University of Cyprus

DEPARTMENT OF BIOLOGICAL SCIENCES

RECONSTRUCTING THE EVOLUTIONARY HISTORY OF TERRESTRIAL ISOPODS (ISOPODA: ONISCIDEA) AT DIFFERENT PHYLOGENETIC LEVELS

DOCTOR OF PHILOSOPHY DISSERTATION

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Reconstructing the evolutionary history of terrestrial isopods (Isopoda: Oniscidea) at different phylogenetic levels

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The present doctoral dissertation was submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy of the University of Cyprus. It is a product of original work of my own, unless otherwise mentioned through references, notes, or any other statements.

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ΠΕΡΙΛΗΨΗ

Τα χερσαία ισόποδα (Oniscidea) παρουσιάζουν ορισμένες ιδιότητες που τα καθιστούν μοναδικά στην παγκόσμια βιοποικιλότητα, προσφέροντας ερευνητικές ευκαιρίες σε πολλά βιολογικά πεδία, όπως η εξέλιξη, η οικολογία, η οικοτοξικολογία, η βιοακουστική και η οικοφυσιολογία. Με περισσότερα από 3700 περιγεγραμμένα είδη, τα Oniscidea είναι η μεγαλύτερη και ταυτόχρονα η μόνη χερσαία υπόταξη ισοπόδων. Η μετάβαση από τη θάλασσα στην ξηρά πιθανότατα έλαβε χώρα κατά τη διάρκεια της Λιθανθρακοφόρου περιόδου, ~300 Mya. Τα χερσαία ισόποδα είναι οι πιο επιτυχημένοι αποικιστές του χερσαίου περιβάλλοντος εντός των Καρκινοειδών, παρουσιάζοντας μια σειρά από ποικίλες μορφολογικές, οικολογικές και συμπεριφορικές προσαρμογές που τους επέτρεψαν να κατακτήσουν τη χέρσο. Είναι αξιοσημείωτο το γεγονός ότι τα αρτίγονα είδη αντιπροσωπεύουν σχεδόν όλα τα εξελικτικά βήματα που τους έδωσαν τη δυνατότητα να καταλάβουν τη μεγάλη πλειονότητα των χερσαίων οικοτόπων, συμπεριλαμβανομένων των πολύ αφιλόξενων περιβαλλόντων της ερήμου, των λιμνοθαλασσών και των υπόγειων γλυκών υδάτων, όπου επέστρεψαν δευτερογενώς. Η παρουσία των χερσαίων ισοπόδων εκτείνεται από το επίπεδο της θάλασσας μέχρι και σε πολύ υψηλά υψόμετρα (>4800 m), εκτός από τις πολικές περιοχές.

Παρά τη συνεχή συσσώρευση γενετικών και μορφολογικών δεδομένων, δεν έχουν ακόμα επιλυθεί οι φυλογενετικές σχέσεις μέσα στην ομάδα των Oniscidea ή μεταξύ των υποτάξεων των Ισοπόδων. Στο πλαίσιο αυτής της διατριβής διερευνήθηκε η εξελικτική δυναμική αυτών των ομάδων σε διαφορετικά ταξινομικά επίπεδα. Πιο συγκεκριμένα, εξετάστηκαν οι φυλογενετικές σχέσεις στα ακόλουθα επίπεδα: i) βαθιά φυλογένεση - διερεύνηση σχέσεων μεταξύ των πέντε κύριων κλάδων Oniscidea και στενά συγγενών υδρόβιων υποτάξεων Ισοπόδων, ii) επίπεδο οικογένειας/γένους - διερεύνηση εξελικτικών σχέσεων ανάμεσα στις κύριες οικογένειες των Crinocheta και των γενών σε μια από τις πλουσιότερες σε αριθμό ειδών οικογένειες (Porcellionidae), και iii) είδους/πληθυσμών - διερεύνηση γενετικής ποικιλότητας εντός του είδους *Armadillo officinalis*, καλύπτοντας μεγάλο μέρος της εξάπλωσής του, εστιάζοντας ωστόσο στην ενδο-νησιωτική διαφοροποίηση εντός της Κύπρου.

Με στόχο τη διερεύνηση της εξελικτικής ιστορίας σε αυτή την πολυεπίπεδη προσέγγιση, εφαρμόστηκε η αλληλούχηση κατά Sanger που στοχεύει μια σειρά μιτοχονδριακών (12s, 16s, COI, Cytb) και πυρηνικών (18s, 28s, NAK, PEPCK) γενετικών δεικτών, καθώς και μοναδιαίων

νουκλεοτιδικών πολυμορφισμών (SNP) σε ολόκληρο το γονιδίωμα των οργανισμών, εφαρμόζοντας το πρωτόκολλο ddRADseq.

Στο επίπεδο βαθιάς φυλογένεσης, τα αποτελέσματά μας υπονομεύουν την ευρέως αποδεκτή μονοφυλετικότητα των Oniscidea. Πιο συγκεκριμένα, το αμφίβιο γένος *Ligia* φαίνεται να είναι εξελικτικά πιο συγγενικό με υδρόβια ισόποδα από ό,τι με τα υπόλοιπα χερσαία ισόποδα που συμπεριλήφθηκαν στην ανάλυση αυτή. Λαμβάνοντας υπόψη τα στοιχεία που παρατίθενται και αμφισβητούν την κοινή καταγωγή των Ligiidae, θα μπορούσε να υποστηριχθεί ότι το γένος *Ligidium,* όπως και τα στενά συγγενικά του *Tauroligidium* και *Typhloligidium,* ανήκουν σε μια νέα οικογένεια, την Ligidiidae, που διακλαδίζεται στη βάση του φυλογενετικού δέντρου των Oniscidea. Το παραγόμενο φυλογενετικό μοτίβο των υπόλοιπων χερσαίων ταξινομικών κατηγοριών αντανακλά τη σύνθετη εξελικτική ιστορία της ομάδας όσον αφορά τη μετάβαση από το θαλάσσιο στο χερσαίο περιβάλλον.

Οι φυλογενετικές αναλύσεις σε επίπεδο οικογένειας/γένους αμφισβήτησαν τόσο τη μονοφυλετικότητα της οικογένειας Porcellionidae όσο και του γένους *Porcellio* που συγκαταλέγεται στα πλουσιότερα σε αριθμό ειδών γένη της οικογένειας. Σύμφωνα με την κλαδοχρονολόγιση, αν εξαιρεθούν τα γένη *Leptotrichus* και *Brevurus* που δεν ομαδοποιούνται με τα υπόλοιπα μέλη της οικογένειας, η μονοφυλετική πλέον οικογένεια Porcellionidae φαίνεται να έχει αφρικανική προέλευση που χρονολογείται από το Ολιγόκαινο (~32 Mya).

