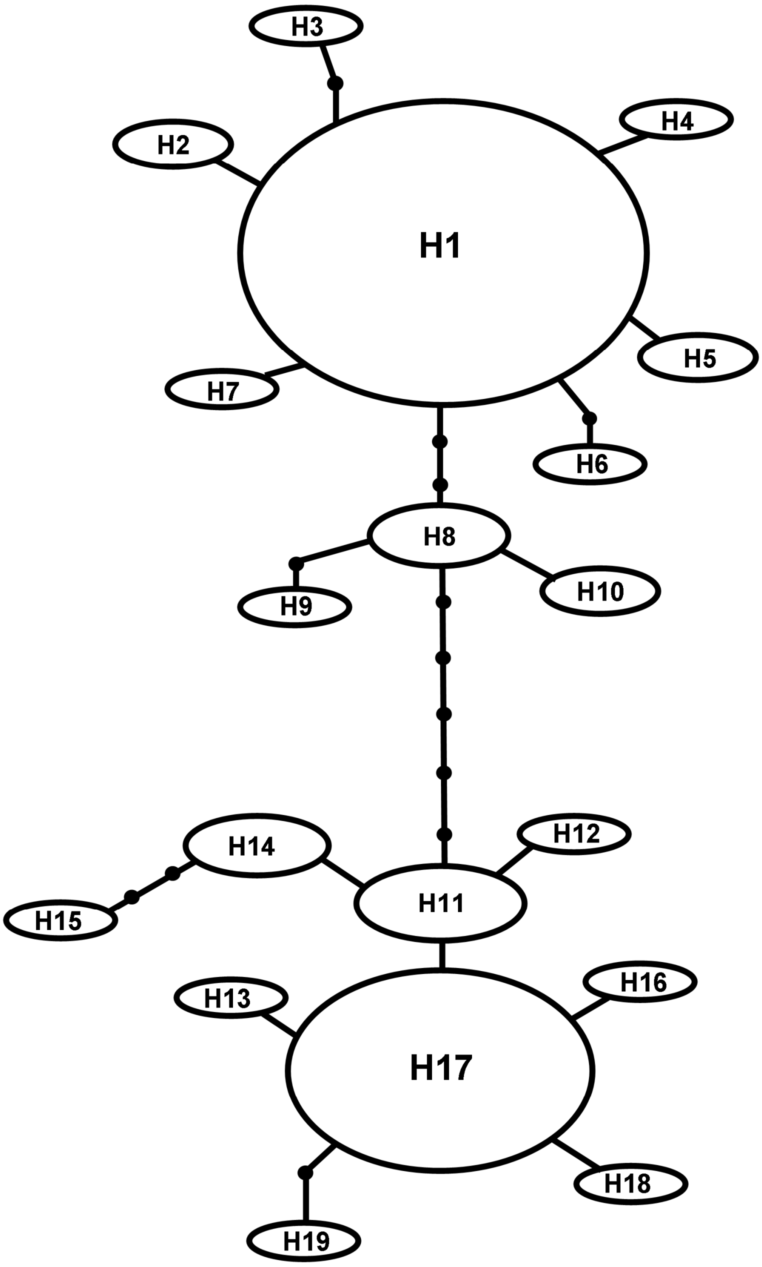

Figure 3: Statistical parsimony network based on 981 bp of the ND1 gene (plus flanking region), 111 sequences. Open circles depict sampled haplotypes; their size is proportional to their frequency. Small filled circles represent missing haplotypes. Connecting lines correspond to single mutational steps.

Inference of dispersal routes

The highest variation among groups was found considering five geographical regions: Central Italy, Southern Italy, Sicily, Northern Balkans and Southern Balkans. Results of the AMOVA series are given in Table 2. This sampling site pooling scheme (Figure 4A) was then used in model selection analyses. Model selection criteria clearly preferred the *Out of Balkans: Otranto Strait* model (AIC = 399, Akaike weight = 0.99) over the remaining five models (cumulative Akaike weight = 0.00; Figure 4B). The selected model implies that the origin of the expansion was on the Balkans and that the migration from the Northern Balkans to Southern Italy most likely occurred in the Strait of Otranto region.

Table 2: AMOVA results. Partitioning of molecular variance within and among sampling sites; significance of variance components was tested by 1 000 permutations. Grouping E maximised the among group variance

Scenarios	**A**	**B**	**C**	**D**	**E**	**F**
Number of regional groups	2	3	3	4	5	6
% variation:						
Among groups	53.35	60.73	72.63	69.73	74.37	68.65
Among populations within groups	42.57	34.44	23.38	25.48	20.81	26.00
Within populations	4.08	4.83	3.99	4.79	4.81	5.35

Grouping A: Italy+Sicily/Malta, Balkans; B: Central Italy, Southern Italy+Sicily/Malta, Balkans;
C: Continental Italy, Sicily/Malta, Balkans; D: Central Italy, Southern Italy, Sicily/Malta, Balkans;
E: Central Italy, Southern Italy, Sicily/Malta, Northern Balkans, Southern Greece; see Figure 4;
F: Western, Eastern Central Italy, Southern Italy, Sicily/Malta, Western Balkans, Eastern Balkans;

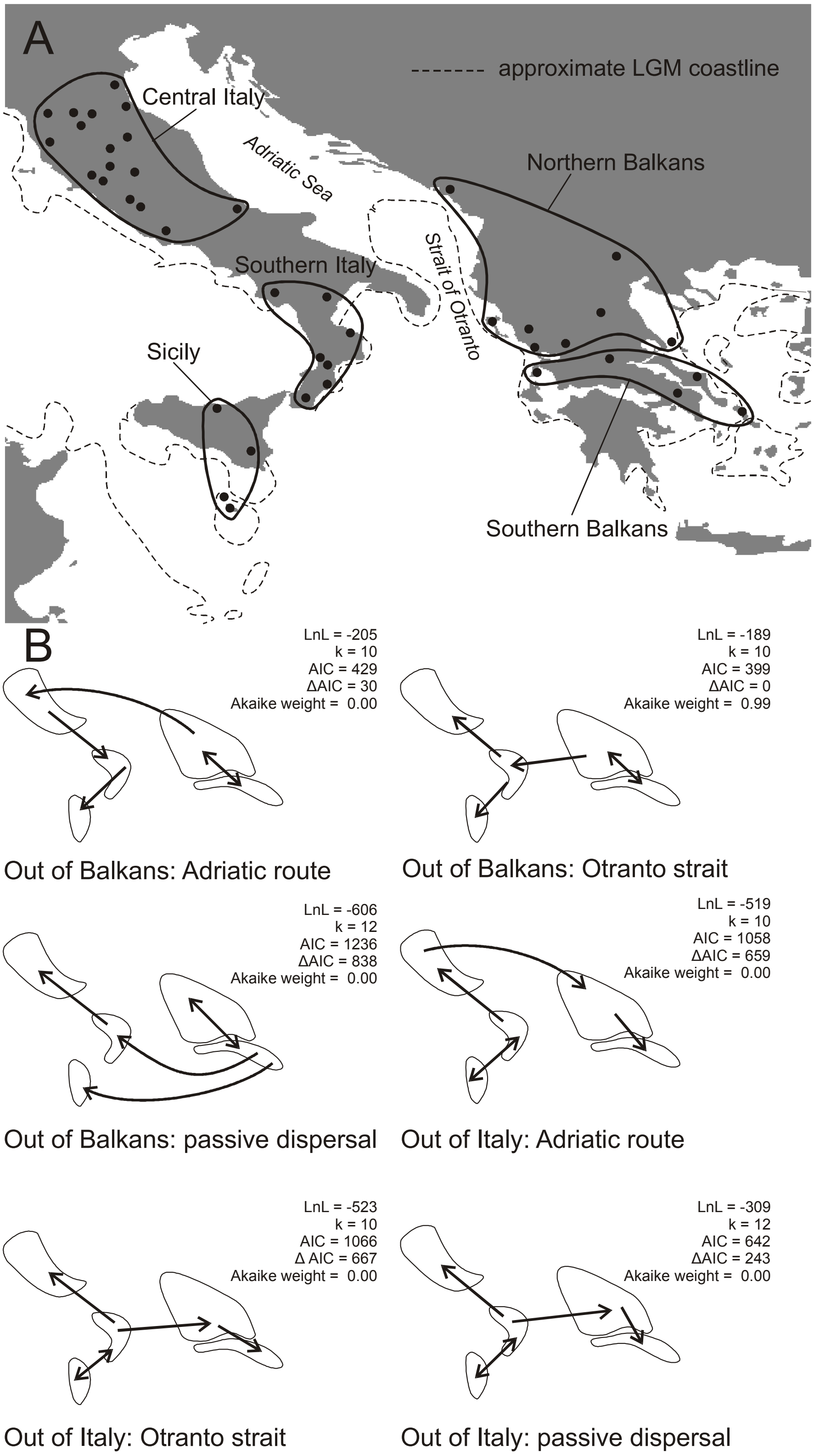

Figure 4: Sampling site clusters (A) and results of the migration hypothesis model selection (B). Arrows indicate the assumed directions of gene-flow. AIC (Akaike information criterion) values measure the fit of the models to the data, taking different parameterisation into account. Note that smaller AIC values indicate better fit.

Demographic analyses

In Mismatch Analysis, the observed number of pairwise differences did not differ significantly from expected values for both demographic and spatial expansion models, even though the spatial model provided a much better fit (Spatial expansion model: SSD p-value = 0.421, Raggedness index = 0.136, Raggedness p-value = 0.610; demographic expansion model: SSD p-value = 0.124, Raggedness index = 0.136, Raggedness p-value = 0.157, Figure 5). The median estimate in the Bayesian skyline plot analysis for the beginning of the expansion was approximately 15 000 years before present (BP) with a 95 % confidence interval ranging from 10 000 years BP to 24 000 years BP. Figure 6 shows the estimated change of effective population size over time with 95 % confidence intervals.

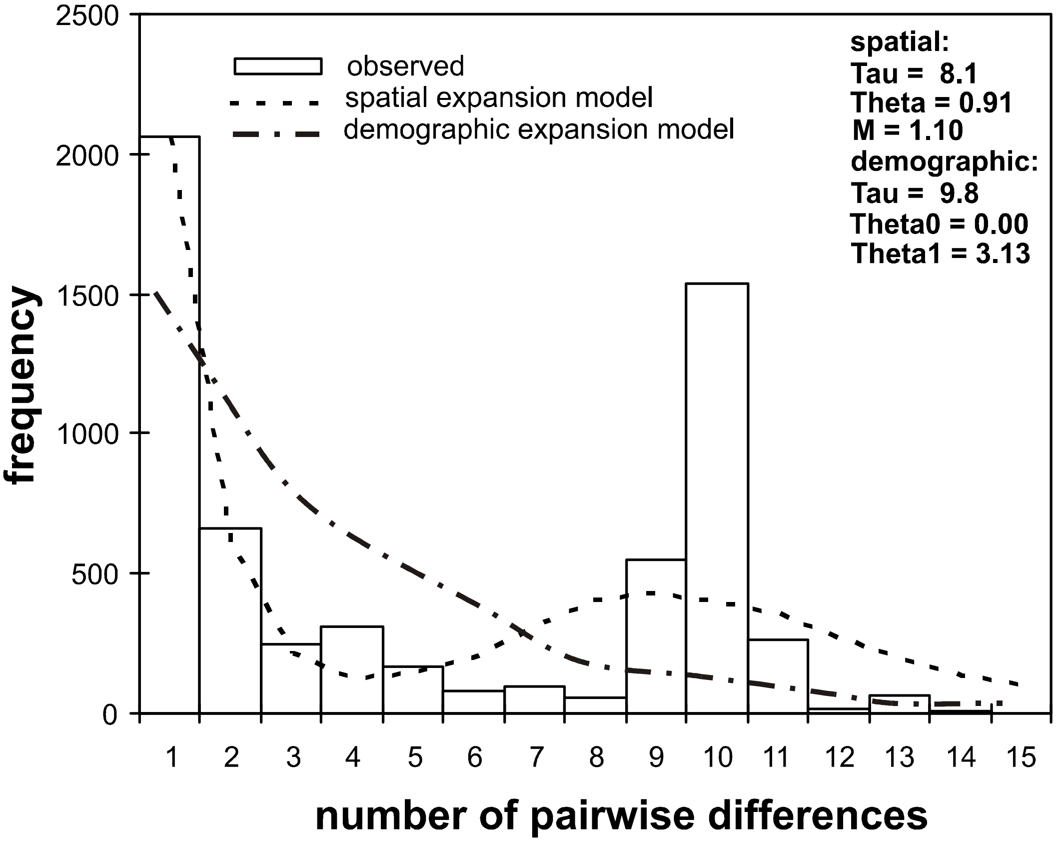

Figure 5: Results of mismatch distribution analyses. Bars represent the observed distribution of pairwise differences among haplotypes of *Potamon fluviatile*. The dotted lines show expected values under the hypothesis of spatial expansion and demographic expansion hypothesis.

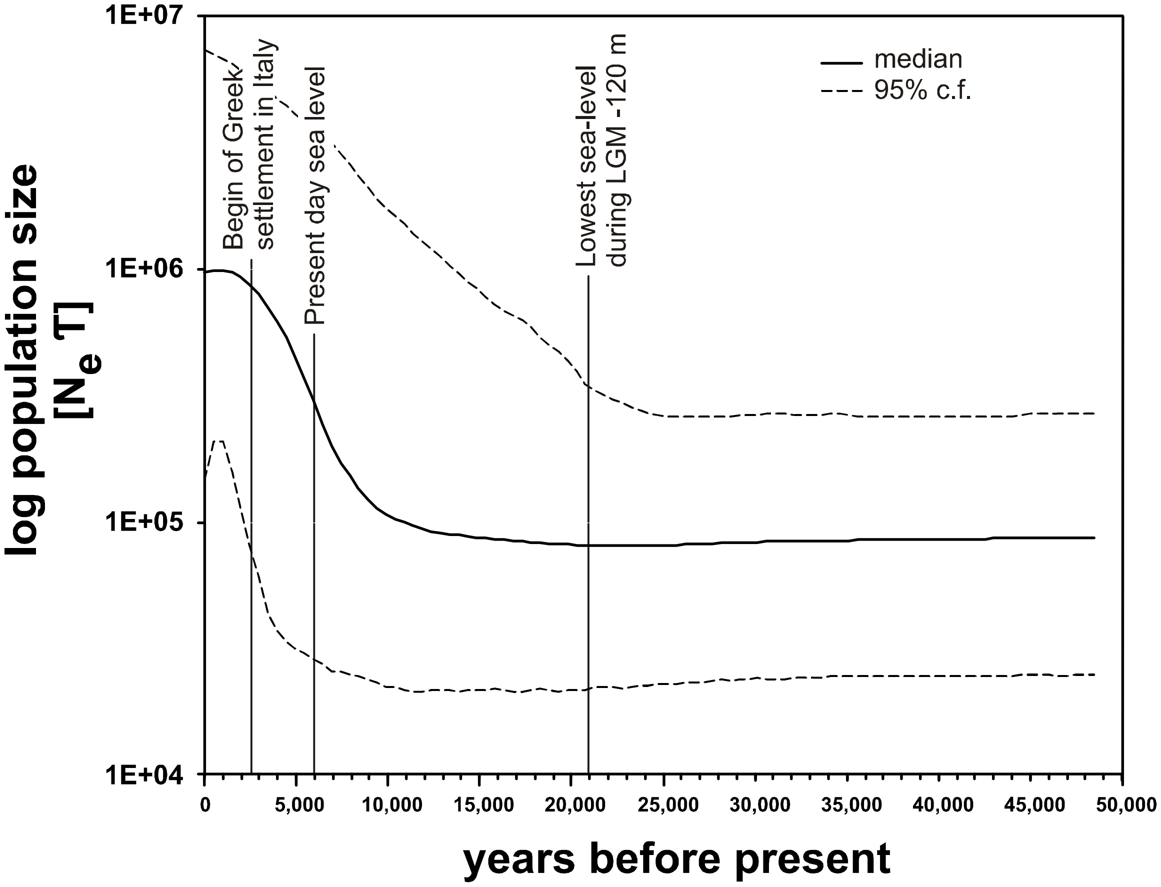

Figure 6: Bayesian skyline plots: evolution of population sizes through time based on Bayesian coalescence. Relevant historic events are indicated.