Εστιάζοντας σε επίπεδο είδους/πληθυσμού, στην περίπτωση του *A. officinalis* αποκαλύφθηκε υψηλή γενετική διαφοροποίηση που δεν υποδεικνύεται από οποιοδήποτε μορφολογικό χαρακτηριστικό από όσα εξετάσθηκαν μέχρι σήμερα. Αποκαλύφθηκαν πέντε κρυπτικοί γενετικοί κλάδοι στην Κύπρο. Η κλαδοχρονολόγηση δείχνει ότι η άφιξη του είδους στο νησί συνέβη περίπου 6 εκατομμύρια χρόνια πριν, πιθανώς υποβοηθούμενη από την Κρίση Αλατότητας του Μεσσηνίου. Επιπλέον, τα αποτελέσματα υπογραμμίζουν τον ρόλο της παλαιογεωγραφίας και της ανθρώπινης παρουσίας στα υπάρχοντα πρότυπα γενετικής ποικιλότητας.

Τα αποτελέσματα που παρουσιάζονται αναδεικνύουν την ανάγκη για επαναξιολόγηση των μορφολογικών χαρακτήρων που χρησιμοποιούνται στην ταξινόμηση των χερσαίων ισοπόδων, λαμβάνοντας υπόψη τα συσσωρευόμενα γενετικά δεδομένα. Έτσι θα οδηγηθούμε σε πιο αξιόπιστες και περιεκτικές φυλογενέσεις που θα μας επιτρέψουν να περιγράψουμε την

περίπλοκη εξελικτική ιστορία αυτής της μοναδικής ομάδας που κατάφερε με επιτυχία να κατακτήσει τη χέρσο.

ABSTRACT

Terrestrial isopods (Oniscidea) exhibit some unique properties that make them an excellent case within global biodiversity, offering research opportunities on many biological fields, such as evolution and ecology, but also ecotoxicology, bioacoustics and ecophysiology. With more than 3,700 described species, Oniscidea is the largest and at the same time the only terrestrial isopod suborder. The sea-land transition most probably took place during the Carboniferous, ~300 Mya. Terrestrial isopods are the most successful colonizers of the terrestrial realm among Crustacea, exhibiting a series of diverse morphological, ecological, and behavioural adaptations that allowed them to conquer land. Remarkably, extant species represent almost all evolutionary steps that allowed them to occupy the whole range of terrestrial habitat types, with a few exceptions, including the very harsh desert environments, salt lakes, and subterranean freshwaters, where they have secondarily returned. Oniscidea presence extends from the sea level to very high elevations (>4800 m), excluding polar regions.

Despite the constantly accumulating genetic and morphological data, we still lack a comprehensive and robust phylogeny of Isopoda suborders, hence also of Oniscidea. Within the framework of this thesis, the evolutionary dynamics of this group were examined at different taxonomic levels. More precisely, phylogenetic relationships were examined at the following levels: i) deep phylogeny - relationships among the five main Oniscidea clades and with closely related aquatic Isopoda suborders, ii) family/genus level - relationships among some of the major Crinocheta families and among genera of one of the species-richest families (Porcellionidae), and iii) species/population - among *Armadillo officinalis* species populations, covering a large part of its distribution but focusing mostly on intra-insular divergence within Cyprus.

Aiming to reconstruct the evolutionary history along such a multilevel approach, a series of mitochondrial (12s,16s, COI, Cytb) and nuclear (18s, 28s, NAK, PEPCK) markers, as well as genome-wide SNPs were retrieved, employing Sanger and ddRADseq sequencing, respectively.

At the deep phylogeny level, our results undermine the widely accepted monophyly of Oniscidea. More specifically, the amphibious genus *Ligia* appears to be evolutionary more closely related to aquatic isopod taxa than the rest of the terrestrial isopods included in the analysis. Considering the evidence against the monophyly of Ligiidae presented herein, we

suggest the assignment of *Ligidium* and of the closely related genera *Tauroligidium* and *Typhloligidium* to a new family, Ligidiidae, that possesses a basal position within Oniscidea phylogeny. The produced phylogenetic pattern of the remaining terrestrial taxa reflects the complex evolutionary history of the group in view of the transition from the marine to the terrestrial realm.

Time-calibrated phylogenetic analyses at family/genus level questioned both the monophyly of Porcellionidae and *Porcellio,* one of the family's richest genera. Excluding the genera *Leptotrichus* and *Brevurus* that are not grouped with the rest of family members, the now monophyletic Porcellionidae seems to have an African origin that dates back to the Oligocene (~32 Mya).

Focusing on species/population level, in the case of *A. officinalis* we found high genetic divergence, not suggested by any of the morphological traits examined so far. The presence of five cryptic genetic lineages on Cyprus was revealed. Cladochronological dating indicates that the species arrival on the island occurred ~6 Mya, probably facilitated by the Messinian Salinity Crisis. Furthermore, results highlight the role of paleogeographic history and of human presence in shaping patterns of genetic diversity.

Our results call for a re-evaluation of morphological characters traditionally used in terrestrial isopod taxonomy, under the light of the constantly accumulating genetic data. This way, we can attain more robust and comprehensive phylogenies which will allow us to describe the complex evolutionary history of this unique group that managed to conquer land.

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CHAPTER 1

Overview

Overview

Taxonomic diversity and phylogenetic relationships within Isopoda

Comprised by more than 10,300 species Isopoda is the largest Peracarida order exhibiting a variety of remarkable morphological and ecological adaptations that allowed them to conquer almost all biomes around the globe, as they can be found in oceans, terrestrial and freshwater habitats (Wilson, 2007, 2009). Based on different morphological characters, alternative evolutionary relationships have been proposed among the eight "main" Isopoda lineages proposed by Wägele (1989), indicating the ambiguous relationships within the group. The phylogenetic placement of Oniscidea within Isopoda has been repeatedly revised, suggesting either a sister group relationship with Calabozoidea or with Asellota, or with Valvifera+Sphaeromatidea+Anthuridea+Cymothoida, indicating either a basal or an apical position within Isopoda (Brusca and Wilson, 1991; Schmalfuss, 1989; Schmidt, 2008; Wägele, 1989).

Among the scarce Crustacea groups that managed to conquer terrestrial habitats, the vast majority of taxa that are independent from the aquatic environment, are found in Oniscidea. From an ecological point of view, terrestrial isopods, largely feeding on plant decaying material, are considered to be one of the most important detritivore soil invertebrates in certain habitat types where they occur in high population densities (Lavelle, 1997). Oniscidea have a key role as ecosystem engineers especially in arid regions of North Africa and Asia, and in tropical and temperate ecosystems where they are an important element of the decomposer communities, acting as litter transformers with a vital role in soil food webs along with fungi and bacteria (David and Handa, 2010; Sfenthourakis and Hornung, 2018; Shachak and Yair, 1984).

According to the most updated published list, Oniscidea includes 3,710 species, 527 genera and 37 families, and is the only terrestrial and the largest of the 11 currently identified Isopoda suborders (Hornung, 2011; Schmalfuss, 2003; Schotte, 1995; Sfenthourakis and Taiti, 2015). Regions around the Mediterranean Sea are the globally species richest in Oniscidea, hosting a considerable number of endemic species (Sfenthourakis and Hornung, 2018). According to estimations that take into account: i) a number of species hosted in subterranean habitats and caves that remain to be sampled and described, and ii) the use of molecular taxonomic tools

which are anticipated to reveal cryptic species, the total number of Oniscidea might be raised up to 7,000 (Sfenthourakis and Taiti, 2015).