3.4. Discussion

Out of the Balkans

Model selection has the advantage to test different, biologically reasonable, competing hypotheses simultaneously, instead of testing each of them against a more or less arbitrary null model (Johnson and Omland 2004). Here the "*Out of Balkans: Otranto Strait*" hypothesis received overwhelming support by the genetic data, compared to the competing models (Figure 4). We recognise, however, that the models were constructed to contrast certain hypotheses and that the true gene-flow model may thus not be represented. The data pointed to a Balkanic origin of the species and an expansion of *P. fluviatile* from the Northern Balkans to Southern Italy. The origin of the species on the Balkans, most likely in southern Greece, was also supported by the phylogenetic analyses, which placed haplotypes from Euboea, Andros and Stephanos at the root (Figure 2).

Even though species status was not contested, the results confirmed the absence of infraspecific taxonomic units (Brandis et al. 2000) or cryptic species in the examined populations of *P. fluviatile*, which can never be *a priori* excluded (Pfenninger and Schwenk 2007). Highest diversity within and among sampling sites was found in Greece, despite the low number of individuals analysed. Genetic diversity was surprisingly low on the Italian Peninsula, which could have been a consequence of a genetic bottleneck caused by the inferred expansion (Donnelly et al. 2001; Duran et al. 2004; Hawks et al. 2000). The hypothesis of an over-land expansion, from the Balkans to northern Italy, as proposed by Pretzmann (1987a; 1995), was rejected. With the favoured scenario also the passive dispersal hypothesis becomes unlikely, as Greek settlers colonised southern Italy and Sicily from southern Greece (Cerchiai et al. 2004). The finding of haplotypes on the islands of Malta grouping with northern or southern Italian specimen (Figure 3) is an indication of human transport. However, an alternative explanation could be differential lineage sorting.

Population expansion in the Holocene

Mismatch analyses and Bayesian skyline plot estimates confirmed that a population expansion took place (Figure 5). As expected by an expansion into a new territory, the spatial expansion model best fitted the data (Figure 4). Bayesian skyline plot estimates indicated that this expansion occurred most likely in the late Pleistocene (~15 000 years BP) after the last glacial maximum (LGM). However, the upper 95 % confidence interval does not rule out an expansion during lowest sea-levels at LGM. During the LGM the sea level was approximately -125 m compared to present conditions (Fleming et al. 1998). The shallow Adriatic Sea was dry land during that time and also the Otranto Strait was considerably smaller (~20 km) (Figure 4A). The sea level rose continuously since then to 50 m below current level 10 000 years BP (Fleming et al. 1998), still exposing considerable areas of land. This leaves two dispersal possibilities between the Northern Balkans and Southern Italy: crossing the Strait of Otranto (e.g. by rafting on logs) or migrating along the coastal fringe. As potential intermediate populations are extinct and the sampling of the Northern Balkans was unfortunately not dense, we can not distinguish between these two possibilities. The lower 95 % confidence interval of 10 000 years BP, however, excludes the hypothesis of an anthropogenic introduction of *P. fluviatile* to Italy by Greek colonists (Pretzmann 1987a; 1995) as the estimated expansion date lays millennia before their settlement began. Also an earlier anthropogenic introduction by other cultures is unlikely, as evidence for maritime activities in the western Mediterranean dates back only to 6 000 years BP (Broodbank 2006).

The temporal inferences depend crucially on the calibration of the molecular clock. Based on three lines of evidence, however, we are confident that our calibration is reasonable. First, the Miocene split of the North African species *P. algeriense* from European species of the genus is undisputed (Brandis et al. 2000). Secondly, in reaction to recent criticism (Donoghue and Benton 2007; Heads 2005; Hickerson et al. 2003), we have used a wide molecular clock prior accounting for dating uncertainty. And third, the resulting rate corresponds well to accepted mitochondrial clock estimates of other crustaceans and arthropods (Brower 1994).

Conclusions

Applying a statistical framework recently introduced in phylogeography on a genetic dataset of *Potamon fluviatile*, we could locate the origin of the species on the Balkans and made a natural expansion into Italy plausible. These results provide a new basis for the discussion whether the species should be included in national conservation management in Italy.

Chapter 4

Temporal speciation pattern in the western Mediterranean genus *Tudorella* P. Fischer, 1885 (Gastropoda, Pomatiidae) supports the Tyrrhenian vicariance hypothesis

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Abstract

The land snail genus *Tudorella* shows a peculiar disjunct distribution around the western Mediterranean coasts. Despite high phenotypic plasticity, only two species with a disputed number of subspecific taxa are currently recognised. We delimited the species with mitochondrial (COX1 and 16S) and nuclear (ITS-1) markers based on the unified species concept and suggested that there are eight species in the genus, two of them currently undescribed. Applying Bayesian phylogenetic model selection, we tested four different biogeographic hypotheses that could be causal for the current distribution pattern of extant *Tudorella* species. A scenario involving vicariance events resulting from the repeated splits of the Tyrrhenian plate with subsequent dispersal events over land bridges during the Pliocene received greatest support in the data.

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4.1. Introduction

It is a major challenge of biodiversity research to understand the processes responsible for the current distribution of species. Particularly taxon ranges of organisms with low dispersal capacities, but disjunct distributions, like terrestrial or freshwater organisms separated by the sea, require dispersal hypotheses that are compatible with the taxon's biology (Jesse et al. 2009). In these cases the processes of vicariance and dispersal as dominant forces underlying biogeographical patterns are often controversially discussed (Austin et al. 2003; Givnish et al. 2004; Yoder and Nowak 2006). Dispersal theory's main principle is that of "centres of origin", from which taxa spread, became isolated and speciated (Dobzhansky 1937; Mayr 1954). In contrast, the central assumption of vicariance theory is that ancestral taxa were widespread and speciation was caused by subdivision of the ancestral range (Sparks and Smith 2005; Yoder and Nowak 2006).

The land snail genus *Tudorella* P. Fischer, 1885 has a distribution that raises the question whether dispersal or vicariance were the prevailing processes, as the genus range comprises areas currently separated by the Mediterranean Sea. It is thus an example for the debated biogeographical dilemma that Northern Africa is faunistically and botanically more related to southern-western Europe than to the rest of Africa, to which it is geographical connected (Jeannel 1952). The genus occurs along the western Mediterranean coasts from the Iberian peninsula to Malta in Southern Europe and from Morocco to Libya in Northern Africa (Giusti and Manganelli 1984; Sacchi 1958) This disjunct distribution, accompanied by a high level of phenotypic variation and sexual dimorphism (Martínez-Ortí and Robles 2005), has long since encouraged authors to describe new taxa, mostly on the subspecific level, within the genus (Pallary 1898; Potiez and Michaud 1838; Sowerby 1847). Apart from *Tudorella ferruginea*, all populations are therefore generally attributed to *Tudorella sulcata* (Draparnaud 1805) with the number of subspecific entities varying in dependence of the author from one (Giusti et al. 1995) to five (Sacchi 1958). *Tudorella* has a quite covert life style. The snails are most of the time deeply borrowed in soil and debris under calcareous rocks. They are active only under suitable (i.e. moist) weather conditions. These behavioural characteristics render the rather large snails to unlikely candidates for the suggested long range over-sea passive dispersal processes of snails (wind, bird migration, rafting on logs), apart from anthropogenic dispersal.

The origins of the genus *Tudorella* can be traced in the fossil record to the beginning of the Miocene (Véla et al. 2008). Unfortunately, it is not possible to attribute these fossilised shells to extant lineages, which precludes a fossil calibration of a molecular clock. This time frame includes several potential, mutually not exclusive distribution scenarios that do not require transoceanic dispersal. These start with the substantial tectonic processes, including the split, dislocation and connection of continental micro-plates, shaping the Western Mediterranean basin between the Strait of Gibraltar and Malta in the last 30 million years (Gueguen et al. 1998; Rosenbaum et al. 2002a). Though debated in detail, the scenario that during the Oligocene terranes now found in Calabria, Sicily, Corsica, Sardinia, Kabylies (Algeria), Balearic Islands and Rif range in Morocco were part of the Tyrrhenian plate situated on the Southern European and Iberian margin is now widely accepted. From the late Oligocene on (~30 million years ago; Mya) this plate split from mainland Europe. By 22 Mya latest, the component parts of the plate were already separated from each other, except for the Betis/Rif region that stuck together until 12 Mya. About 10 Mya, the joining of the Rif region with Northern Africa brought them into secondary contact with the Kabylies region, having attached to this continent some 5 millions years earlier. All other terranes reached their current positions approximately in the late Pliocene (Rosenbaum et al. 2002a). Intriguingly, these terranes coincide largely with the current distribution of the genus *Tudorella.* Another major event, connecting most of the current distribution by land bridges and thus providing the opportunity of land based dispersal, was the Messinian Salinity Crisis in the Pliocene (Duggen et al. 2003; Hsü 1972). However, during this short time interval between 5.9 and 5.3 Mya (Krijgsman et al. 1999), the Mediterranean Basin may not have completely dried out (Cipollari et al. 1999; Manzi et al. 2005) and at least the lower parts of the area may not have supported much life (Quézel 1995). Also the major Pleistocene sea level changes (Rohling et al. 1998) might have constituted opportunities to link several but not all current distribution areas. Lastly, as mentioned above, a relatively recent anthropogenic introduction from a single source area is also a possible scenario.

Our goals for the present study were thus twofold:

First, we aimed to delimit comprehensively the Western Mediterranean taxa of the genus *Tudorella*, applying the unified species concept of De Queiroz (2007). We have chosen this species concept, because it comprises the biological species concept without the need to prove actual reproductive isolation. In short, the unified species concept equates species with separately evolving metapopulation lineages, which is appropriate in a case of allopatric relations.

Second, we evaluated the relative support of several hypotheses on the temporal and thus biogeographical splitting pattern of the identified evolutionary lineages, using a Bayesian phylogenetic model selection approach.

4.2. Material and Methods

Sampling

The present study covered all putative taxonomical entities and all described occurrences of the genus *Tudorella* in the western Mediterranean region (Figure 1). Forty-two sequences were made specifically for this study. Data on Algerian, French, Iberian, Moroccan and Maltese localities were integrated from previous studies (Martinez Orti et al. 2008; Véla et al. 2008). For each sampled population, several individuals were preserved in 70-90 % alcohol and with few exceptions, two to five specimen per locality were analysed. As potential outgroup species in Bayesian analyses *Pomatias rivulare* (Eichwald 1829), *P. elegans* (O.F. Müller 1774), *Leoni mammiliaris* (Lamarck 1822) and two species of the genus *Cochlostoma* Jan 1830 were included.

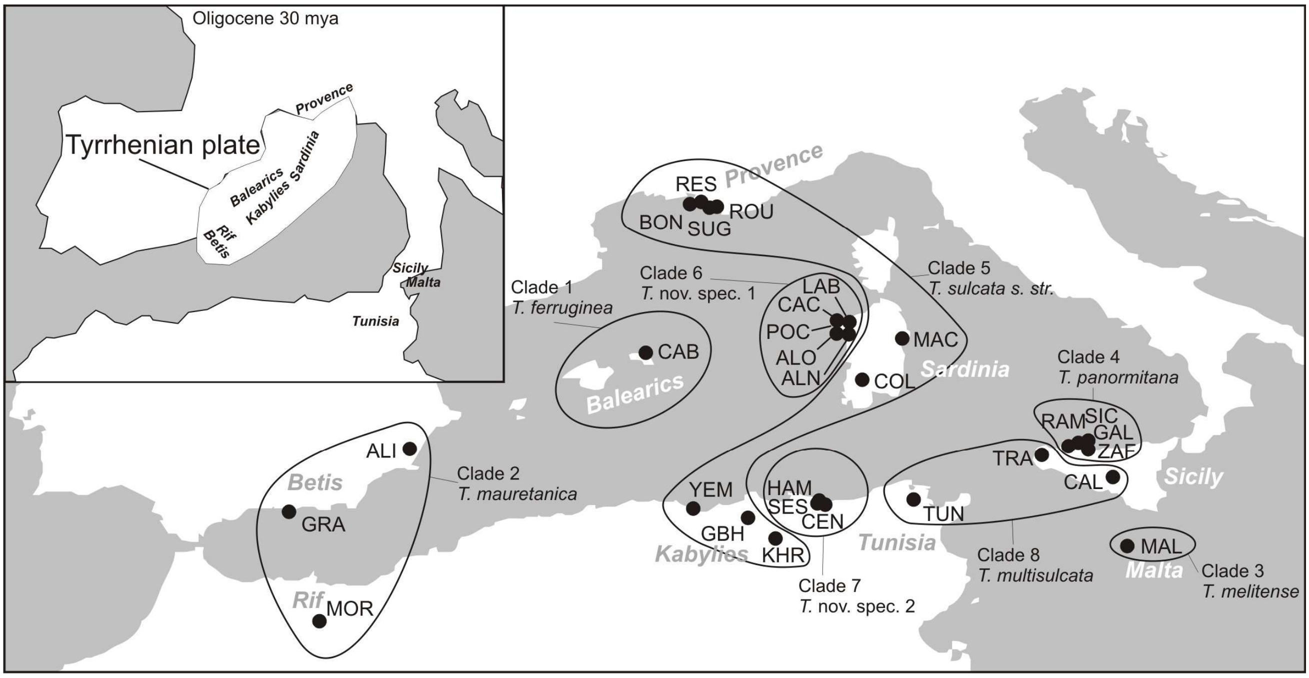

Figure 1: Sampling sites and clade distributions of *Tudorella* across the Western Mediterranean. This represents also the current knowledge on the distribution of the genus, since most recently and securely confirmed localities were sampled. The names of the regions discussed are indicated in italics. The insert shows the presumed Oligocene distribution of the current distribution areas (modified from Rosenbaum et al. 2002a,b).

Molecular analyses

The operculum was forced open without damaging the shell and a small part of the foot muscle was taken. The shell, operculum fragment and remaining tissue were preserved for further studies and eventual museum deposition. DNA extraction was conducted with 10 % w/v laundry detergent solution following the protocol of Bahl and Pfenninger (1996). For 57 individuals, a 670 bp segment of the cytochrome oxidase subunit I gene (COX1) was PCR-amplified. A mostly overlapping set of 46 individuals was also characterised for a section of the nuclear ITS-1 locus (368 bp). For 24 selected individuals representing the major evolutionary lineages, a 473 bp fragment of the mitochondrial ribosomal gene 16S rRNA was additionally sequenced. PCR conditions and primers were adopted from the study of Pfenninger et al. (2005). PCR products were purified using Pure Link™ PCR Purification Kit (invitrogen, Carlsbad, CA, USA). Ten nanograms per sample were subjected to cycle sequencing using the ABI Prism Big Dye terminator kit (Perkin-Elmer, Norwalk, CT, USA). Sequencing reactions were electrophoresed on an ABI 377 automated DNA sequencer. Sequences were deposited in GenBank (see Table 1).