Why are terrestrial isopods a special case to study?

The unique character of the group is highlighted by the fact that it is probably the only low-rank taxon that includes living representatives of almost all stages of the invasion into land from the marine realm (i.e., they include amphibious, littoral, supra-littoral, extremely hygrophilous, riparian, mesophilous, xerophilous, and even desert-living species, including lots of cave-dwelling ones). Excluding extremely high elevations (>4800m) and polar regions, isopods are found in a wide and extremely diverse range of terrestrial habitats (Beron, 1997; Hornung, 2011; Schmidt, 2008; Sfenthourakis and Taiti, 2015). Furthermore, the group also consists of amphibious, even aquatic species, found in salt lakes or subterranean freshwaters, which have originated from terrestrial ancestors (Tabacaru, 1999; Taiti and Xue, 2012).

The invasion to land became feasible after the evolution of some unique innovative morphological and physiological adaptations that allowed Oniscidea to leave the marine environments. These autapomorphies include: i) the water conducting system that plays a role in thermoregulation, in respiration by keeping wet the epithelial lung cells, and in excretion, ii) the development of pleopodal lungs, necessary for gas exchange in terrestrial environments, and iii) the cotyledons of the marsupium, that provide water, oxygen, and nutrients to the eggs, offering the ability to develop a closed brood-pouch (Hoese, 1981, 1982; Hoese and Janssen, 1989; Hornung, 2011). Further morphological changes compared to marine taxa include the reduced body size, the water resistant cuticle and the development of diverse surface structures (Bursell, 1955; Holdich, 1984; Schmalfuss, 1978a). Beyond these features, Oniscidea also exhibit a wide range of ecomorphological and behavioural characteristics related to habitat selection, foraging and drought resistance, which are compliant with the terrestrial environment (Hornung, 2011).

Considering the fact that they have developed unique adaptations to land, parallel to those of evolutionary more recent and more complex groups (such as egg-feeding apparatus in the marsupium or closed pleopodal lungs, features analogous to the placenta and the vertebrate lungs, respectively), make the resolution of their phylogeny an exciting task that can shed light to many important evolutionary, functional and ecological questions.

Alternative terrestrialization scenarios and Oniscidea taxonomy

Based on the fossil record and available phylogenetic studies, experts date the origin of Oniscidea in the Carboniferous (~300 Mya; Broly et al., 2013). According to the predominant scenario, a single invasion to land took place in the past, since the monophyly of Oniscidea is well supported by numerus described synapomorphies (Erhard, 1995, 1996, 1997; Schmalfuss, 1989; Schmidt, 2008; Tabacaru and Danielopol, 1996). Among the most "convincing" Oniscidea autapomorphies are: 1) the water conducting system, 2) the very short (not reduced in length) pleotelson, slightly longer than one pleon segment, 3) an antennula with less than four articles, 4) the absence of the mandibular palp, 5) two groups of setae on the mandible, one growing on the *lacinia mobilis*, 6) the presence of only one moveable sclerite on the basis of the second maxilla, 7) a single coxal sclerite on the maxilliped, 8) non-subchelate first pereopod, 9) sexually-dimorphic first pleopod pair, and 10) the occurrence of scale-setae on tergites (Schmidt, 2008).

Regarding within-group classification, terrestrial isopods are divided into five major clades (Figure 1). The more basal ones include species with the most 'primitive' characters regarding terrestrial life, whilst the more apical clades include species that exhibit the most advanced adaptations regarding drought resistance and hence the ability to live totally independent from the aquatic environment (Erhard, 1996). It is worth noticing that (with some exceptions) representatives of the first two more basal clades, Diplochaeta and Tylida, include amphibious species living at the littoral zone (Santamaria et al., 2013; Schmalfuss, 1978b; Schmidt, 2008). On the other hand, the richest and more derived clade, Crinocheta, exhibits the most complex adaptations to the terrestrial environment, including species living in arid areas, even deserts (Schmalfuss, 1998; Sfenthourakis and Hornung, 2018). Crinocheta and Synocheta are consistently forming a well-supported monophyletic sister group based on both genetic data and morphology (Schmidt, 2008). This group is sister to the poorest Oniscidea major clade Microcheta (Mesoniscidae), represented by only two congeneric species that are distributed at the central-eastern part of the European subcontinent (Schmalfuss, 2003). Although in the past Mesoniscidae were considered as closely related with Synocheta, the monophyletic origin of Crinocheta and Synocheta seems to be unambiguous (Dimitriou et al., 2019; Tabacaru and Danielopol, 1996).

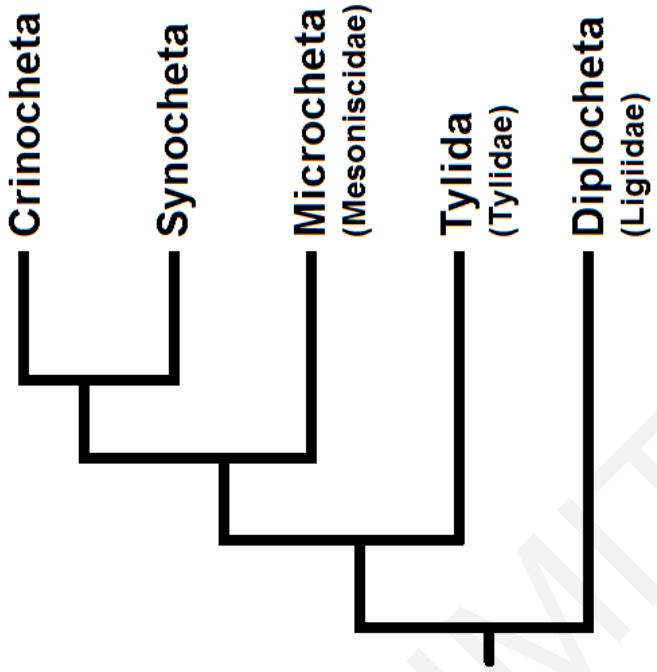

Figure 1. Schematic phylogenetic relationships of main Oniscidea lineages (after Erhard, 1996).

If we take for granted that Oniscidea are monophyletic, and that all evolutionary steps that allowed them to leave the aquatic environment are found in the extant taxa, we could assume that the marine ancestors were morphologically closer to Ligiidae and/or Tylidae. Special attention was given to the genus *Ligia* Fabricius, 1798 which is supposed to represent an intermediate form between the marine ancestors and the modern terrestrial isopods (Hornung, 2011). Focusing on the physiological, morphological and behavioral characteristics of *Ligia*, we could imagine how the ancestral forms of Oniscidea may looked like (Schmidt, 2008; Figure 2).

Alternatively, Vandel (1965) proposed the independent colonization of land by three distinct genetic lineages: a) "tylienne" (Tylida), restricted to coastal habitats, b) "trichoniscienne" (Trichoniscidae + Styloniscidae), restricted to humid micro-habitats, and c) "ligienne" including all other groups, originating from an ancestor similar to the modern amphibious genus *Ligia*. Based on a single Tylidae trait, the clearly distinct coxal plates from tergites, Tabacaru and Danielopol (1996) supported that this is the clade that is evolutionary more closely related to aquatic ancestors. Regardless the scenarios about the phylogenetic origins of extant Oniscidea, it is widely accepted that the transition from the marine to the terrestrial environment was direct, not including a freshwater step (Carefoot and Taylor, 1995; Little, 1990; Schmalfuss, 2005).