Table 1: Locality codes, geographical coordinates and GenBank accession numbers of *Tudorella* specimens and outgroups haplotypes

Sampling site	Code	Latitude	Longitude	GenBank COX1	GenBank 16S	GenBank ITS-1
Orihuela, Cañada de la Estaca, Alicante, Spain	ALI	38°05'01"N	00°56'37"W	EF053235	EF053250	
Alghero, La Nurra, Sardinia, Italy	ALN1,2,3	40°33'11"N	08°19'15"E	GQ370434 GQ370435	GQ370422	GQ370413
Alghero, Via Oristano, Sardinia, Italy	ALO1,2	40°34'31"N	08°19'39"E	GQ370436	GQ370422	GQ370414 GQ370415
Bonnieu, Bouches-du-Rhône, France	BON1,2,3	43°20'42"N	05°01'32"E	GQ370446		GQ370396 GQ370397
Cabo de los Caballeros, Menorca, Spain	CAB1,2,3,4	40°05'14"N	04°05'27"E	GQ370423		GQ370390
Capo Caccia, Sardinia, Italy	CAC1	40°48'56"N	08°48'53"E	GQ370449		
Caltanissetta, Sicily, Italy	CAL3,4	37°29'19"N	14°03'49"E	GQ370437	GQ370420	GQ370411

Table 1 (continued)

Sampling site	Code	Latitude	Longitude	GenBank COX1	GenBank 16S	GenBank ITS-1
Rass el Hamra «Centaurea», Annaba, Algeria	CEN1,2,4,5,6	36°57'51"N	07°46'38"E	GQ370428 GQ370429 GQ370430		GQ370410
Collinas, Medioi Campidano, Sardinia, Italy	COL1	39°38'29"N	08°50'24"E			GQ370402
Capo Gallo, Palermo, Sicily, Italy	GAL1,2,3,4	38°13'15"N	13°19'00"E	GQ370424 GQ370425		GQ370405
Gorges de Ben Harun, Jijel / Mila, Algeria	GBH1,4,5	36°36'07"N	06°16'58"E			GQ370403
El Tajo del Escalate, Motril, Granada, Spain	GRA	36°44'40"N	03°31'01"W	EF053234	EF053249	
Rass el Hamra «Argiles», Annaba, Algeria	HAM1,2,3,4	36°57'42"N	07°46'25"E	EF053241 EF053242 GQ370433	EF053256E F053257	GQ370409
El Khroub, Constantine, Algeria	KHR1	36°16'17"N	06°54'22"E	GQ370440		GQ370391
Lago di Baratz, Sardinia, Italy	LAB1	40°48'54"N	08°48'07"E	GQ370450		
Cirkewwa, Malta Island, Malta	MAL1, 2	35°59'14"N	14°19'42"E	EF053236 EF053237	EF053251E F053252	GQ370407
Taourirt, Rif, Morocco	MOR	32°41'57"N	09°04'29"W	EF215453	EF215452	
Capo Rama, Palermo, Sicily, Italy	RAM2,3	38°08'00"N	13°03'30"E	GQ370426 GQ370427		GQ370406
Resquiadou, Bouches-du-Rhône, France	RES1,2,3	43°21'15"N	05°16'53"E	GQ370447		GQ370398
Roucas-Blanc, Bouches-du-Rhône, France	ROU1,2,3	43°16'28"N	05°22'33"E	GQ370448		GQ370399 GQ370400
Monte Albo, Nuoro, Sardinia, Italy	MA	40°27'21"N	09°31'24"E	EF053245	EF053260	
Porto Conte, Sardinia, Italy	POC1,2	40°48'00"N	08°48'00"E	GQ370451		
Rass el Hamra «Seseli», Annaba, Algeria	SES1,2,4	36°57'43"N	07°46'35"E	GQ370431 GQ370432		GQ370408
Monte Gallo, Palermo, Sicily, Italy	SIC1, 2	38°10'50"N	13°16'22"E	EF053238 EF053239	EF053253E F053254	
Sugiton, Bouches-du-Rhône, France	SUG1,2,3,4,5	43°12'55"N	05°26'45" E	EF053243 EF053244 GQ370444 GQ370445	EF053258E F053259	GQ370401

Table 1 (continued)

Sampling site	Code	Latitude	Longitude	GenBank COX1	GenBank 16S	GenBank ITS-1
Trapani, San Vito lo Capo, Sicily, Italy	TRA1	38°07'28"N	12°44'41"E	GQ370438	GQ370421	GQ370412
Ghar el Melh, Tunisia	TUN1,2	37°10′31′′N	10°11′03′′E	GQ370439		
Yemma Gouraya, Bejaia, Algeria	YEM1,2,3,4	36°46'10"N	05°05'02"E	GQ370441 GQ370442 GQ370443		GQ370392 GQ370393 GQ370394 GQ370395
Capo Zafferano, Palermo, Sicily, Italy	ZAF3	38°06'30"N	13°32'00"E			GQ370404
Outgroups						
Cochlostoma patulum, Luberon, Vaucluse, France		43°48′55′′N	05°13′50′′E		GQ370416	
Cochlostoma septemspirale, , Luberon, Vaucluse, France		43°48′44′′N	05°13′49′′E		GQ370417	
Pomatias rivulare, Marmaris, Turkey		36°51′16′′N	28°16′10′′E		GQ370418	
Pomatias elegans: Roucas-Blanc, Bouches-du-Rhône, France		43°16'28"N	05°22'33"E		GQ370419	
Leonia mammillaris: Pilar de la Horadada, Alicante, Spain		37°51'53"N	00°48'35"E	EF053231	EF053246	

Phylogenetic reconstruction

The orthologous DNA sequences were initially aligned using the default settings of CLUSTAL X (Larkin et al. 2007) and optimised by eye. In an initial analysis, we used the COX1 and ITS-1 data sets to identify evolutionary lineages. For the former marker, a 99.9 % credible set of phylogenetic trees was estimated with the program Mr. Bayes vers. 3.1.2 (Ronquist and Huelsenbeck 2003) by sampling the tree space using a Metropolis coupled Monte Carlo Markov chain, implementing a GTR + Γ + I model of sequence evolution (where GTR denotes General Time Reversible, Γ is the shape parameter of the gamma distribution and I the proportion of invariant sites), because the most parameter-rich model makes most use of the Bayesian approach. The parameter space was not constrained by *a priori* expectations, but estimated during the runs. Initial runs as well as a posterior inspection of the likelihoods in the final run showed that a burn-in phase of 10 000 generations was largely sufficient for both analyses to allow the likelihood values to reach convergence. The chain was run for 10 000 000 generations and sampled every 100th generation. A rooted majority consensus tree was computed from the sampled trees, excluding the trees sampled in the burn-in phase. For the ITS-1 data set, we applied a Maximum Parsimony search with 1 000 bootstrap replicates in MEGA4 (Tamura et al. 2007), because mainly insertions and deletions appeared to be informative, while the remaining sequence variation was rather negligible. As both coding each insertion/deletion position as new character and each insertion/deletion as single character yielded the same results, we present here only the first option to illustrate the sequence divergence.

The concatenated COX1 and 16S datasets, including five outgroups, were also subjected to a Bayesian analysis. The parameters used were identical to the previous analyses with the exception that the Markov chain was run with a separate instance of the GTR + Γ + I model for each marker. Outgroup status was assigned to *Cochlostoma patulum* (Draparnaud 1801).

Phylogenetic model selection

We used hypotheses on the temporal splitting pattern of the *Tudorella* lineages in conjunction with the knowledge on the geological history of the Western Mediterranean (Rosenbaum et. al. 2002a,b) for a Bayesian model comparison approach. Given the posterior probabilities of the inferred clades, we assumed the existence of the following most recent common ancestors (mrca) and their successive branching: The ancestor of all *Tudorella* lineages in the dataset (mrca *Tudorella*, split 1 in Figure 4), the mrca of the sister taxon to *T. ferruginea* (Lamarck 1822) (mrca FerrSister, split 2 in Figure 4), the mrca of the sister taxon to *T. mauretanica* (Pallary 1898) (mrca MauSister split 3 in Figure 4) and the mrca of the three *T. sulcata s.l.* lineages (SL, split 4 in Figure 4).

As argued above, it is reasonable not to take the possibility of pre-anthropogenic over-sea dispersal in these land snails into account. Therefore, we assumed that the colonisation of unoccupied areas required the existence of direct land connections. We furthermore assumed that the minimal date of a split between lineages is given by the geological separation of the respective harbouring land masses. If a new lineage occupied a newly connected land mass, we assumed that the split from the ancestral lineage occurred after the connection in the course of the dispersal, thus given us a maximum age for the respective split. Figure 1 gives an overview of the temporal and spatial pattern of land connections or isolation of areas that currently harbour *Tudorella* lineages, according to Rosenbaum et al. (2002a,b). We then fitted the inferred phylogeny according to the assumptions detailed above on this area connection matrix. This resulted in four distinct hypotheses concerning the temporal splitting pattern:

Messinian Radiation:

The mrca of *Tudorella* lived in southwest Europe. When the Tyrrhenian plate broke off about 25 Mya, *T. ferruginea* was isolated on the Balearics (split 1). The sister lineage on the continent spread and diverged over North Africa to Sicily, Malta and Sardinia during the Messinian Salinity Crisis (Krijgsman et al. 1999; MSC: 5.33 – 5.96 Mya, split 2-4), when all areas in question were connected by land bridges.

Rif-Rafting:

The extant Tudorella lineage was completely on the Tyrrhenian plate that broke off mainland Europe. First, *T. ferruginea* split off on the Balearics 25 Mya the latest (split 1). The mrca of FerrSister rafted on the Betis/Rif terrane. When this terrane attached to Northern Africa about 10 Mya, the lineage had from there on the possibility to spread to the East and diverge from *T. mauretanica* (split 2 and split 3). The earliest possibility to reach Sardinia was the onset of the MSC, whose end also marks the minimum age for split 4.

Kabylies Crossing:

The extant Tudorella lineage was completely on the Tyrrhenian plate that broke off mainland Europe. First, *T. ferruginea* split off on the Balearics 25 Mya the latest (split 1). The remaining plate split the latest 21 Mya, isolating *T. mauretanica* on the Rif/Betis terrane (split 2). The mrca of MauSister crossed the Mediterranean on one of the Kabylies-fragments and had the possibility to spread and diverge to the East (Tunisia, Sicily and Malta) after this area attached to Northern Africa 15 Mya (split 3). The colonisation of Sardinia was achieved during the MSC.

Sardinia Sailing:

The extant *Tudorella* lineage was completely on the Tyrrhenian plate that split/broke off mainland Europe. First, *T. ferruginea* split off on the Balearics 25 Mya the latest (split 1). The remaining plate split the latest 21 Mya, isolating *T. mauretanica* on the Rif/Betis terrane (split 2). The mrca MauSister sailed on Sardinia. When this island was connected to Sicily during the MSC, the lineage dispersed and diverged to there, Malta and Northern Africa (split 3 and 4).

These hypotheses are visualised in Figure 5. We compared the fit of these temporal diversification hypotheses on the molecular dataset under the assumption of a relaxed molecular clock model of DNA sequence evolution using Bayesian factors. With the software BEAST vers. 1.4.8 (Drummond et. al. 2006), we constrained the divergence time of the splits according to the above scenarios, respectively. We applied uniform priors on the Time to the Most Recent Common Ancestor (tmrca) of the respective clades, since we had no information or plausible assumption when during a possible period the actual lineage split occurred. The data was partitioned into 16S and COX1. For each partition, we used a separate GTR + Γ + I model with four rate categories. We used an uncorrelated lognormal relaxed clock model. As tree prior, a Yule speciation model was chosen. We ran the MCMC chain for $2x10^7$ generations and sampled every 1000th tree, discarding the first 2 000 as burn-in. This assured an effective sampling size (ESS) of at least 1 500 for all relevant parameters. Both chain convergence and ESS were monitored using the software Tracer vers. 1.4.1 (Rambaut and Drummond 2007). From the tree-file output, we calculated the harmonic mean of the sampled tree log likelihoods, excluding the burn-in. This harmonic mean is an estimator of the marginal log likelihood of the model with respect to the prior. The difference of two marginal log likelihoods is the Bayes factor (BF) between them. A BF larger than 3 indicates substantial, over 10 strong and above 30 very strong support for the respective hypothesis. Additionally, a model without temporal restrictions on the clades was estimated for comparison.

4.3. Results

Species delimitation with mitochondrial and nuclear markers

The unrooted phylogram derived from Bayesian analysis of the COX1 data (Figure 2) showed eight divergent clades within the genus *Tudorella*, each with a Bayesian posterior probability above 0.87. The most divergent group (Bayesian posterior probability 1.00, clade 1) corresponded to the well known Balearic endemic *T. ferruginea*. The remaining seven clades are generally attributed to the polytypic *T. sulcata sensu lato*. A second branch represented the clade from SE-Spain and NE-Morocco (clade 2). This clade was also highly supported (post. prob. 1.00). One strongly distinct unit was found on Malta (post. prob. 1.00, clade 3), another (post. prob. 0.93, clade 4) originates from northern Sicily (near Palermo). One divergent group comprised individuals sampled from Central-Northern Algeria, South-Eastern France and several localities from Sardinia (post. prob. 1.00, clade 5). The last three closely related units (sequence divergence 0.012 - 0.021) were well supported clades (post. prob. ranged between 0.87 and 0.94) from Sicily and Tunisia (clade 6), Sardinia (clade 7) and North-Eastern Algeria (clade 8), respectively.

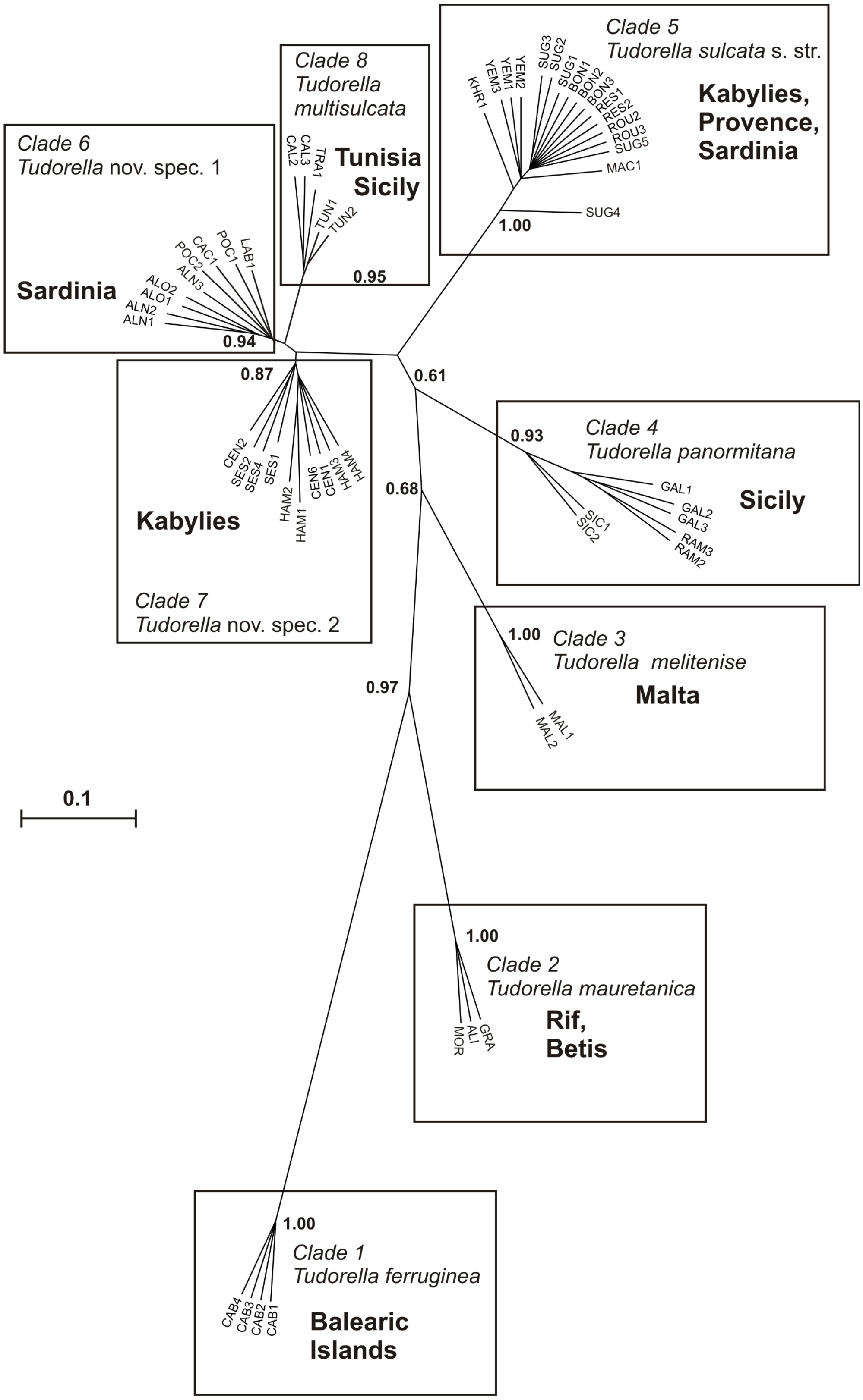

Figure 2: Unrooted majority rule consensus tree of the 99% credible set sampled by the Markov-chain in Bayesian analysis for the COX1-fragment. Numbers on nodes indicate their Bayesian posterior probability.

The second phylogram (Figure 3) was obtained from sequencing nuclear ribosomal DNA from the first internal transcribed spacer (ITS-1) region. All clades previously identified with COX1, with the exception of the individuals from SE-Spain and NE-Morocco from which no ITS-1 sequences could be obtained, were also forming well defined units.

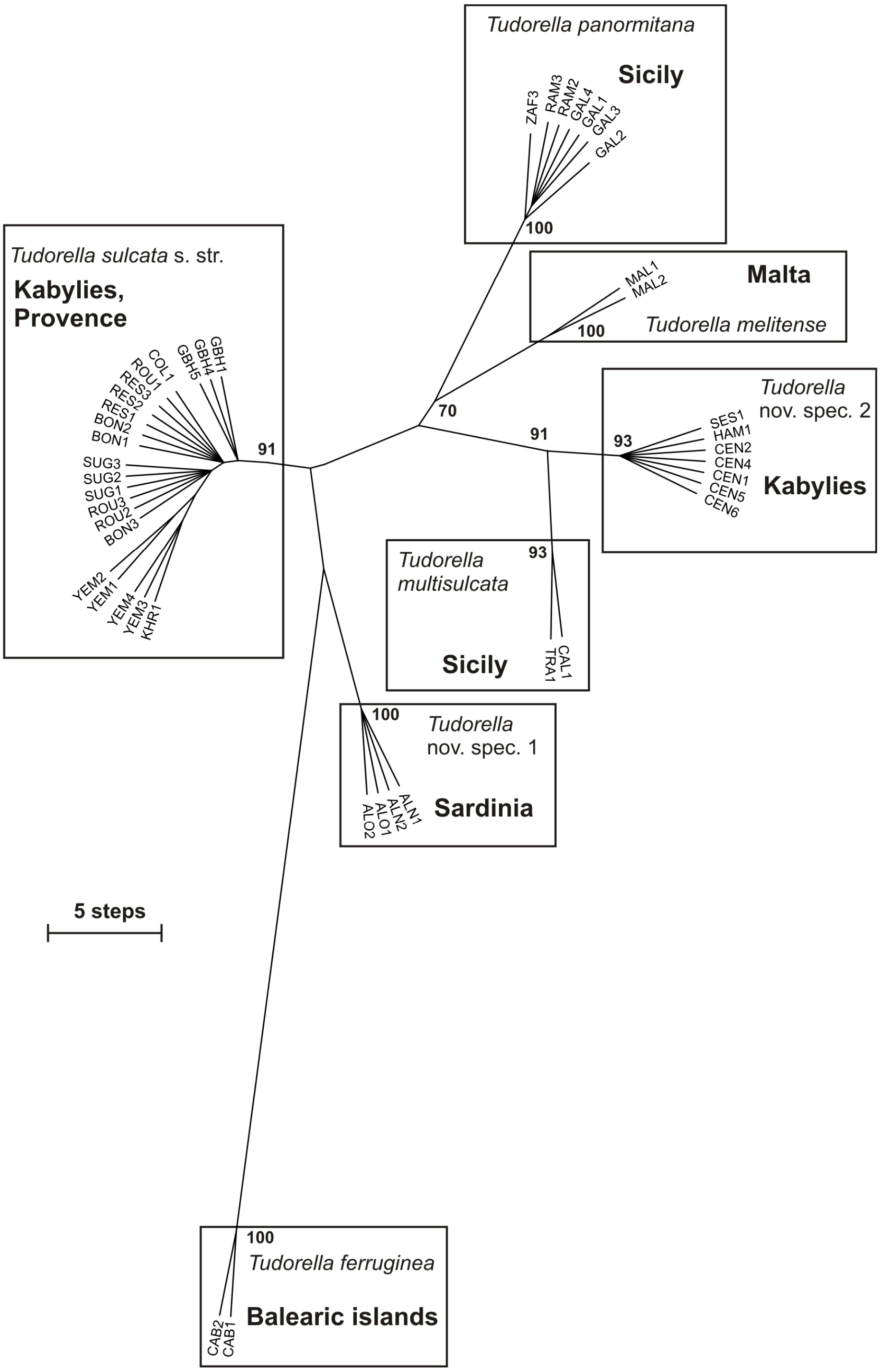

Figure 3: Unrooted majority rule consensus tree calculated from the most parsimonious trees for the ITS1-fragment. Numbers on nodes indicate their bootstrap proportion.

The delineated entities could be partially ascribed to existing taxa, applying the criterion that the describer must have remarked a specific distinctness of the taxon in question in the same area where we located the respective clade:

- Clade 1 corresponded to *Tudorella ferruginea* (Lamarck 1822), the type species of the genus. It is endemic to the Balearic Islands of Mallorca and Minorca.
- Clade 2 was described as *Tudorella mauretanica* (Pallary 1898). It is restricted to areas around the Alboran Sea: SE Spain, NE Morocco and NW Algeria, *i.e.* the Betis and Rif region (Martinez-Orti et al. 2008) where it was initially described.
- Clade 3 was congruent to *Tudorella melitense* (Sowerby 1847), endemic to the Maltese Islands of Malta and Gozo (Giusti et al. 1995).
- Clade 4 could be attributed to *Tudorella panormitana* (Sacchi 1954) which is endemic to northern Sicily near Palermo.
- Clade 5 was identified as *Tudorella sulcata* sensu stricto (Draparnaud 1805) because this clade includes the Provencal type locality of *T. sulcata* (Draparnaud 1805).
- Clade 6 with its distribution restricted to Western Sardinia could not be matched to one of the available names and was thus identified as a new species (*Tudorella* nov. spec. 1).
- Clade 7 is known from a single region in north-eastern Algeria (Annaba). No available name is corresponding to this local entity. Consequently, it was termed as *Tudorella* nov. spec. 2.
- For Clade 8, also occurring in Tunisia, *Tudorella multisulcata* (Potiez and Michaud 1838) is the oldest available name for localities of Western and Southern Sicily.

Phylogenetic hypothesis

A Bayesian analysis of the concatenated mitochondrial markers (16S and COX1), resulting in an alignment of 1143 positions was performed (Figure 4). The nuclear marker ITS-1 was not included because integration of the non-*Tudorella* outgroup taxa would have required many additional insertion/deletions, rendering a credible overall alignment impossible.

Tudorella was distinct from the genera *Pomatias* Studer 1789 and *Leonia* Gray 1850 that belong to the same family Pomatiidae. The splitting order within *Tudorella* stayed partially unresolved, as evidenced by two polytomies (Figure 4). The remaining splits, however, showed four well supported unequivocal monophyla. First, the Balearic *T. ferruginea* split from the remaining *Tudorella* species. Next, *T. mauretanica* split from the *T. sulcata* species complex. From the latter, *T. melitense* seemed to have speciated first. However, the next lower level node had relatively weak support (0.71 / 0.66, posterior probability / bootstrap proportion) so that this divergence order remains uncertain. The relation between *T. panormitana*, *T. sulcata* s. str. and the *T. sulcata s. l.* complex could not be resolved. This was also the case for the three clades within the *T. sulcata s.l.* complex. A *post-hoc* power analysis (Braun and Kimball 2001; Walsh et al. 1999; Walsh and Friesen 2001) indicated that the combined COX1 and 16S data set of 1143 bases should have been sufficient to resolve sequential branching that occurred during an interval of at least 6.3 % (± 1.2 %) of the total divergence time between the lineages. This indicated that the radiation of some *Tudorella* lineages indeed occurred during a relatively short time interval, if not simultaneously, and seemed not due to a poor resolution caused by a lack of data.

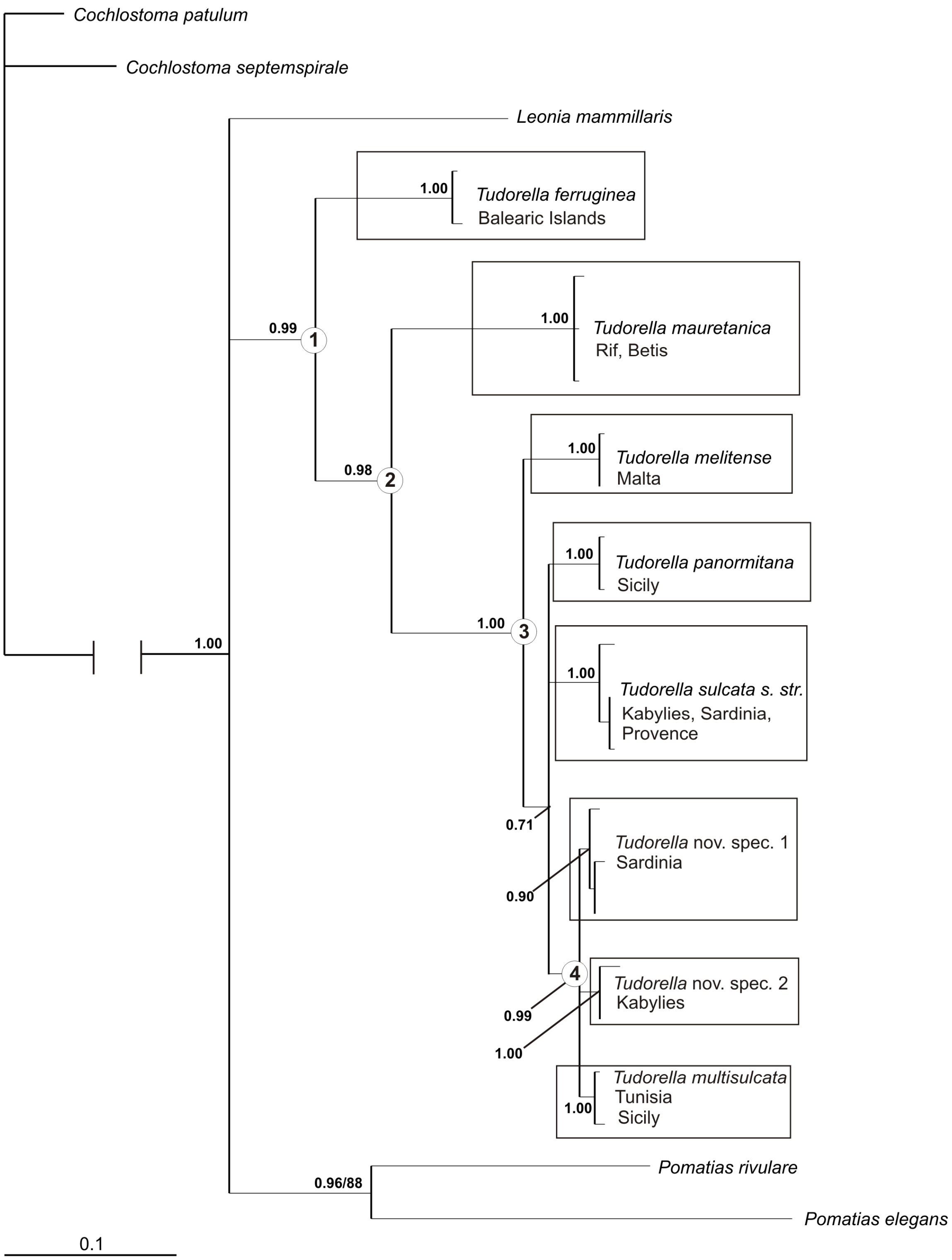

Figure 4: Majority rule consensus tree of the 99% credible set sampled by the Markov-chain in Bayesian analysis for the concatenated 16S and COX1 dataset, rooted with *C. patulum*. Numbers on nodes indicate their Bayesian posterior probability/boostrap proportion, respectively. The encircled numbers indicate splitting events referred to in the text.

Bayesian phylogenetic model selection

The current distribution of the genus *Tudorella* was best explained by geologic processes shaping the western Mediterranean since the Oligocene/Miocene. Figure 5 shows land connections of the current distribution areas in the last 25 Mya. From the tested hypotheses, *Kabylies Crossing* received best support in the data (marginal log likelihood = -3779.70). This model had substantial support over *Rif-Rafting* (BF = 6.16) and *Sardinia Sailing* (BF = 6.33) and very strong support over the *Messinian Radiation* scenario (BF = 90.34). The chosen model was not much worse than a temporally unconstrained model (marginal log likelihood = -3772.68). All possible scenarios required explaining the presence of *T. sulcata* either in the Provence and Sardinia or Provence and Kabylies with recent anthropogenic dispersal.

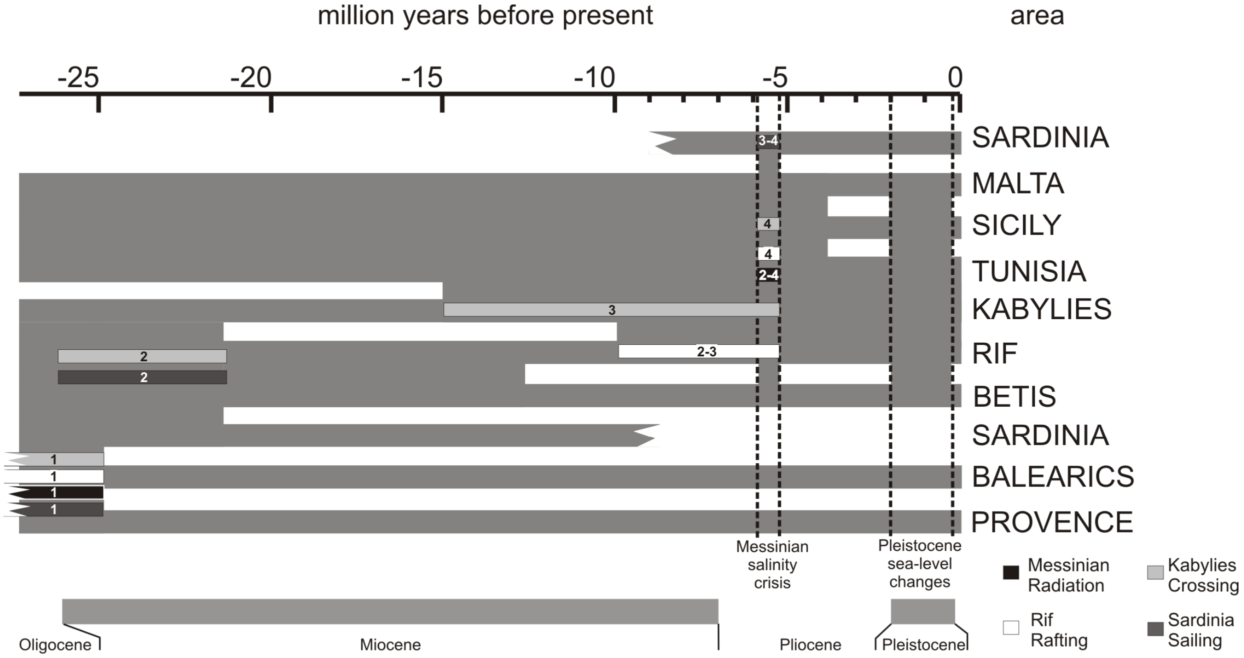

Figure 5: Land connections between current distribution areas of *Tudorella* since the late Oligocene. Connections are indicated by grey coloured areas. Please note that Sardinia occurs twice, because of the complicated reticulate area relations. Possible splitting periods for respective indicated nodes (numbers in the bars) of the hypotheses tested: *Messianian Radiation* (black bars), *Kabylies Crossing* (white bars), *Rif Rafting* (horizontally hatched bars) and *Sardinian Sailing* (vertically hatched bars).

4.4. Discussion

Species concept and status of lineages in the genus Tudorella

All *Tudorella* samples appeared as a well supported monophyletic taxon. The relation to the single *Leonia* species and the well supported genus *Pomatias*, however, could not be resolved, as a trichotomy at the basis of this clade shows (Figure 4). This result supports the generic distinction of the former "Cyclostoma" into *Tudorella* and *Pomatias* following Picard (1949).

We accomplished species delimitation of the genus *Tudorella* based on a representative sampling across the whole area and analyses of both mitochondrial and nuclear genetic markers. In our analyses, we found eight well supported evolutionary lineages (Figures 2 and 3). We recognised these lineages as eight different species according to the unified species concept (De Queiroz 2007), because these entities i) were reciprocally monophyletic and deeply divergent at two independently inherited marker loci, ii) represented thus independent coalescence processes and iii) showed a non-overlapping allopatric distribution, which indicates absence of gene-flow in these poorly dispersing animals (Figure 1). This finding excludes already the recent anthropogenic dispersal from a single source hypothesis as explanation for the current distribution.

At our knowledge, this species complex is more or less truly cryptic, as it is only partially recognisable by quantitative characters like the mean size of the shell or the shell colour (Véla et al. 2008). Nevertheless, most of these molecularly recognised taxa were previously described at species or subspecies rank based on shell characteristics (Kobelt 1879; Pallary 1898; Potiez and Michaud 1838; Sacchi 1954; Sowerby 1847) except for Clades 6 and 7. The first of these new species of *Tudorella* has a restricted distribution in Western Sardinia, the second is an endemic confined to the Edough peninsula near Annaba, a "continental fossil island" (Lanza 1984) from where also other cryptic animal or plant species are known (Carranza and Wade 2004; Véla and Benhouhou 2007). However, additional studies are necessary to see whether morpho-anatomical characteristics distinguishing the delimited entities can be found. The delineation of these highly divergent cryptic species underlines the importance of the use of molecular markers in the estimation of biodiversity (Pfenninger and Schwenk 2007).