Figure 2. Terrestrial isopods representing the more basal and one of the more apical phylogenetic clades of the taxon. A: *Ligia italica* living at the littoral zone, B: *Armadillo officinalis* inhabiting arid/semi-arid habitats.

Despite the great effort given in collecting samples around the globe and classification, the taxonomic diversity of Oniscidea is not comprehensively described since, according to Sfenthourakis and Taiti (2015), there are still 192 species of uncertain generic assignment. Furthermore, taxonomic rearrangements were repeatedly proposed, as the accepted classification proved to be sensitive in taxonomic practices followed (Sfenthourakis and Taiti, 2015). Morphological datasets including different combinations of traits have resulted in inconsistent patterns of taxonomic diversity at different levels (Schmidt, 2008). On the other hand, phylogenetic analyses based on molecular data in the last two decades showed incongruent patterns (Dimitriou et al., 2019; Lins et al., 2017; Mattern, 2003; Mattern and Schlegel, 2001; Michel-Salzat and Bouchon, 2000; Zou et al., 2020).

Analyses based on morphological and/or molecular evidence are questioning the current classification as well as the validity of traditionally used morphological characters for terrestrial isopod taxonomy. Previously published studies indicated the important effects of taxonomic sampling, outgroup selection, targeted loci, and selected models of nucleotide evolution on constructed phylogenies (Dimitriou et al., 2019; Lins et al., 2017; Zou et al., 2020). Extremely high genetic distances in both nuclear and mitochondrial genetic markers, reaching up to 50.3% between confamiliar genera, and 20.3% between individuals of the same species (Dimitriou et al., 2019; Kamilari et al., 2014; Parmakelis et al., 2008) were found. These results highlight the vivid divergence between even closely related taxa, indicating that we are still far from a comprehensive and fully resolved phylogeny of Oniscidea.

Research aims

The present thesis, as stated at the title, aims to investigate the phylogenetic relationships of Oniscidea at different taxonomic levels, considering the long evolutionary history of the group and the vivid diversification that followed the transition from the aquatic to the terrestrial environment. Sanger as well as ddRAD sequencing were applied to retrieve genetic data from Oniscidea genome, in order to compare phylogenetic patterns from different datasets.

Within the framework of this thesis, sampling effort has focused mostly to Cyprus and Greece. Nevertheless, the collaboration with the two leading specialists of Oniscidea systematics that own major collections of terrestrial isopod material, namely Dr Stefano Taiti at the 'Istituto per lo Studio degli Ecosistemi and Florence University, and Dr Helmut Schmalfuss until recently at the Staatliches Museum für Naturkunde, Stuttgart, provided us access to additional valuable isopod material. In addition, collaborators sent samples also from many distant regions around the world (such as Australia, China, South Africa, Iran, and Russia). Overall, the final isopod collection used consists of >2,300 individuals, representing 209 species in 134 genera and 23 families.

It has to be noted that, despite numerus efforts, it was not possible to retrieve genetic data from a big number of samples, especially those kept in 70% alcohol (or even formaldehyde) for a long time (some of which for >60 years).

The present study focuses on exploring the evolutionary dynamics at three phylogenetic levels, examining the relationships: a) among the five Oniscidea main clades and closely related aquatic relatives (as proposed by previews studies), b) the genetic divergence between the family Porcellionidae and other families in Crinocheta, as well as within-family relationships among genera, and c) local intraspecific divergence using *A. officinalis* populations distributed in Cyprus and neighbouring regions as a case study.

By following such a three-levels approach, in addition to contributing in our understanding of Oniscidea phylogenetic relationships, we aim to offer further insights into problems in terrestrial isopod systematics and evolution, as well as issues stemming from the use of molecular markers compared to established taxonomic views based on morphological characters at all levels of Oniscidea taxonomy.

Here is a brief summary of the three main chapters:

- **Deep phylogeny of the group - Chapter 2**

This chapter attempts to resolve relationships among the main clades that correspond also to the main lineages depicting the transition from the marine to the terrestrial realm.

Considering the long evolutionary history of the group, hence the deep phylogenetic relationships among clades that we aim to resolve, the selection of proper loci exhibiting the desired characteristics, was essential. Nuclear, protein-coding, highly conserved Sodium-Potassium Pump (NAK) and Phosphoenolpyruvate Carboxykinase (PEPCK) genes along with traditionally used 18s and 28s ribosomal DNA regions, were targeted for the resolution of phylogenetic relationships at such a level. This was the first time that these nuclear protein-coding genes were used to resolve phylogenetic relationships among major groups of Oniscidea. Newly designed primers, compatible with all examined taxa, were used in order to compile a dataset including the same gene regions across all taxa. At the final dataset, all five Oniscidea clades were adequately represented whilst samples from Valvifera, Sphaeromatidae, Asellota, and Phreatoicidea were also included.

A paper titled "Genetic evidence against monophyly of Oniscidea implies a need to revise scenarios for the origin of terrestrial isopods" in which we investigated the phylogenetic relationships among the five major terrestrial isopod clades and closely related aquatic taxa, was published in *Scientific Reports* (Dimitriou et al., 2019). Based on the phylogenetic results, scenarios regarding the transition from aquatic to terrestrial environment are also discussed. The findings of this work are questioning the predominant scenarios about the origin of terrestrial isopods, the monophyly of certain main lineages, and the validity of traditionally used morphological characters.

- **Family/ Genus-level phylogeny - Chapter 3**

The third chapter is focusing on the resolution of relationships among major families, given that accumulating data are challenging the monophyly of established taxa. Employing four nuclear (18s, 28s, NAK, PEPCK) genetic markers, as well as the mitochondrial cytochrome oxidase I (COI) and 16s rRNA, (16s) loci, a subset including 14 (out of 19) Porcellionidae family genera was created. These analyses aimed to explore the phylogenetic relationships among the genera of Porcellionidae, one of the richest Oniscidea families (with >330 known species), and examine its genetic affinities with genera in the families Armadillidiidae,

Agnaridae, and Trachelipodidae, which are assumed to be closely related to Porcellionidae. Specimens of the presumably more distant families Scyphacidae and Philosciidae were included as outgroups. Divergence times among the studied taxa were calculated based on a calibrated molecular clock using gene-specific substitution rates. The monophyly of the species-richest genus of the family, *Porcellio*, was also examined. This chapters' findings were published in a paper titled "A molecular phylogeny of Porcellionidae (Isopoda, Oniscidea) reveals inconsistencies with present taxonomy" (Dimitriou et al., 2018).

- **Species/Population level - Chapter 4**

Evolutionary processes at local scale (at the population/species level), are explored within the framework of this chapter which focuses on divergence patterns and speciation processes. Individuals of the species *A. officinalis*, distributed all over the circum-Mediterranean countries and the western Black Sea coasts, were collected from many sites across Cyprus, while individuals from Israel, Greece, Turkey, Italy, and Tunisia were included in the final dataset, in order to investigate genetic differentiation among populations. This part of my thesis aims to clarify the evolutionary dynamics of the targeted species after its establishment on the island of Cyprus. More precisely, the relationships among local populations and those from neighboring countries that are possible sources of introduction of the species on the island, are investigated. Sanger and ddRAD sequencing were applied in order to retrieve genetic data from specific targeted genes and genome-wide single nucleotide polymorphisms (SNPs).

Results provided valuable information both at the population-level and the shallow-phylogeny level. Constructed phylogenies, population genetic analyses and cladochronological estimation for the studied populations, revealed a vivid diversification of the focal taxon on the island and, at the same time, highlighted the importance of historical processes on determining current patterns of biodiversity. Possibly cryptic putative new species for science were identified in this morphologically invariant 'species', indicating within-island speciation events. A careful in-depth morphological analysis of the respective populations is underway, in search for possible phenotypic characters supporting results of molecular evidence.

The results of this work promote our understanding of Oniscidea evolutionary history, leading to a reconsideration of terrestrialization scenarios and a re-evaluation of current

taxonomy, as well as of traditionally used morphological characters. Beyond these, provided protocols for amplifying proposed genes with Sanger sequencing, applicable to a significant number of Oniscidea taxa, could facilitate future research on related fields. Finally, this is the first study of its kind that uses ddRAD sequencing to gather NGS data aiming to resolve phylogenetic and population genetic relationships within Oniscidea.

CHAPTER 2

Genetic evidence against monophyly of Oniscidea implies a need to revise scenarios for the origin of terrestrial isopods

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Genetic evidence against monophyly of Oniscidea implies a need to revise scenarios for the origin of terrestrial isopods

ABSTRACT

Among the few crustacean taxa that managed to inhabit terrestrial environments, Oniscidea includes the most successful colonizers in terms of species richness and abundance. However, neither morphological traits nor molecular markers have definitively resolved phylogenetic relationships among major Oniscidea clades or established the monophyly of the taxon. Herein, we employed the highly conserved, nuclear protein-coding genes Sodium-Potassium Pump (NAK) and Phosphoenolpyruvate Carboxykinase (PEPCK), along with the traditionally used 18s and 28s ribosomal RNA genes, in an attempt to clarify these questions. Our dataset included sequences representing all major Oniscidea clades and closely related aquatic taxa, as suggested by previous studies. We applied Bayesian Inference and Maximum Likelihood methods and produced a robust and fully resolved phylogenetic tree that offers strong evidence against the monophyly of Oniscidea. The amphibious genus *Ligia* appears to be more closely related to representatives of marine suborders, while the phylogenetic pattern of the remaining Oniscidea implies a complex history of the transition from the marine environment to land. With the exception of the basal clade, all other established major clades have been recovered as monophyletic, even though relationships within these clades call for a revised interpretation of morphological characters used in terrestrial isopod taxonomy.

* Dimitriou, A. C., Taiti, S., & Sfenthourakis, S. (2019). Genetic evidence against monophyly of Oniscidea implies a need to revise scenarios for the origin of terrestrial isopods. *Scientific Reports*, 9(1), 1-10.

INTRODUCTION

Among the 11 suborders currently identified in Isopoda, Oniscidea is the only terrestrial suborder and by far the richest, comprising more than 3,700 described species (Schmidt, 2008; Sfenthourakis and Taiti, 2015). Despite their generally limited dispersal abilities and their ancestors' dependence on aquatic environments, they managed to extend their presence all over the globe and inhabit most types of habitats, including deserts (Schmalfuss, 1998; Sfenthourakis et al., 2020; Sfenthourakis and Taiti, 2015). According to current taxonomy, terrestrial isopods are divided into five main clades, with the more basal ones exhibiting behavioural, ecological and morphological traits related to aquatic environments (Schmalfuss, 1989; Schmidt, 2008). The more apical clades are generally more species-rich and more diverse, reflecting acquisition of vital adaptations to terrestrial environments that allowed them to conquer a wide range of habitats (Hornung, 2011; Schmalfuss, 1989; Sfenthourakis and Taiti, 2015).

According to the most widely accepted phylogeny based on morphological traits, proposed by Erhard (1996), Oniscidea are divided in five major clades based on their morphological adaptations to terrestrial life and, hence, their dependence on the aquatic environment. In more detail, Diplocheta, is the most basal clade, exhibiting a series of morphological characters that suggest the form of the possible marine ancestor (Hornung, 2011). The two apical sister-clades are Crinocheta and Synocheta, while Microcheta constitutes their very species-poor sister-clade and Tylida have a more basal position in-between Microcheta and the 'less terrestrial' basal Diplocheta. Schmidt (2008) proposed a more elaborate classification, reflecting assumed phylogenetic relationships, according to which there is a basal split into Ligiidae and Holoverticata, which in turn split into Tylidae and Orthogonopoda, which consists of *Mesoniscus* Carl, 1906 and Euoniscoidea. The latter comprises the two major clades Synocheta and Crinocheta. Some of the most important characters that differ among taxa belonging to the major basal clades of Oniscidea are shown in Figures 1-4. In particular, Figures 1 and 2 show characters of the major genera in Ligiidae, Figure 3 shows one of the two genera in Tylidae, and Figure 4 shows the only genus in Microcheta.

The phylogenetic position of Oniscidea within Isopoda has been based mainly on morphological characters with controversial results so far, even regarding their monophyly (Brusca and Wilson, 1991; Schmalfuss, 1989; Vandel, 1943; Wägele, 1989). Brusca and Wilson (1991) proposed Calabozoidea as sister group of Oniscidea, while Tabacaru and Danielopol

(1996) suggested Valvifera as the sister group. Dreyer and Wägele (2002) conducted a molecular phylogeny based on one nuclear DNA marker and proposed Scutocoxifera as a monophyletic clade including Oniscidea, Valvifera, Sphaeromatidea, Anthuridea and Cymothoida, with Oniscidea as the basal clade in the group.

The monophyly of Oniscidea has been supported by several, presumably well-documented synapomorphies (Erhard, 1997, 1996, 1995; Schmalfuss, 1989; Schmidt, 2008; Tabacaru and Danielopol, 1996). The most important of these are: (1) the water conducting system, formed by scales on the ventral side of coxal plates, (2) the relatively short pleotelson, (3) an antennula with less than four articles, (4) the absence of the mandibular palp, (5) the occurrence of setae on the mandible in two groups, one growing on the *lacinia mobilis*, (6) the presence of only one moveable sclerite on the basis of the second maxilla, (7) a single coxal sclerite on the maxilliped, (8) a non-subchelate first pereopod, (9) a sexually-dimorphic first pleopod, and (10) the occurrence of scale-setae on tergites. Nevertheless, Michel-Salzat and Bouchon (2000), based on mtDNA markers and a similarity-based tree, suggested that *Ligia* Fabricious, 1798 (Diplocheta, Ligiidae) is closer to Valvifera, and *Tylos* Audouin, 1826 (Tylida) to Sphaeromatidea than to the other Oniscidea. A more recent study by Lins et al. (2017) arrived at similar conclusions, using a Bayesian Inference approach in the analysis of two datasets, one consisting of 18s and 28s rRNA and COI sequences, and one comprising 13 mitochondrial protein-coding genes, but for a limited number of specimens. In both cases, *Ligia* and Tylida (included only in the first dataset) were not included in the statistically well-supported group formed by the rest of Oniscidea. Unlike Tylida, represented by *Tylos* and *Helleria* Ebner, 1868, whose close evolutionary relationship has strong statistical support, the monophyly of Ligiidae is not well supported.