Biogeography of Tudorella

We relied on a Bayesian phylogenetic model selection approach to distinguish among several biologically plausible temporal diversification models by measuring the fit of the data to the models. A better fit of data in the present case means less variation in sequence evolution rate along the branches of the phylogeny, i.e. less introduced rate changes to fit the observed branch lengths to the applied temporal diversification pattern. The advantage of such approaches to hypothesis testing is the possibility to test several models simultaneously, instead of testing a single hypothesis against a null model. However, one has to be aware that all model selection approaches measure solely the relative support of the models tested by the data – regardless whether the true model was among them or not. Here, we have taken only biogeographic diversification models into account that did not require prehistoric transoceanic dispersal, because this appeared biologically most plausible for the targeted species.

All models involving some speciation due to vicariance events during the Miocene received significantly better support than a rapid radiation during the comparatively short MSC, the only event potentially connecting the areas in question by land in their more or less current positions. A necessary assumption, of course is, that all terranes implicated were not completely submerged during any time of the process. This is, however, a quite reasonable assumption, since all areas harbour mountains of at least 1 000 m altitude and their appearance is usually attributed to the alpidic orogenesis prior to their detachment from Europe (Schmid et al. 1997).

Among these well supported models, the *Kabylies Crossing* scenario best fitted the data. This scenario is also supported by the fossil record insofar as *Tudorella*-like shells appeared first in Europe and subsequently in the strata of the respective regions only after the inferred arrival period (Véla et al. 2008). Unfortunately, the fossil remains cannot be reliably associated to extant lineages, which rendered a molecular clock calibration with them impossible. However, we found current disjunct distributions of *Tudorella* populations clearly belonging to the same coalescence process as evidenced by not reciprocally monophyletic relations and even shared haplotypes (*T. mauretanica* in Spain and Morocco, *T. multisulcata* in Sicily and

Tunisia and *T. sulcata* in Algeria, Sardinia and Provence). These disjunct populations belong therefore to the respectively same species according to the applied criteria and their respective most recent common ancestor is presumably younger than the geologic events taken into account. Therefore these distribution patterns require additional studies as both the current sample strategy and sampling size do not allow distinguishing between relatively recent vicariance events in the late Pliocene or Pleistocene and a postglacial anthropogenic introduction.

The current distribution pattern of the *Tudorella* species in the Western Mediterranean is therefore most probably not the result of active or passive dispersal processes, but was caused by vicariance events, followed by subsequent dispersal to areas that came later into contact with the drifting microplates. This study thus supports the hypothesis of Giusti and Manganelli (1984) who forwarded these tectonic events shaping the Western Mediterranean region as cause for the disjunct distribution of several snail taxa. In the northern peri-Tyrrhenian area, this sequence of tectonic events is corroborated by molecular clock analyses of the land snail genus *Solatopupa* (Ketmaier et al. 2006). A recent study on cork oaks found a chloroplast haplotype distribution pattern that was also best explained by the above described tectonic vicariance scenario (Magri et al. 2007). Even though additional studies with more taxa are required, the Tyrrhenian vicariance hypothesis seems to be the emerging explanation for the paradoxical taxonomic composition of North African biodiversity and thus provide a partial explanation for the high endemism rate in the Western Mediterranean Basin (Médail and Diadéma 2009; Verlaque et al. 1997).

Chapter 5

Phylogeography of a land snail suggests trans-Mediterranean Neolithic contact

Ruth Jesse, Errol Véla , Bruno Streit, Markus Pfenninger

Abstract

Fragmented distribution ranges of species with little active dispersal capacity raise the question about their origin and the where and when of either range fragmentation or dispersal. The peculiar distribution of the land snail *Tudorella sulcata* s. str. in Southern France, Sardinia and Algeria is such a challenging case. Statistical phylogeographic analyses with COX1 sequences were used to answer the questions of the species' origin, sequence and timing of dispersal. The origin of the species was on Sardinia. Starting from there, a first expansion to Algeria and then to France took place. Abiotic and zoochorous dispersal could be excluded by considering the species' life style, leaving only anthropogenic translocation. The geographic expansion could be dated to approximately 8 000 years before present with a 95% confidence interval of 10 000 to 3 000 years before present. This period coincides with the Neolithic expansion in the Western Mediterranean, suggesting a role of these settlers as vectors.

Manuscript

5.1. Introduction

Ever since people have had the ability to move around the planet, they have been introducing new species, either intentionally or accidentally, into new geographical areas (Wilson et al. 2009). Likewise, the biodiversity in the Mediterranean Basin was shaped, at least since the Neolithic emergence (ca. 10 500 – 5 000 years before present; BP) by human landscape management and species introductions which usually impacted the indigenous fauna and flora (Zeder 2008). Since the beginning of animal domestication about 10 000 – 9 500 years BP in the Eastern Mediterranean, approximately a millennium after the first domestication of crop plants, both were spread westwards with settlers disseminating the Neolithic culture. From then on, the Mediterranean region has served as sink and source for extensive exchange of biodiversity associated to human use (Zeder 2008).

But also species not directly involved in human exploitation were spread accidentally from the very beginning of human migrations, like weed propagules contaminating crop seeds or blind passengers in ship ballasts (Wilson et al. 2009). In the Mediterranean, it is known that at least nine land snail species were dispersed as an unintentional by-product of bronze-age maritime copper and resin trade (Welter-Schultes 2008). Analysis of land snail shells found in a ship wreck indicated that they were carried off alive with scrub used to cushion heavy freight (Welter-Schultes 2008).

The possibility of (un)-intentional introductions in addition to natural range expansions of a species makes it often difficult to infer the direction and timing of dispersal events (Jesse et al. 2009). Historic records of first sightings of a species in a new area are rare (Pringle and Vellinga 2006), in particular for organisms that are inconspicuous, not a pest or otherwise attracting human attention. Moreover such records are, by definition, non-existing for prehistoric times. In these cases, the (sub)fossil archaeological record can provide an estimate of the minimum time of residence in an area (Olson 2003; Pregill and Steadman 2009). However, not all organisms are prone to fossilisation or their remains cannot be attributed to a particular species with the necessary certainty. To overcome these difficulties, various methodological approaches based on the distribution of genetic variation have been developed in the past 25 years. The rationale behind these phylogeographic approaches is the reconstruction of the demographic history of the extant populations from DNA sequence data (Emerson et al. 2001).

The distribution of the land snail *Tudorella sulcata* s. str. in Southern France, Sardinia and Algeria, areas that are currently separated by an ocean and that were last in direct contact about 30 million years ago, raises the suspicion that this species could have been at some point in the past subject to passive dispersal. The rather large snail has a low active dispersal capacity and a covert life style, burrowed mostly in the soil under rocks and shrubs, except for activity phases during wet weather periods (Vela et al. 2008). The first record of the species in literature is its description from Provence by Draparnaud in 1805, thus setting the minimum age for the introduction to Southern France. Due to the presence of cryptic species, it is unfortunately not possible to attribute fossilised shells to extant species in the genus *Tudorella* (Pfenninger et al. 2009; Vela et al. 2008). Consequently, we used statistical phylogeographic analyses to answer two major questions:

i) Where is the origin of the land snail species *Tudorella sulcata* and what was the colonisation sequence?

ii) When were the respective areas invaded?

5.2. Material and Methods

Molecular analyses

The present study covered all known occurrences of the species *Tudorella sulcata* sensu stricto (Draparnaud 1805) as described in Pfenninger et al. (2009). For each of the 28 sampled locations, several individuals were preserved in 90 % alcohol and with one exception, two to five specimen per locality were analysed. Further information about the sampling sites is given in Table 1. For DNA extraction a part of the foot muscle was taken. Genomic DNA was extracted using the CTAB method (Doyle and Doyle 1987). For 138 individuals, a 604 bp segment of the cytochrome oxidase subunit I gene (COX1) was PCR-amplified. PCR conditions and primers were adopted from the study of Pfenninger et al. (2005). PCR products were purified using Pure Link™ PCR Purification Kit (invitrogen, Carlsbad, CA, USA). Ten nanograms per sample were subjected to cycle sequencing using the ABI Prism Big Dye terminator kit (Perkin-Elmer, Norwalk, CT, USA). Sequencing reactions were electrophoresed on an ABI 377 automated DNA sequencer.

Haplotype phylogeny

The statistical parsimony (SP) cladogram was constructed using TCS vers. 1.21 (Clement et al. 2000). The connection limit was set at 95% and gaps were treated as fifth character state. The few loops that occurred in the constructed network were resolved according to the rules detailed in Pfenninger and Posada (2002).

Phylogeographic Model Selection (PMS)

To compare the relevance of nine explicit dispersal hypotheses, we applied a model selection approach (Johnson and Omland 2004; Stephens et al. 2007), introduced into phylogeography by Pfenninger and Posada (2002) and further developed in Depraz et al. (2008). The 28 sampling sites were pooled into three geographic groups (Southern France (F), Western Sardinia (S) and Algeria (A), Figure 1). Taking each of the three regions as potential origin in turn, we evaluated i) the hypothesis that each of the other two regions were independently colonised from there and ii) that either one of the two regions was colonised first and the remaining region from there. This resulted in nine hypotheses, which were translated into the corresponding gene-flow matrices.

The maximum likelihood migration rate matrix of each model was then estimated using MIGRATE-N version 2.3 (Beerli and Felsenstein 2001). The first genealogy was started with a random tree. Initial theta and migrant values were generated from an F_{ST} calculation. A static heating scheme with four different temperatures was applied. We ran ten short chains with $4x10^4$generations each, from which 1 000 trees were recorded in regular intervals after a burn-in phase of 20 000 generations. These were followed by three long chains of 10^5 generations, from which 10^3 trees were sampled after a burn-in period of $2x10^3$ generations. Parameter estimates were gained from the combination of the last chains. Log likelihood estimates cannot be directly compared over different runs with MIGRATE-N. We therefore ran a final analysis with an unconstrained migration model using the likelihood-ratio-test option to gain likelihood estimates that were comparable between the different hypotheses and their parameter sets. We used these estimates and the number of free parameters in each model to calculate the Akaike Information Criterion (AIC, Akaike 1974) and resulting Akaike weights to obtain measures of relative hypothesis support.

Demographic analysis

Dating demographic expansions associated with geographic expansions, the demographic history of the species was performed with the extended version of the Bayesian Skyline Analysis (Drummond et al. 2005) implemented in BEAST 1.5.2 (Drummond and Rambaut 2003). As site evolution model, we have chosen the General Time Reversible model with empirical base composition, a gamma distribution of rate heterogeneity with four rate categories and invariant sites. As we are dealing with intraspecific date, we have chosen a strict molecular clock model with a fixed rate of $4.28x10^{-8}$ changes per site and year, as estimated for this *Tudorella* lineage (Pfenninger et al. 2009). As tree model, the Extended Bayesian Skyline Model for mitochondrial data with linear growth between population size change events was applied with a UPGMA generated tree as starting point. The prior for the number of population size changes was a Poisson distribution with a mean of two, as we were expecting two expansion events (see results). A uniform distribution between 10^4 and

10^{12} with an initial size of 10^6 was set for the demographic population mean prior, as no information on the actual population size of *T. sulcata* was available. Initial runs indicated that it was necessary to run 4×10^7 generations of the Monte Carlo Markov Chain, sampling every 1 000th generation, to obtain effective sampling sizes above 500 for all estimated parameters.

Table 1: Study specimens with origin, total number of individuals per population / region, geographical coordinates and haplotype diversity per population / region.

Sampling sites	**Number of Individuals**	**Latitude**	**Longitude**	**Haplotype diversity**
France	60			0.067
Anse de Bonnieu	5	N 43° 20.668'	E 5° 01.407'	0.200
Bonnieu	7	N 43° 20.700'	E 5° 01.533'	0.286
Couroune	7	N 43° 19.700'	E 5° 04.450'	0.143
La Redonne	6	N 43° 20.127'	E 5° 11.906'	0.333
Mejean	2	N 43° 19.857'	E 5° 13.162'	0.500
Port Miou	6	N 43° 12.367'	E 5° 30.758'	0.167
Resquiadou	3	N 43° 21.250'	E 5° 16.833'	0.333
Roucas-Blanc	6	N 43° 16.467'	E 5° 22.550'	0.167
Sausset les Pins	7	N 43° 19.800'	E 5° 05.790'	0.143
Sugiton	11	N 43° 12.917'	E 5° 26.750'	0.182
Algeria	28			0.143
Bejaia	8	N 36° 45.279'	E 5° 04.568'	0.375
El Khroub	1	N 36° 16.283'	E 6° 54.367'	1.000
Yemma Gouraya	19	N 36° 46.167'	E 5° 05.033'	0.105
Sardinia	50			0.160
Capo Mannu	4	N 39° 01.859'	E 8° 22.765'	0.250
Florinas	2	N 40° 39.370'	E 8° 38.610'	0.500
Genna Maria	5	N 39° 37.944'	E 8° 51.284'	0.200
Is Arenas	4	N 40° 03.985'	E 8° 30.025'	0.500
Is Arutas	3	N 39° 57.194'	E 8° 24.390'	0.667
Lago del Cuga	3	N 40° 36.825'	E 8° 30.402'	0.333
Mara	4	N 40° 27.241'	E 8° 35.580'	0.500
Mari Ermi	2	N 39° 57.884'	E 8° 26.333'	0.500
Morgongiori	3	N 39° 42.209'	E 8° 45.084'	0.667
Padria	3	N 40° 24.240'	E 8° 38.130'	0.333
Romana	2	N 40° 28.645'	E 8° 36.120'	1.000
San Marco	4	N 40° 11.830'	E 8° 28.860'	0.250
Santa Catarina	4	N 40° 06.550'	E 8° 29.310'	0.250
Thiese	3	N 40° 31.770'	E 8° 42.160'	0.333
Zuchinu	4	N 40° 43.760'	E 8° 25.880'	0.750

5.3. Results

Phylogenetic relationships

We obtained 138 COX1 sequences of 604 bp length. All sequences aligned unambiguously and translated without stop codons. Statistical parsimony analysis resulted in a network of 13 haplotypes, defined by 12 singleton mutations and five parsimony informative (PI) sites (Figure 1). Haplotype diversities for the three geographical regions are: Sardinia (in grey) 0.160 %, Algeria (in black) 0.143 % and France (in white) 0.067 %. Haplotype sequences are registered in GenBank, accession numbers are GU385953 – GU385966.

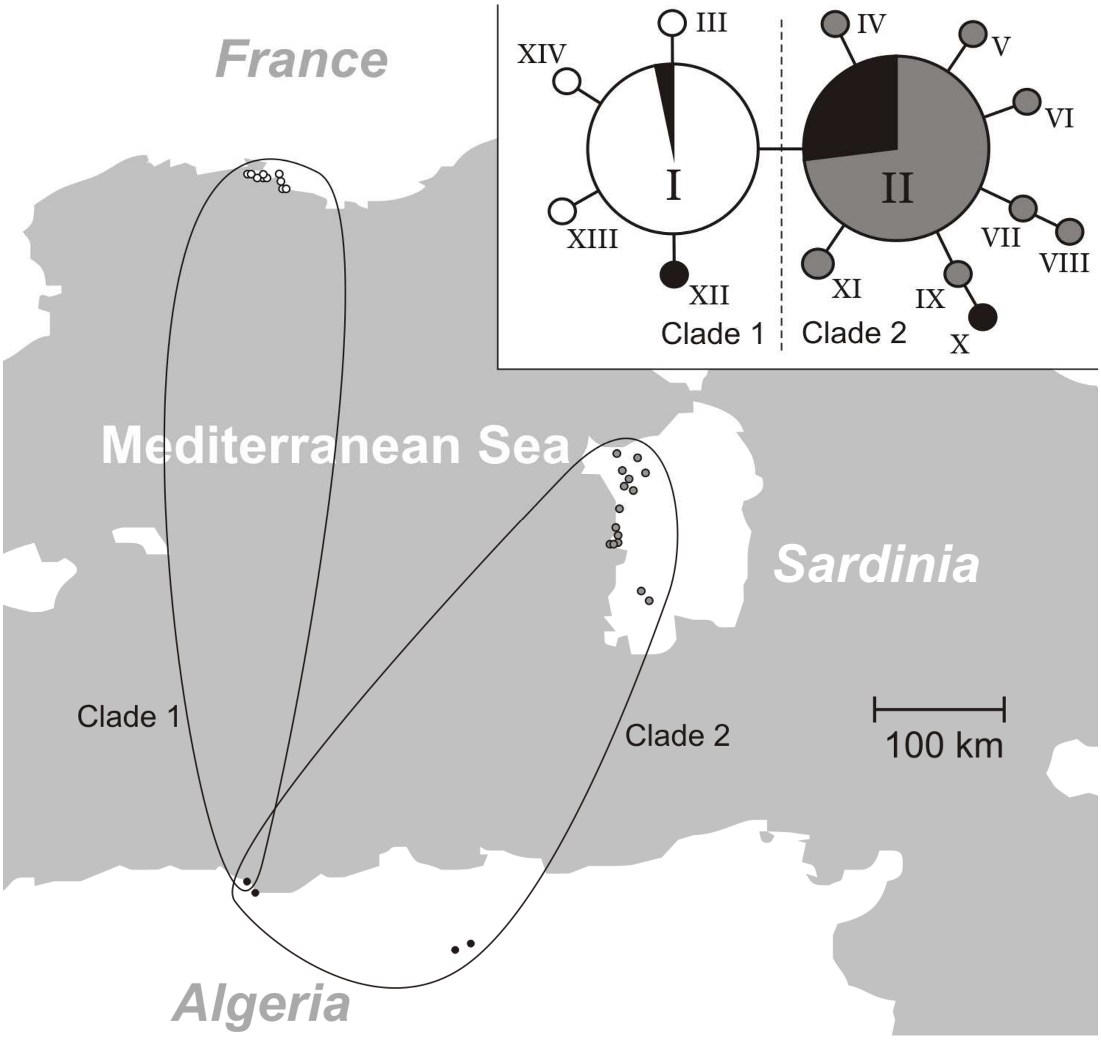

Figure 1: Clade distribution of 138 COX1 haplotypes of *Tudorella sulcata* s. str. superimposed on the sampling sites. The inset shows the statistical parsimony network with the two-step clade structure, based on 604 bp of the COX1 gene. Circles depict sampled haplotypes, their size is proportional to their frequency. Connecting lines correspond to single mutational steps. Haplotypes occurring in France are white, Algeria black and Sardinia grey.