Furthermore, based solely on morphological characters, Vandel (1957, 1965) had proposed a repetitive invasion of isopods from aquatic to terrestrial environments that happened at least three times. More specifically, Vandel (1957, 1965) had suggested that terrestrial isopods should be divided into three lineages: (i) “Tylienne” (=Tylida - restricted to coastal areas), (ii) “Trichoniscienne” (=Trichoniscidae + Styloniscidae? - restricted to humid micro-habitats), and (iii) “Ligienne”, which includes all remaining taxa that originated from an ancestor similar to the modern amphibious genus *Ligia*. The hypothesis that Tylida is more closely related to aquatic ancestors than the rest of Oniscidea was also supported by Tabacaru and Danielopol (1996). Nevertheless, this hypothesis was based exclusively on a single

morphological character (i.e., clearly distinct coxal plates from tergites, see Figure 3A). Overall, it is widely believed that the transition from marine to terrestrial environment was direct, without an intermediate freshwater stage (Carefoot and Taylor, 1995; Little, 1990; Schmalfuss, 2005).

Herein, we aim to investigate the phylogenetic relationships among major clades of Oniscidea, in order to evaluate the validity of current taxonomy and discuss issues related to the origins of terrestrial isopods. For this purpose, in addition to the traditionally used 18s and 28s ribosomal RNA genes, we also targeted the highly conserved, thus suitable for the resolution of deep phylogenies, protein-coding Sodium-Potassium Pump (NAK) and Phosphoenolpyruvate Carboxykinase (PEPCK) genes (Anderson et al., 2004; Friedlander et al., 1996; Tsang et al., 2008).

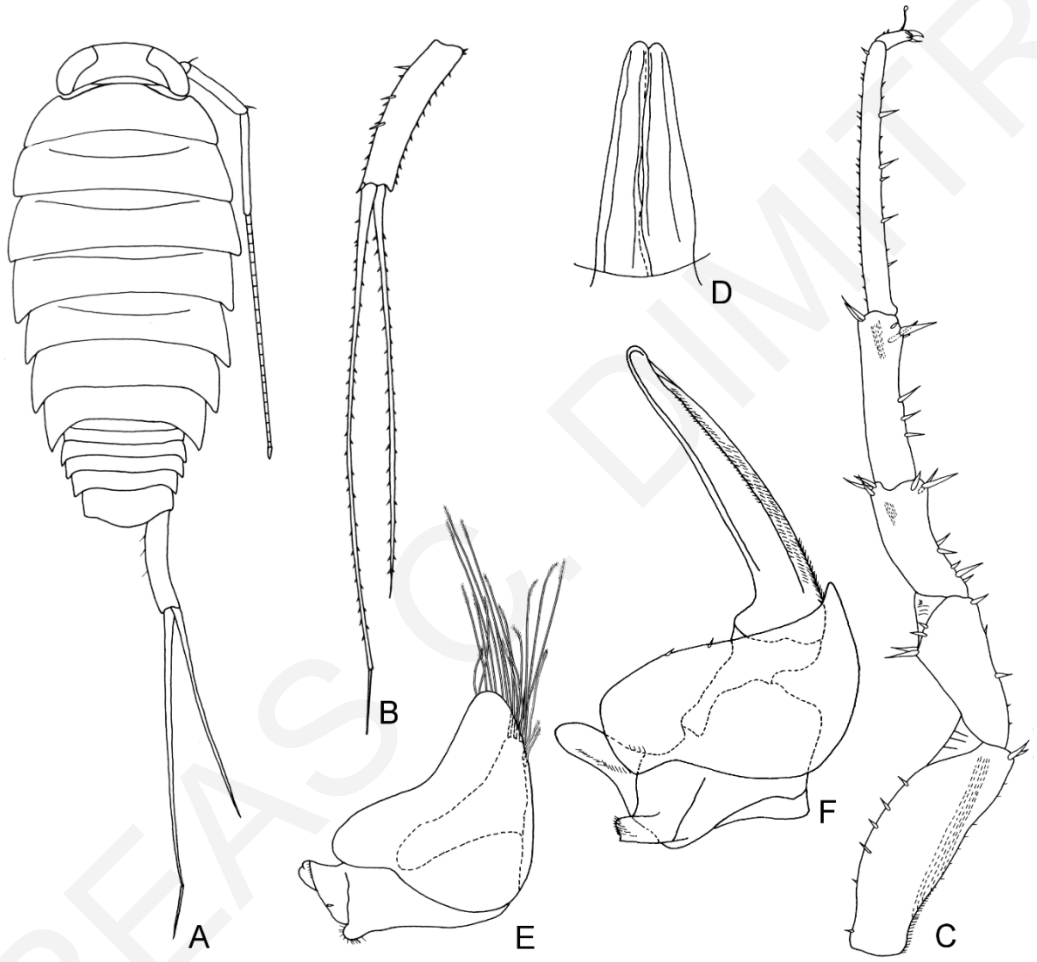

Figure 1. *Ligia italica* Fabricius, 1798 from Giannutri Island, Tuscany, Italy, ♀: (A) adult specimen, dorsal; (B) uropod. ♂: (C) pereopod 7; (D) genital papilla; (E) pleopod 1; (F) pleopod 2. Figures drawn by Taiti using the method by Montesanto (Montesanto, 2016, 2015).