Inference of dispersal routes

Model selection criteria preferred the S>A>F model (AIC = -33.2, LnL = 21.6) over the remaining eight models (Table 2). The best supported model implies that the origin of the expansion was on Sardinia. Starting from there a first expansion to Algeria and a second expansion from Algeria to France took place.

Table 2: Results of the migration hypothesis model selection. AIC values measure the fit of the models to the data, taking different parameterisation into account. Note that smaller values indicate better fit. A model suggesting origin in Sardinia, followed by expansion to Algeria and subsequent colonisation of France (S>A>F) received best support.

Scenario	LnL	No. of parameters	AIC
full model	21,6	9	-25,2
S>A, S>F	-6,0	5	22,0
F>S, F>A	-63,9	5	137,9
A>F, A>S	-37,7	5	85,4
F>S>A	-6,0	5	22,0
A>S>F	-64,0	5	138,0
S>F>A	-63,9	5	137,9
A>F>S	-37,7	5	85,4
S>A>F	21,6	5	-33,2
F>A>S	-55,6	5	121,2

Demographic analyses

The median estimate in the Bayesian skyline plot analysis for the beginning of population growth was approximately 8 000 years BP with a 95% confidence interval ranging from 10 000 to 3 000 years BP. Figure 2 shows the estimated increase of effective population size with 95% confidence intervals.

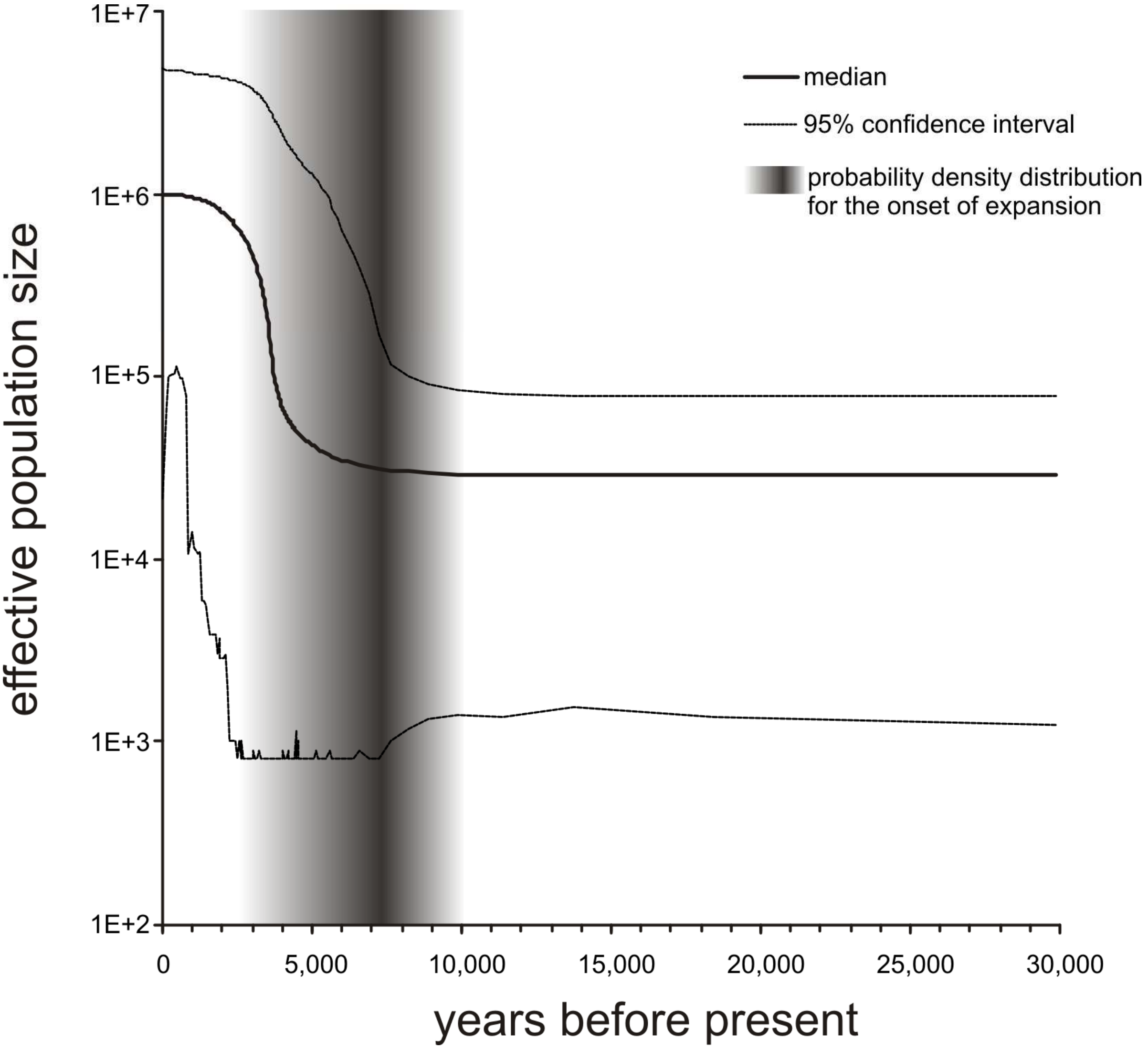

Figure 2: Bayesian skyline plot: historical development of population sizes through time based on Bayesian coalescence analyses. The analysis suggests a population increase between 3 000 and 10 000 years BP with the highest probability density around 8 000 years BP.

5.4. Discussion

Statistical phylogeography is a powerful tool to answer questions about a species' origin, dispersal routes and timing of expansions in a rigorous statistical framework and has been successfully established in the last years (Cordellier and Pfenninger 2009; Depraz et al. 2008; Jesse et al. 2009; Yannic et al. 2008). However, such analyses demand a sampling of the whole range of the focal species. The distribution range of *Tudorella sulcata* comprising Southern France, Western Sardinia and Algeria, is well know due to a previous study by Pfenninger et al. 2009 on the range wide analysis of the genus and could thus be comprehensively sampled.

Expansion sequence

The occurrence of Algerian haplotypes both on Sardinia and in France and the lack of shared haplotypes between France and Sardinia argue either for an Algerian origin or a connection of the areas through this country (Figure 1). To distinguish among all nine possible expansion sequence models by measuring the fit of the data to the models, we relied on Phylogeographic Model Selection (PMS). The advantage of this alternative to classical hypothesis testing is the possibility to evaluate several models simultaneously instead of testing repeatedly single hypotheses against a null model (Johnson and Omland 2004). In our analysis the data strongly supported an origin of *Tudorella sulcata* on Sardinia and an expansion from there first to Algeria and subsequently to France. Sardinia as origin of the species was also indicated by the highest haplotype diversity in that region. The lack of support for France as origin of the species is also compatible with the results of the biogeographical analysis (Pfenninger et al. 2009).

The somehow counter-intuitive dispersal sequence suggested by our analysis, in addition to the species' biology makes a natural expansion very unlikely. Dispersal by log-rafting in the sequence Sardinia-Algeria-France is most implausible when considering the surface circulations in the Western Mediterranean Sea. Dense water from the Tyrrhenian Sea is transported in a current flowing from Sardinia northwards to Corsica and France and counter clockwise along the French and Spanish coasts until reaching Northern Africa near the Strait of Gibraltar (Millot 1999). The Algerian Current is going 3 – 5 km per day eastwards in anticlockwise circulation along the northern continental slope, making drift from Algeria to France under regular conditions highly unlikely as it would require the snails to survive a several week long journey exposed to saltwater.

The maximum reported dispersal distance of light objects (< 450 g) by extreme strong winds is 130 km (Nathan et al. 2008). As the distances between Sardinia and Algeria (ca. 250 km) as well as Algeria and France (ca. 700 km) exceed this maximum by far, it is reasonable to dismiss the hypothesis of wind-borne dispersal.

The pattern observed in our analysis thus suggests transportation through biological vectors. Migrating birds are potential over-sea vectors for small invertebrates, but rather for freshwater species (Bilton et al. 2001; Boag 1986). Usually eggs or larval stages are transported, attached to feathers of water fowl. *Tudorella sulcata* lives mostly buried in the soil and contact to migrating birds is hardly imaginable. Even though important bird migration routes run between central Africa and Europe, transport by birds is thus unlikely for this land snail.

An ancient expansion (e.g. during the Messinian Salinity Crisis 5.96 ± 0.20 Mya; Krijgsman et al. 1999) was a priori unlikely in the light of the shallow divergence of the populations and the COX1 molecular clock rate estimated from phylogenetic analysis (Pfenninger et al. 2009). After exclusion of the possible alternatives, anthropogenic passive dispersal remains as sole plausible mechanism.

Timing of the expansion

As a colonisation of new areas is logically linked to demographic expansion, the dating of the latter can give a time frame for these events. Bayesian skyline plot estimates suggested that a population expansion in *Tudorella sulcata* started between 10 000 and 3 000 years ago (Figure 2). This broad interval excludes historic times on one end and Pleistocene dispersal on the other. The highest probability density of the estimate lied around 8 000 years BP. This period coincides with the beginning of the Neolithic period in the Western Mediterranean. Coming from the Near East (Levant), Neolithic colonists started around 10 500 years BP to migrate first into the Balkans and then continuously colonised the European continent in expansion waves westwards during the following 4 500 years, with a peak of migration around 8 000 years BP (Davison et al. 2006; Turney and Brown 2007; Zeder 2008). First sea crossings in the Mediterranean are documented already for the Mesolithic Age by obsidian originating from the Cycladic island of Melos found in Mesolithic sediments of the Franchthi cave on the Peloponnesus (Broodbank 2006). Numerous obsidian artifacts found in Italy, Southern France, Eastern Spain, Croatia, Greece, Tunisia and Algeria document the efficiency of trans- oceanic transport during the Neolithic period (Tykot 2002). The simultaneousness of the demographic expansion with this phase of early human expansion and the exclusion of other factors suggests indeed that Neolithic settlers or traders were acting as vectors for the snails. Genetic analyses of Sardinian human populations connect Sardinia and Northern Africa through early human migrations and strengthen the above scenario (Calò et al. 2008; Tobias 2002). The secondary expansion from Algeria to France is more difficult to date because the installation of the rather small population there has not left traces in the demography. In Southern France, the first Neolithic coastal settlements date to 7 700 – 7 600 years BP (Zeder 2008). Contacts between the French and Algerian coasts have been numerous since then. However, the private haplotypes found in France (Figure 1) argue for a relatively early introduction.

Without further archaeological evidence, it is impossible to determine the purpose the snails were transported for. Unintentional shipping as in the case of the Uluburun wreck (Welter-Schultes 2008), transportation as trading good or simply because the light pink, regularly sculptured shells have a quite attractive appearance, can be imagined.

Conclusions

This study indicates that also the population histories of non-domesticated animals and plants may give hints on the direction and timing of early human expansion routes. It suggests that modern invasions, such as the well-known zebra-mussel proliferation in freshwaters worldwide (Ricciardi et al. 1998) or the invasion of the Asian tiger mosquito in Europe since 1979 (Adhami and Reiter 1998), are only the last instances of a long series of anthropogenic introductions, shaping the distribution of extant biodiversity.

General Discussion

The present study investigated Mediterranean freshwater crabs of the genus *Potamon* and land snails of the genus *Tudorella* in three contexts: taxonomy, biogeography of the genus and particular species. The main findings I want to summarise and discuss in the following paragraphs.

Taxonomy

By analysing the genetic relatedness of individuals grouped in the same taxonomic unit according to morphological characters (morphospecies), I intended to detect either congruence of characters or cryptic diversification. In *Potamon* as well as *Tudorella* I was able to identify genetic lineages that could not be attributed to known species or subspecies, thus showing considerable cryptic diversification in both groups.

Previous to my studies, nine species in the genus *Potamon* were recognised in the Mediterranean region (Brandis et al. 2000). I could distinguish five additional evolutionary lineages: three clades formerly described as subspecies (*P. hippocrate*, *P. karpathos* and *P. kretaion*) and two new clades (*P. pelops* and *Potamon* sp.).

In the genus *Tudorella* the two species *T. ferruginea* and *T. sulcata* were generally recognised (Giusti et al. 1995; Velá et al. 2008). The presented genetic analyses resulted in eight lineages in the genus: the two above mentioned species, four clades attributed to former (sub) species (*T. mauretanica*, *T. melitense*, *T. panormitana* and *T. multisulcata*) and two new evolutionary lineages (*Tudorella* nov. spec. 1 / 2).

I propose that the genetic lineages described in Chapters 1 to 5 should be treated as separate species as they fulfil the requirements described in two recent theoretical approaches to species identification: the unified species concept of De Queiroz (2007) and the taxonomic circle introduced by DeSalle et al. (2005). In the unified species concept a species is per definition a separately evolving metapopulation lineage. One species criterion detected by a discipline like DNA taxonomy, morphology, mating studies or ecology can be sufficient evidence for lineage identification. The taxonomic circle, on the other hand, requests for evidence from at least two disciplines to support species status.

All genetic lineages described in this study represented separately evolving units, as they were monophyletic and showed a deep genetic differentiation by analysis with at least two markers, thereby meeting the rules of De Queiroz (2007). Further, they were allopatric as their ranges did not overlap, so that gene-flow between lineages seemed unlikely. That made geographical separation a secondary line of evidence for species identification. The only exceptions were the lineages *Potamon hippocrate* and *Potamon kretaion* which co-occur on the island of Crete. However, as these two genetic clades were separated for more than 3 Ma and cluster in different subgenera (see Chapter 1, Figure 4), their status as separate species is not questionable.

To determine how many of the 11 new genetic lineages in the two genera represent cryptic species, additional morphological analyses have to be conducted. Cryptic species are two or more distinct evolutionary lineages which were recognised as only one species because of their high phenotypic similarity. Morphological differences between such species are in general not detectable by traditional systematic methods (Bickford et al. 2006; Pfenninger and Schwenk 2007). Especially by working with preserved material in ethanol like I did in my studies, subtle differences in the outer appearance (e.g. colour patterns) can be no longer recognisable (Knowlton 1986). For my study genera the traditional morphological characteristics in use were all quantitative: shell size and colour in *Tudorella* (Martínez-Ortí et al. 2008), carapace morphometrics and shape of first gonopods in *Potamon* (Brandis et al. 2000). The seven genetic lineages which could be assigned to former (sub) species showed morphological differences to raise the suspicion of separate evolution in the first place. Therefore, I suppose that these clades will less likely be characterised as cryptic species. It has to be verified if their quantitative differences fall in the regular phenotypic variation within species or are sufficient for morphological species delimitation.

In my study on *Potamon pelops* (Chapter 2) it has been demonstrated that the morphological characters in use were not sufficient for separating species in the genus *Potamon.* Differences in the morphology of the first gonopods (used for species delimitation in freshwater crabs) fell in the morphological range described for the sister species *P. fluviatile* (Chapter 2, Figure 2). So far, the only detectable difference between both species, except the genetic diversification, was spermatophore morphology. While in *P. fluviatile* only cleistospermia had been accounted for (Guinot et al. 1997), *P. pelops* was shown to produce cleisto- as well as coenospermia. This finding, in addition to the genetic differentiation and allopatric ranges, supported the status of *Potamon pelops* as individual species in the genus *Potamon*. Due to the fact that spermatophore morphology has not been used as a criterion for species description before and nothing is known about the variability or seasonality of this character, *Potamon pelops* was characterised as a cryptic species in *Potamon*.

Taxonomy was not the main issue in this thesis, but prerequisite for all further analyses. Molecular analyses increased the resolution in the taxonomy significantly, but further morphological investigations are necessary to determine the extent of cryptic diversification in both genera.

Biogeography of the genera

At the genus level I wanted to find out if biogeographical patterns in the two genera mirror the geological history of the Mediterranean region, where the origin of the genus was and if vicariance or dispersal was the main factor shaping these patterns.

To achieve this, I made use of Bayesian Phylogenetic Model Selection (PMS), a statistical framework established in biogeography research in recent years (Cordellier and Pfenninger 2009; Depraz et al. 2008, Klaus et al. 2010; Yannic et al. 2008). The advantage of this method to classical hypothesis testing is the possibility to test different, biologically reasonable, hypotheses simultaneously by measuring the fit of the data to the models, instead of testing each model against a null model (Johnson and Omland 2004). However, all model selection approaches measure solely the relative support of the models tested by the data – regardless whether the true model is among them or not. Therefore, it is of great importance to take a species' ecology and its dispersal abilities into account before creating expansion models.

In the study on the genus *Potamon* in the Aegean region (presented in Chapter 1), I used PMS to test if the formation of the Mid-Aegean Trench (12-9 Mya; Dermitzakis and Papanikolaou 1981) coincided with the split separating the genus in a western and an eastern group. The model selection approach, as well as the temporal estimation of the phylogeny, suggested that the east-west split occurred after formation of the Mid-Aegean trench (8.3–5.5 Mya).

This result implied that freshwater crabs successfully dispersed across this geographical barrier which had a strong influence on the biogeography of other invertebrate species in the Aegean (Parmakelis et al. 2005; Simaiakis and Mylonas 2008). The crabs could have surmounted the trench by rafting or migration through the basin during the late Messinian.

However, most lineages in the genus *Potamon* originated in the Pleistocene between 3.96 and 0.30 Mya (Chapter 1, Figure 4). In this period the sea-level in the Mediterranean fluctuated by up to 125 m and the Aegean islands were repeatedly connected and disconnected by possible dispersal corridors (Fleming et al. 1998). For some insect species on the Aegean islands it had been assumed that their present biogeography was caused by these sea-level changes (Allegrucci et al. 2009; Fattorini 2002). As I found only one island endemic (*Potamon kretaion*) in the course of my studies, it seemed that vicariance events caused by sea-level fluctuations had not been the main factors shaping the present biogeography of *Potamon*. Alternatively the Pliocene/Pleistocene climate change itself could have caused speciation. As freshwater crabs need humid conditions for successful migration over land (Gherardi et al. 1988a,b) the establishment of the Mediterranean type of climate with marked seasonality and stable summer draughts between 3.2 and 2.8 Mya could have resulted in restricted dispersal between single drainage systems and speciation of isolated populations therein. This period of climate change was assumed to have influenced also other taxa which are depended on adequate levels of humidity like Mediterranean plants (Bittkau and Comes 2009) and water frogs (Çiğdem et al. 2010). With the present data set it was not possible to identify the origin of the genus *Potamon*, but most likely the Aegean region was colonised by crabs coming from Asia Minor and/or the Middle East. This result is congruent to the gradient in potamid freshwater crab diversity which decreases from east to west in Asia and Europe.

In contrast to *Potamon*, land snails of the genus *Tudorella* seem to deserve their reputation as poor dispersers (further reading in Chapter 4). Considering the ecology of the snails, I assumed in all models tested by PMS that the colonisation of unoccupied areas required the existence of land bridges. I designed three different hypotheses based on Tyrrhenian micro-plate movements (Rosenbaum et. al. 2002a,b) and one model assuming that the snails colonised the western Mediterranean actively during the MSC (Krijgsman et al. 1999). The hypothesis best supported by PMS estimated that all species of the genus *Tudorella*, occurring in the western Mediterranean region at present, originated on the Iberian Peninsula and were drifted through the area by Tyrrhenian micro-plates. Their diversification and temporal splitting pattern mirrored the Tyrrhenian plate movements and vicariance seems to have been the most important factor in shaping the biogeography of this genus. As proposed for speciation caused by vicariance events, similar biogeographic patterns could be found in other poor dispersing species co-occurring in the western Mediterranean, e.g. several other snail taxa (Giusti and Manganelli 1984) and the cork oak (Magri et al. 2007). The biogeography of *Tudorella* gave no indication for speciation through active dispersal to new areas. The land snails did not expand their ranges considerably from the regions the micro-plates connected to (e.g. in the Rif and Kabylies regions) and different species occurring in close vicinity, as on Sardinia and Sicily, show no overlap in their ranges.

In summary the studies on genus level illustrate that invertebrates with poor dispersal abilities occurring in the same area do not need to have an analogue phylogeographic past. As the biogeography and dispersal potential of *Potamon* and *Tudorella* resembled at a first glance, it seemed probable that both groups had been influenced similarly by past geological and climatic events in the Mediterranean region. But PMS analyses reconstructed highly divergent expansion histories for these two study genera.

Phylogeography of Potamon fluviatile *and* Tudorella sulcata *sensu stricto*

In the studies concentrating on single species, I analysed populations covering the entire range, to answer questions about the origin of the taxa, their routes and timing of range expansion and to estimate if humans were involved as vectors for long distance dispersal.

Previous to my studies both species had already raised interest in reconstructing their dispersal history because of their disjunct distributions. In Italy the presence of freshwater crabs during Greek and Roman periods is well documented through archaeological remains (Brandis 1997). Like sheep, goats and crop plants which were spread in the course of the Neolithic expansion in Europe (Zeder 2008), *Potamon fluviatile* was assumed to have been transported to Italy in the course of Greek colonisation (Pretzmann 1987, 1995), as it likewise was intensively used for human consumption until the last century. As *Tudorella* was never used for human consumption, this species was not expected to be transported in the course of human migration and a colonisation of its range possibly during the MSC originating from Africa was proposed (Véla et al. 2008). My analyses suggested contrary that *Potamon* was the one actively expanding its range and *Tudorella* was transported by Neolithic settlers.

My studies on both species were based on a dense sampling of populations and could with this data, additionally to Bayesian model selection, date the expansion into new areas by demographic analyses. Bayesian skyline analysis (Drummond et al. 2005) works on the assumption that the colonisation of a new area (geographical expansion) is followed by a rise in the number of individuals (demographic expansion) as populations spread in the new habitat. Therefore, temporal estimation of past raise in effective population size with the help of a molecular clock can give a time frame for expansion events. After the revision of taxonomy and phylogeographical analyses on genus level, the ranges of the study species were known and could be comprehensively sampled. Molecular clocks for both species were calibrated by geological events. For *Potamon fluviatile* it was calculated by the sequence divergence between *P. fluviatile* and *P. algeriense* which probably split during the MSC (Brandis et al. 2000). For *Tudorella sulcata* s. str. sequence divergences between different species in the genus could be combined with the well dated Tyrrhenian break up to calculate the mutation rate (see Chapter 4).

The combination of PMS and demographic analyses resulted in a detailed scenario of the biogeographical history in both species:

The freshwater crab *Potamon fluviatile* has currently a disjunct distribution on the Balkans and in Italy/Malta. To estimate the route of dispersal six biological reasonable expansion models, taking into account two possible origins and three different colonisation routes, were tested (Chapter 3, Figure 4). PMS suggested that the origin of the species was on the Balkans and the route of dispersal in the region of the Strait of Otranto. Demographic analysis estimated a time frame of 10 000 – 24 000 years with highest probability at 15 000 years ago for the expansion to Italy. This time frame made over-sea transport by humans improbable as earliest seafaring in the Mediterranean was documented for 6 000 years BP (Broodbank 2006). Similar to the inference at genus level, the route and timing of expansion in this species pointed to active dispersal during times with favourable conditions. In the late

Pleistocene (around 15 000 years BP) the sea-level in the Mediterranean was considerably lower than at present (Fleming et al. 1998) and crabs could have crossed the only 20km wide sea passage by rafting or migration along the Adriatic coastline, which at that time was located in this area.

The species *Tudorella sulcata* sensu stricto is presently distributed in three regions separated by the Mediterranean Sea: Southern France, Sardinia and Algeria (Chapter 5, Figure 1). The choice of expansion models in this study was non-ambiguous as crossing the basin is the only way to connect these three areas. Hence I took each region as origin in turn with either expansion to one or the other region independently or colonisation of one region first and the second from there (stepping stone expansion). Model selection supported the latter expansion type with a Sardinian origin of the species and expansion from Sardinia to Algeria and then to France (Chapter 5, Table 2). The colonisation sequence alone made natural expansion by wind, bird migration or rafting unlikely. The median estimate in the Bayesian skyline plot analysis for the beginning of population expansion was approximately 8 000 years BP with a 95% confidence interval ranging from 10 000 to 3 000 years BP. That time frame made human transport in the course of the Neolithic expansion likely. However, the results presented in Chapter 5 have to be considered preliminary. All described studies on the phylogeography, model selection and demographic analyses were conducted solely with the mitochondrial marker COX1. As phylogeographical analyses based on a single locus are considered insufficient (Brito and Edwards 2009), the extension of this study is still in progress. After next generation sequencing of the whole *Tudorella sulcata* sensu stricto genome, I was able to design several nuclear markers (e.g. for heat shock proteins). With these additional markers I will repeat all analyses presented in Chapter 5 in a multilocus approach.

In summary an analogue trend as found at genus level was shown for the species. Freshwater crabs seem to be more mobile and made use of favourable conditions for active dispersal. *Potamon fluviatile* expanded its range to the Italian peninsula when the decrease in sea-level reduced the width of the Strait of Otranto and accordingly moved the coastline southwards. Land snails are more restricted in their dispersal and relied on vectors, in the case of *Tudorella sulcata* s. str. on human transport. For their dispersal both species have taken different routes as intuitively assumed and by reconstructing the time frame of their expansion the question of human involvement could be answered independently from archaeological remains.

Conclusions

The presented studies showed that it is impossible to reconstruct a species' expansion history from looking at its current distribution pattern alone. Just as well is it insufficient to predict a species' future movements on that basis. Every species moves individually and often counterintuitive even though they seem to have the same preconditions and dispersal opportunities. This thesis demonstrated the importance of studying biogeographical patterns with molecular and statistical methods. But one has to keep in mind that to get reasonable results, the ecology and taxonomy of the study species and the geological history of their range must be considered before proposing hypotheses. Consequently, the state of the art methods in molecular and statistical analyses will never be independent from the "old" disciplines ranging from ecology to morphology.

Perspectives

This thesis gave an overview about the taxonomy and dispersal history of the Mediterranean genera *Potamon* and *Tudorella*. Especially on taxonomy and species level new questions emerged in the course of this study.

Eleven new detected genetic lineages were delimited by molecular analyses but only *Potamon pelops* was further analysed morphologically and described as a species. To put taxonomical uncertainties to rest, all these lineages should be checked for morphological differences. For *Potamon kretaion* so far only two individuals were studied, thus for detailed molecular and morphological analyses more specimens should be obtained.

As the reproductive apparatus does not differ significantly between the cryptic species *P. pelops* and its sister species *P. fluviatile* but spermatophore morphology does, it would be interesting to test if mating between the sister species is possible. Spermatophore morphology should be evaluated as a new marker for species delimitation in potamid freshwater crabs.

As I raised species numbers for both genera considerably and some of the newly recognised species have small ranges, conservation considerations should be taken. Especially the island endemics *Tudorella melitense* on Malta, *Tudorella* nov. spec. 1 on Sardinia, *Potamon pelops* on the Peloponnesus and *Potamon kretaion* on Crete are threatened by habitat degradation and water management. Detailed genetic studies on population level would help to find hotspots of diversity as well as the origin and expansion mode of the species, necessary information to make informed conservation decisions.

After species delimitation it became clear that several species of both genera have disjunct distribution ranges and would therefore be interesting candidates for further analyses at species level. In the genus *Potamon* the species *P. rhodium, P. potamios* and *P. hippocrate* showed a fragmented distribution. *P. rhodium* and *P. hippocrate* are present on Aegean islands as well as the surrounding mainland, *P. potamios* along the same coastline but in fragmented, far distanced regions. In the genus *Tudorella* the two species *T. mauretanica,* present at the coasts of Southern Spain and Northern Africa, and *T. multisulcata,* distributed in Tunisia and Sicily, are of special interest. As I pointed out in the studies on *P. fluviatile* and *T. sulcata* s. str., the expansion history of each species is unique and these species could give additional insight on dispersal strategies in the two invertebrate genera *Potamon* and *Tudorella*.

In this thesis the focus was on reconstructing the past of two organism groups to find out the origin, route and time frame of expansion in their area of distribution. But the knowledge of a species' past dispersal mechanisms can help to predict their expansion in the future. The statistical framework presented here will be of use in studies modelling species ranges in the context of climate change (like in Cordellier and Pfenninger 2009) or can be helpful in the risk assessment and control of invasive species (Estoup and Guillemaud 2010).

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Deutsche Zusammenfassung (German summary)

Arten, die nur begrenzt ausbreitungsfähig sind, aber trotzdem ein großes Verbreitungsgebiet, unterbrochen von geographischen Barrieren (z.B. Meere, Berge oder Wüsten), besiedelt haben, stellen für biogeographische Studien besondere Herausforderungen dar.

Die Rekonstruktion der Verbreitungsgeschichte einer solchen Art kann durch einen unzureichenden Datensatz oder taxonomische Unklarheiten zusätzlich erschwert werden. Oft ist es der Fall, dass sich Arten von denen angenommen wurde, dass sie ein großes, fragmentiertes Gebiet besiedeln, als ein kryptischer Artkomplex herausstellen. Da kryptische Arten per Definition morphologisch schwer zu identifizieren sind, sind molekulare Analysen notwendig, um die Phylogenie zweifelsfrei aufzuklären. Diese ist dann Voraussetzung für alle weiteren Analysen zur Rekonstruktion von Route und Zeitverlauf vergangener Ausbreitungsereignisse. In der Phylogenie einer Art mit einem großen, unterteilten Verbreitungsgebiet können zwei Extreme auftreten:

1) Wenn kaum genetische Unterschiede zwischen Individuen aus weit entfernten Populationen auftreten, ist es wahrscheinlich, dass die einzelnen Gebiete in genetischem Austausch stehen. Eine Möglichkeit, um über geographische Barrieren und große Distanzen hinweg genetischen Kontakt aufrecht zu erhalten, ist passiver Transport. Vektoren hierfür können Wind, Wasserströmungen, Tiere oder auch Menschen sein. Viele im Süßwasser lebende Wirbellose sind dafür bekannt, sich mit Hilfe von Insekten, Amphibien, Reptilien und Wasservögeln oder angeheftet an Boote auszubreiten. Diese Beispiele verdeutlichen, dass Kenntnisse über die Ökologie einer Art, ihres Lebensraums und ihrer Interaktionen mit anderen Arten Voraussetzung für qualifizierte Annahmen über mögliche Verbreitungswege sind.

2) Wenn es große genetische Distanzen zwischen Populationen einer Art gibt, kann dieses biogeographische Muster die geologische Geschichte der Region widerspiegeln. Geologische und klimatische Ereignisse, wie zum Beispiel Plattentektonik oder die Eiszeiten, können Barrieren für Migration hervorbringen und genetische Unterschiede in isolierten Populationen sammeln sich an. Genaue Kenntnisse über die Geologie und das Klima des

Verbreitungsgebietes sind daher ebenso essentiell wie das Wissen über die Tiere selbst, um die Geschichte einer Art zu rekonstruieren.

Es wird oft diskutiert, ob Vikarianz oder Migration die biogeographischen Muster von Arten mit geringem Ausbreitungspotential mehr beeinflussen. Die Vikarianztheorie geht davon aus, dass ursprüngliche Taxa weitverbreitet waren und Artbildung durch Fragmentierung des ursprünglichen Gebietes verursacht wurde. Im Gegensatz dazu besagt die Ausbreitungstheorie, dass es Ursprungsgebiete gab, von denen aus Arten sich verbreitet haben, danach isoliert wurden und so getrennt evolviert sind.