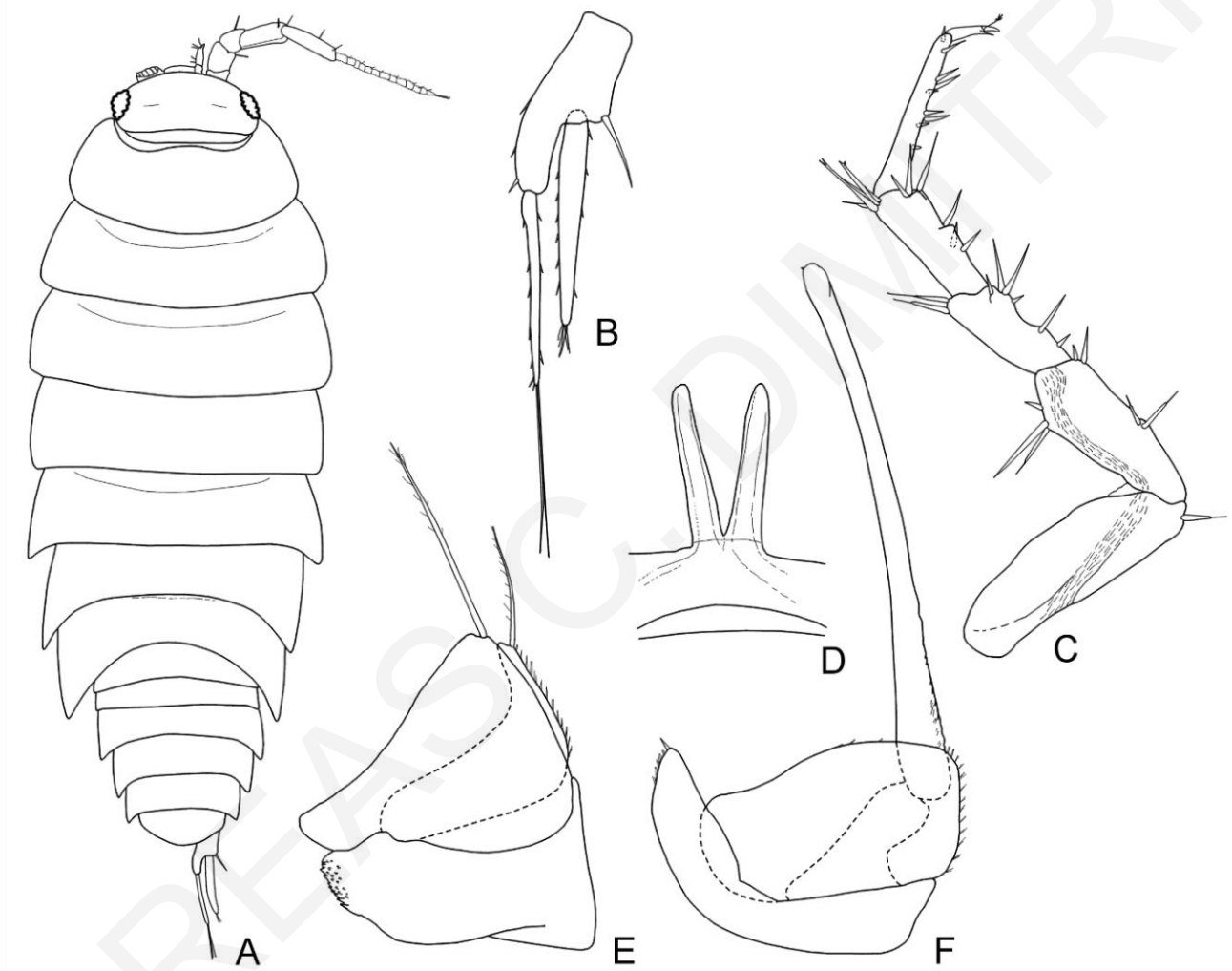

Figure 2. *Ligidium germanicum* Verhoeff, 1901 from Cardoso, Tuscany, Italy, ♀: (A) adult specimen, dorsal; (B) uropod. ♂: (C) pereopod 7; (D) genital papilla; (E) pleopod 1; (F) pleopod 2. Figures drawn by Taiti using the method by Montesanto (Montesanto, 2016, 2015).

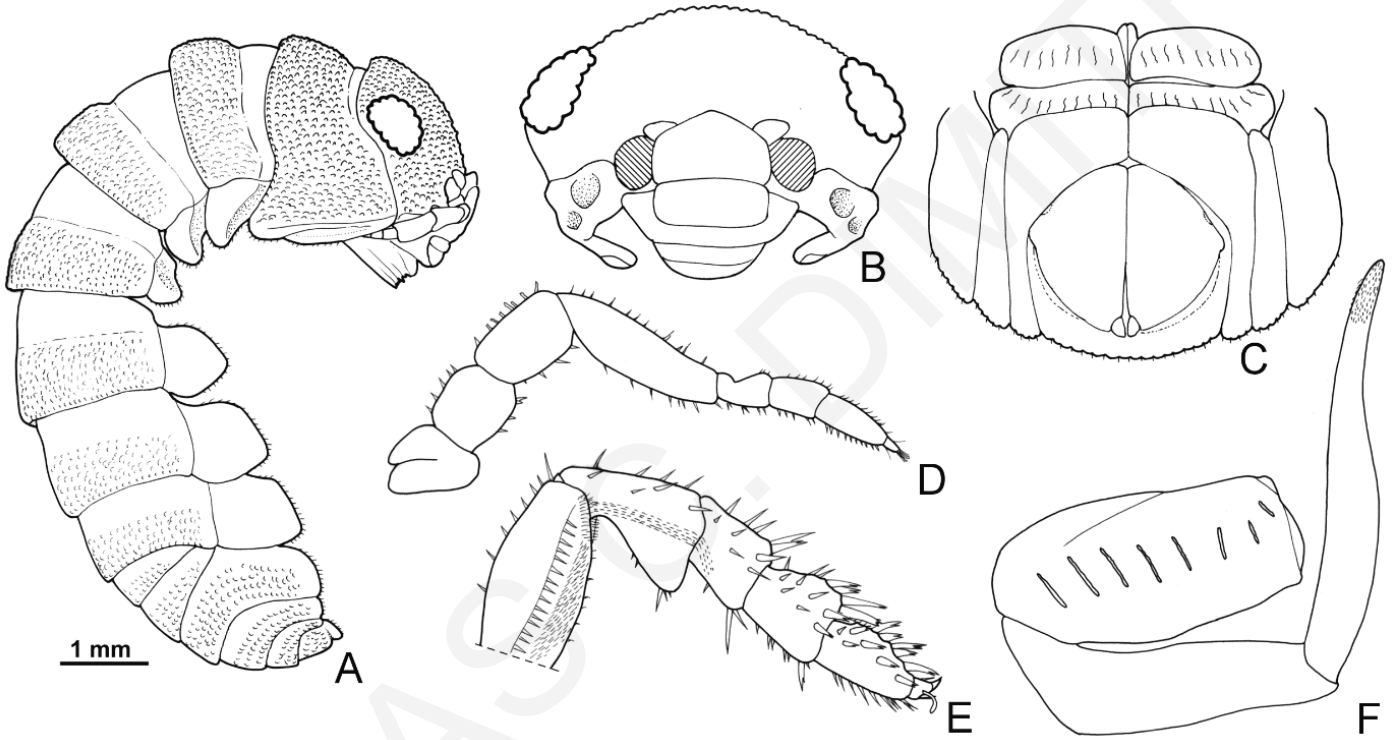

Figure 3. *Tylos albidus* Budde-Lund, 1885 from KudaBandos, Maldives, ♂: (A) adult specimen, lateral; (B) cephalon, frontal; (C) pleon and uropods, ventral; (D) antenna; (E) pereopod 7; (F) pleopod 2. Figures from Taiti (Taiti, 2014).