Der Mittelmeerraum ist, aufgrund seiner komplexen geologischen Geschichte und hoher Biodiversitäts- und Endemismusraten, eine Modellregion für biogeographische Studien. In dem Gebiet kam es in der geologischen und klimatischen Geschichte der letzten 30 Millionen Jahre zu mehreren Ereignissen, welche die Artbildung von Tieren und Pflanzen, durch Vikarianz oder durch die Möglichkeit zur Ausbreitung, verursacht haben könnten. Besonders einschneidende Vikarianzereignisse waren zum Beispiel die Fragmentierung der Tyrrhenischen Platte und der Ägäischen Inseln sowie die Bildung des Mittelägäischen Grabens. Zusätzlich kam es in der geologischen Geschichte der mediterranen Region auch zu Ereignissen, welche die Ausbreitung von terrestrischen und limnischen Arten begünstigt haben, wie die Messinische Krise oder die eiszeitlichen Meeresspiegelschwankungen. Wie schon beschrieben, stellt passiver Transport eine besonders gute Möglichkeit für Invertebraten dar, weit entfernte Regionen zu erreichen. Die unabsichtliche Verschleppung von Arten über menschliche Transportwege ist derzeit die häufigste Art von passivem Transport in den dicht besiedelten Gebieten des Mittelmeerraums und begann schon vor etwa 10 000 Jahren während der neolithischen Expansion.

In der vorliegenden Arbeit konzentrierte ich mich auf mediterrane wirbellose Tierarten, welche sich als Konsequenz ihrer Lebensweise nur schlecht ausbreiten können. Nichtsdestotrotz haben es Süßwasserkrabben der Gattung *Potamon* und Landschnecken der Gattung *Tudorella* geschafft, große Gebiete zu besiedeln, die heute durch das Mittelmeer getrennt sind. Für beide Gruppen wurde spekuliert, dass Menschen an ihrer Ausbreitung beteiligt waren. Es war mein Ziel die biogeographischen Muster dieser beiden Gattungen zu analysieren und abzuschätzen, ob Menschen tatsächlich Vektoren ihrer Ausbreitung waren. Meine Analysen fanden auf drei Ebenen statt: Taxonomie, Gattung und Art.

1) Als Voraussetzung für alle weiteren Analysen, erstellte ich einen Stammbaum durch Sequenzierung molekularer Marker, um evolutionäre Linien abzugrenzen. Die Fragestellungen auf taxonomischer Ebene waren:

- Sind die morphologisch beschriebenen Arten kongruent zu genetischen Linien?
- Kann kryptische Artbildung in den untersuchten Gattungen nachgewiesen werden?

2) Ich untersuchte die Biogeographie auf Gattungsebene indem ich alle Arten, die im Mittelmeerraum vorkommen, analysierte. Auf Gattungsebene war es mein Ziel, folgende Fragen zu beantworten:

- Spiegelt sich im Artverbreitungs- und zeitlichen Aufspaltungsmuster die geologische und klimatische Geschichte der Region wider?
- Wo liegt der Ursprung der mediterranen Arten?
- Waren Vikarianz- oder Ausbreitungsereignisse die maßgeblichen Faktoren, die die heutige Biogeographie beeinflusst haben?

3) Ich rekonstruierte die Phylogeographie einzelner Arten im Detail, indem ich die genetische Populationsstruktur untersuchte. Folgende Fragen stellte ich hierbei:

- Wo hat die Art ihren Ursprung und wie hat sie sich verbreitet?
- Wann hat diese Expansion stattgefunden?
- Waren Menschen an der Ausbreitung über große Entfernungen beteiligt?

Die ersten drei Kapitel beschäftigen sich mit Süßwasserkrabben der Gattung *Potamon*.

In Kapitel 1 wird die Biogeographie der Gattung *Potamon* im Mittelmeerraum beschrieben. In einem DNA-basierten Ansatz untersuchte ich die Taxonomie aller Arten der Region mit zwei mitochondrialen (COX1 und ND1) und einem nukleären Marker (28S) und konnte 14 Linien (davon vier bisher unbeschrieben) identifizieren. Nach der Artabgrenzung rekonstruierte ich die Phylogenie und das zeitliche Muster der Artaufspaltung. In der Gattung teilen sich die Arten in eine westliche und eine östlich Gruppe auf. Diese Aufspaltung fand etwa vor 8,3 bis

5,5 Millionen Jahren statt, in einem Zeitraum in dem sich das mediterrane Klima in Richtung erhöhter Trockenheit und Saisonalität veränderte. Des Weiteren testete ich verschiedene Hypothesen zur Aufspaltungen der Arten in der Phylogenie mit *Phylogenetic Model Selection* (PMS). Die meisten Arten entstanden im Pleistozän, zeitgleich mit den eiszeitlichen Meeresspiegel- und Temperaturschwankungen. Durch eine phylogenetische Arealanalyse habe ich verschiedene Modelle getestet, um abzuschätzen ob die Ägäis als Ursprung oder Sammelbecken für die heutigen europäischen Süßwasserkrabben fungierte. Das Szenario einer Ausbreitung aus Kleinasien in den ägäischen Raum und weiter nach Afrika stellte sich als am wahrscheinlichsten heraus. Die Ägäis scheint somit Sammelbecken der heutigen Süßwasserkrabbendiversität zu sein und der Ursprung der Gattung lag vermutlich in Kleinasien oder dem Mittleren Osten.

In Kapitel 2 wird die kryptische Art *Potamon pelops*, welche im Verlauf einer früheren Studie über *Potamon fluviatile* (Herbst 1785) entdeckt wurde, beschrieben. *Potamon pelops* kommt isoliert auf dem Peloponnes vor und entwickelte sich wahrscheinlich durch allopatrische Artbildung, als vor 11,8 bis 3,4 Millionen Jahren der Golf von Korinth entstand. Durch genetische Analysen zweier mitochondrialer und eines nukleären Markers und zusätzliche morphologische Untersuchungen des Reproduktionsapparates und der Spermatophoren, konnte die Art taxonomisch eingeordnet werden. Morphologisch unterscheidet sich *P. pelops* nicht signifikant von *P. fluviatile*, erst bei der Untersuchung der Spermatophoren wurden Unterschiede sichtbar: in der Art *P. fluviatile* treten nur Cleistospermien auf, während bei *P. pelops* Coeno- und Cleistospermien nachgewiesen werden konnten.

Kapitel 3 beschäftigt sich mit der Phylogeographie der Art *Potamon fluviatile*. Diese Art ist in Italien und auf Malta sowie auf dem Balkan in Serbien, Albanien und Griechenland verbreitet. Durch die Untersuchung von Individuen aus dem ganzen Verbreitungsgebiet mit zwei mitochondrialen Markern (COX1 und ND1), rekonstruierte ich die Phylogenie der Art. Diese Informationen konnte ich für einen PMS-Ansatz nutzen, um die wahrscheinlichste Ausbreitungsroute zwischen den getrennten Gebieten nachzuvollziehen. Ich testete sechs verschiedene Modelle gegeneinander und das Modell mit Ursprung auf dem Balkan und Ausbreitung in der Region der heutigen Strasse von Otranto, erhielt die größte statistische Unterstützung. In einer *Bayes'schen Skyline Plot Analyse* rekonstruierte ich das

Populationswachstum von *Potamon fluviatile* und konnte die Expansion auf einen Zeitraum vor etwa 15 000 Jahren festlegen. Während dieser Zeit, nach dem letzten eiszeitlichen Maximum, war der Meeresspiegel in der Adria noch erheblich niedriger als heute, die Straße von Otranto nur 20 km breit und die Küste verlief nur wenig nördlich davon. Das waren günstige Vorrausetzungen für die Krabben, um Italien zu erreichen.

Die letzten beiden Kapitel befassen sich mit terrestrischen Schnecken der Gattung *Tudorella*.

In Kapitel 4 beschreibe ich die Taxonomie der Gattung *Tudorella* basierend auf der Analyse zweier mitochondrialer (COX1, 16S) und eines nukleären Markers (ITS-1). Die Identifizierung der einzelnen Arten war Voraussetzung für alle weiteren Untersuchungen zur Biogeographie der Gattung. Ursprünglich waren zwei Arten in der Gattung bekannt, durch meine genetischen Analysen konnten acht Linien identifiziert werden. Durch PMS testete ich, ob die Schnecken sich während der Messinischen Krise aktiv ausbreiteten oder passiv durch die Fragmentierung und Verdriftung der tyrrhenischen Mikroplatten. Die beste Unterstützung erlangte ein Szenario mit Artbildung durch Vikarianz, resultierend aus wiederholter Fragmentierung der tyrrhenischen Platten mit darauf folgender Ausbreitung über Landbrücken im Pliozän.

Kapitel 5 beschreibt die Phylogeographie der Art *Tudorella sulcata sensu stricto*, welche an den Küsten Südfrankreichs, Sardiniens und Algeriens beheimatet ist. Durch Analyse von Populationen aus allen drei Regionen mit dem mitochondrialen Marker COX1 erstellte ich die Phylogenie der Art. Ich testete neun verschiedene Ausbreitungsmodelle durch PMS, um den Ursprung und die Reihenfolge der Ausbreitung nachzuvollziehen. Die Schnecken scheinen sich ausgehend von Sardinien erst nach Algerien und von dort nach Südfrankreich ausgebreitet zu haben. Durch diese Expansionsfolge ist eine Ausbreitung durch natürliche Vektoren wie Wind, Strömungen und Zugvögel sehr unwahrscheinlich. Der Zeitpunkt der Migration konnte durch eine demographische Analyse auf vor etwa 8 000 Jahren abgeschätzt werden und fällt somit in die Epoche der neolithischen Expansion in Europa. Somit spielte passiver Transport durch den Menschen in der Verbreitungsgeschichte dieser Schnecken wahrscheinlich eine entscheidende Rolle.

Als Resultat meiner taxonomischen Untersuchungen schlage ich vor, dass alle genetischen Linien beschrieben in den Kapiteln 1 bis 5 als eigenständige Arten behandelt werden. Sie erfüllen alle Kriterien, die in den beiden analytischen Ansätzen zur Artidentifizierung, dem „Taxonomischen Kreis" von DeSalle *et al.* (2005) und dem „Einheitlichen Artkonzept" von De Queiroz (2007) vorgestellt wurden. Alle genetischen Gruppen aus meinen Untersuchungen repräsentieren eigenständig evolvierende Linien, da sie monophyletisch sind und eine tiefe genetische Separierung aufweisen. Ihre Verbreitungsgebiete überschneiden sich nicht und somit ist Genfluss zwischen den Linien unwahrscheinlich.

Auf den ersten Blick wirken die Biogeographien der Gattungen *Potamon* und *Tudorella* sehr ähnlich und da die Organismengruppen etwa gleiche Ausbreitungsfähigkeiten besitzen, würde man auch ähnliche Besiedelungsgeschichten erwarten. Meine Untersuchungen auf Gattungs- und Artebene zeigten allerdings, dass sie sehr unterschiedliche Wege zur Ausbreitung nutzten. Süßwasserkrabben stellten sich als die mobilere Gruppe heraus und nutzten günstige Umstände für ihre aktive Ausbreitung. Landschnecken scheinen eingeschränkter in ihren Ausbreitungsmöglichkeiten zu sein und wurden passiv mit Hilfe von Vektoren, zum Beispiel schwimmende Mikroplatten oder der Mensch, verbreitet.

Als Fazit dieser Dissertation kann ich festhalten, dass es unmöglich ist, die Ausbreitungsgeschichte einer Art durch bloße Betrachtung der aktuellen Biogeographie zu rekonstruieren. Arten breiten sich individuell und unvorhersehbar aus, auch wenn es für den Betrachter den Anschein hat, dass ihre Ausbreitung denselben Voraussetzungen unterliegt. Die vorliegende Arbeit hat verdeutlicht, wie wichtig es ist, statistische Analysen zur Untersuchung von Biogeographien heranzuziehen. Aber dabei sollte man immer bedenken, dass man, um verlässliche und biologisch vernünftige Resultate zu erzielen, die Ökologie und Taxonomie der Studienobjekte sowie die geologische Geschichte des Gebietes mit einbeziehen muss.

Curriculum Vitae

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Employment

10/2010 – 01/2011	Research associate, **Biodiversity and Climate Research Centre** (BiK-F), Frankfurt am Main
10/2006 – 09/2010	Research associate, **Goethe University Frankfurt am Main**, Department of Ecology and Evolution

Education

Since 10/2006	**PhD** student at the **Department of Ecology and Evolution**, Goethe University Frankfurt am Main **Prof. Dr. Bruno Streit** *Natural or human mediated – Biogeography of widespread Mediterranean invertebrates with poor dispersal capacities*
09/2005 – 09/2006	**Diploma thesis** **University of Regensburg**, Institute of Zoology **Prof. Dr. Jürgen Heinze** *Population Genetics of the Endangered Freshwater Crab* Potamon fluviatile *in Italy*
10/2001 – 09/2006	**University of Regensburg** Study of Biology with the main subjects: Zoology, Genetics and Medical Microbiology

Publications

Jesse et al. (2010) Evolution of freshwater crab diversity in the Aegean region (Crustacea: Brachyura: Potamidae). *Molecular Phylogenetics and Evolution*, in press

Kuch, **Jesse** et al. (2010) Klimawandel und Krankheiten – Welche Rolle spielen neue Erreger und Überträger? *Natur und Museum* **140**, 238-241

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Jesse et al. (2009) Disjunct distribution of the Mediterranean freshwater crab *Potamon fluviatile* – natural expansion or human introduction? *Biological Invasions* **11**, 2209-2221

Conference presentations

04/2010	Graduiertentreffen der Deutschen Zoologischen Gesellschaft, Studiengruppe Evolutionsbiologie, Freiburg, Germany Oral presentation (honourable mention talk, third prize)
10/2009	World Conference on Biological Invasions and Ecosystem Functioning, Porto, Portugal; Oral presentation
11/2008	International Symposium: The Asian Tigermosquito „*Stegomyia albopicta*“ – Monitoring and modelling of its distribution area in relation to its bionomy and climatic factors, Speyer, Germany
09/2007	Tagung der Deutschen Gesellschaft für Limnologie, Münster, Germany Poster presentation
08/2007	13th Annual Meeting of PhD Students in Evolutionary Biology, Lund, Sweden; Oral presentation
03/2007	Tagung deutschsprachiger Crustaceologen, Senckenbergmuseum, Frankfurt/Main, Germany; Oral presentation
02/2007	Graduiertentreffen der Deutschen Zoologischen Gesellschaft, Fachgruppe Evolutionsbiologie, Bayreuth, Germany Poster presentation (second prize)
02/2006	Graduiertentreffen der Deutschen Zoologischen Gesellschaft, Studiengruppe Evolutionsbiologie, Potsdam, Germany Oral presentation

Workshops

02/2010	Achieving goals – How to stay focused on important issues, GRADE
07/2009	Scientific English Writing, GRADE
09/2008	Introduction in the statistic software "R", Graz, Austria
07/2008	Summer Short Course Statistics – Theory and Practice, GRADE
07/2008	Conference Presentation and Communication, GRADE

Teaching experience

SS 2007 – 2008	Systematik & Ökologie – Zoologische Bestimmungsübungen (6SWS), Diversität der Organismen und Lebensräume (6SWS)
WS 2006 – 2008	Ökologie & Evolution der Tiere (6SWS)

International and Research Experience

01/2006 – 02/2006	Practical course in the laboratory of the Dipartimento di Biologia Animale e Genetica, University of Florence, Italy
10/2000 – 04/2001	Au-pair in Sydney, Australia

Grants

GRADE –Goethe Graduate Academy (2009), 643€ travel grant to participate in the BIOLIEF conference in Porto, Portugal

VFF (Vereinigung von Freunden und Förderern der Johann Wolfgang Goethe-Universität) (2007) 3396€ travel grant for a field trip to the Peloponnesus, Greece

Hermann Willkomm-Stiftung (2007-2009): in all 1222€ travel grants for conferences and sampling trips

Languages

German: first language

English: fluent

Frankfurt am Main, 31.01.2011

Erklärung

Ich erkläre hiermit, dass ich mich bisher keiner Doktorprüfung unterzogen habe.

Frankfurt am Main, den 31.01.2011

……………………………….
(Unterschrift)

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorgelegte Dissertation über

Natural or human mediated – Biogeography of widespread Mediterranean invertebrates with poor dispersal capacities

selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass alle Entlehnungen aus anderen Schriften mit Angabe der betreffenden Schrift gekennzeichnet sind.

Ich versichere, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Frankfurt am Main, den 31.01.2011

………………………… (Unterschrift)

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