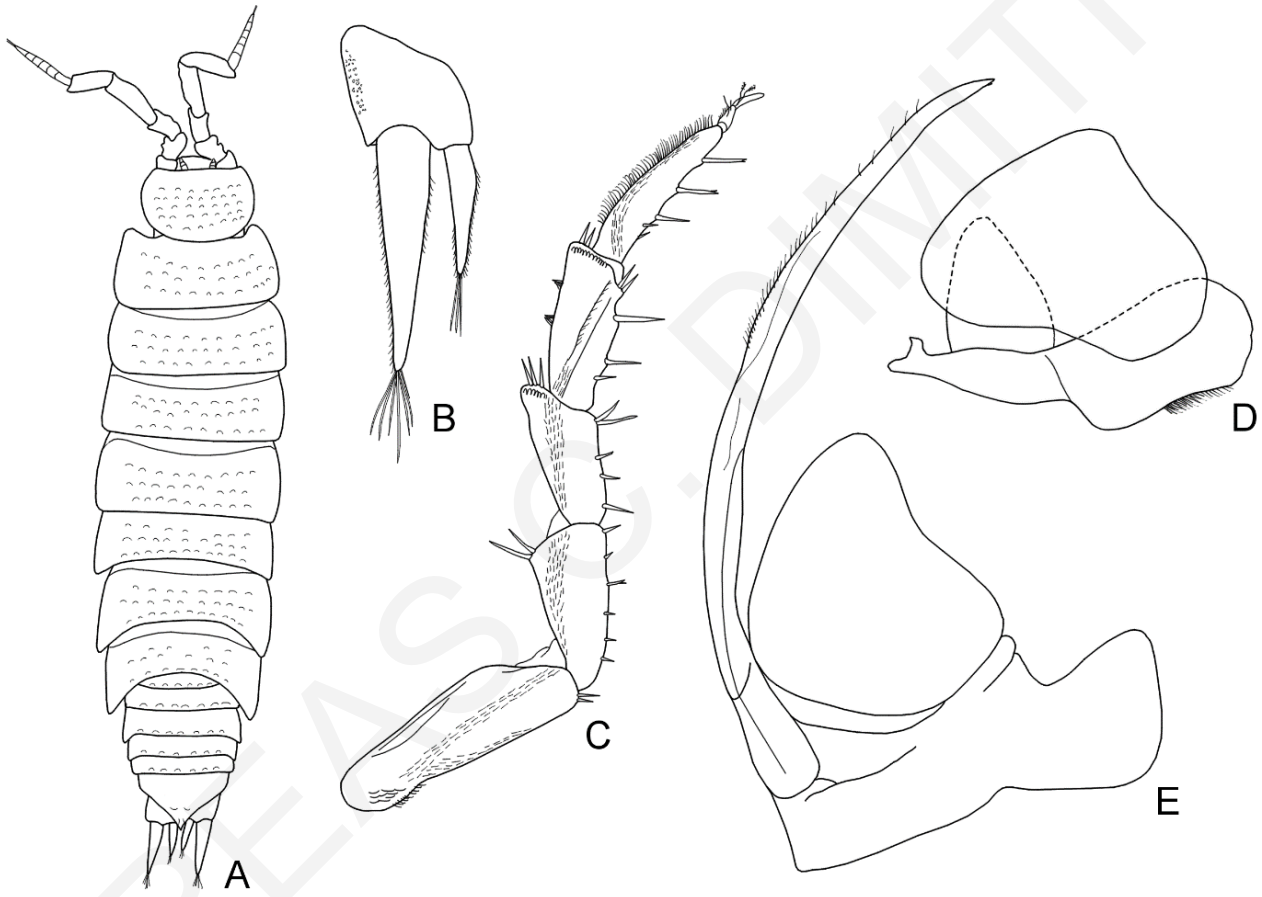

Figure 4. *Mesoniscus alpicola* (Heller, 1858) from San Martino cave, Varese, Lombardy, Italy, ♂: (A) adult specimen, dorsal; (B) uropod, (C) pereopod 7; (D) pleopod 1; (E) pleopod 2. Figures drawn by Taiti using the method by Montesanto (Montesanto, 2016, 2015).

MATERIALS & METHODS

Sample collection

Using both field collecting, deposited and loaned material, we compiled a data set including 34 Oniscidea species, representing 30 genera and 14 families. Moreover, non-Oniscidea specimens of Valvifera (*Idotea*), Sphaeromatidea (*Sphaeroma* Bosc, 1801) and Asellota (*Asellus*) were also included. Colleagues that kindly sent us material are mentioned in the acknowledgements. Freshly collected specimens, as well as the majority of available museum specimens were placed in 96% ethanol until further laboratory procedures, but we also managed to retrieve genetic data from specimens preserved in 70% alcohol for a relatively long period. Detailed information about specimens is given in Table 1.

Amplification of targeted loci

Total genomic DNA was extracted from available specimens using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's proposed protocol. Quality and quantity control of extracted DNA was performed with NanoDrop 2000/200c (Thermo Fisher Scientific Inc., USA). The final concentration was measured in ng /μl and purity was verified with A260/A280nm absorption ratio.

The non-coding nuclear genetic markers 18s and 28s, and the protein-coding Sodium-Potassium Pump (NAK) and Phosphoenolpyruvate Carboxykinase (PEPCK) genetic loci were targeted with common PCR procedures using gene specific primers. Desired regions were successfully amplified using 18Aimod/700 R primer pair for 18s (Raupach et al., 2009), 28sa/28sb for 28s (Whiting et al., 1997) , NAK for-b/NAK rev 2 or NAK for-b/NAK 638 R for NAK (Dimitriou et al., 2018; Tsang et al., 2008) and PEPCKfor/ PEPCKrev (Tsang et al., 2008) and the newly designed PEPCK 545 R (5′- CCR AAG AAN GGY STC ATN GC -3′) for PEPCK. All PCR reactions were carried out in a Veriti thermal cycler (Applied Biosystems, USA). Taking into account the genetically diverse samples, we used a touchdown PCR approach to eliminate aspecific products and save time, opposed to using multiple reactions, specific for different taxa. This way we managed to increase specificity, sensitivity and yield (Korbie and Mattick, 2008). In each case, the final reaction volume was adjusted to 20 μl, including 0.5 U of Kapa *Taq* DNA Polymerase, 3 mM MgCl2, 1X of Kapa PCR buffer A, 0.3 mM dNTP (Kapa) 0.3 μM of each primer and >20 ng of DNA template. The reactions' thermal profile followed Dimitriou *et al*. (2018). Amplicons were purified with a Qiaquick Purification Kit (Qiagen,

Germany) following the proposed instructions. The final products were sent for sequencing of both DNA strands at Macrogen facilities (Amsterdam, The Netherlands).

Data processing

CodonCode Aligner (v. 3.7.1; CodonCode Corp., USA) was used to manually inspect chromatograms, generate assemblages and make edits, where necessary. Our final dataset also included sequences of additional *Ligia* spp. and *Colubotelson thomsoni* Nicholls, 1944 (Phreatoicidea) retrieved from NCBI GenBank. The latter was included to serve as an additional outgroup. In the case of the genus *Ligia*, apart from the data generated in the framework of the present study, a chimeric sequence combining data from all targeted genes from the congeneric species *L. oceanica* (Linnaeus, 1767), *L. hawaiensis* (Dana, 1853) and *L. exotica* Roux, 1828 was included in our analyses. In this way, we manage to verify the phylogenetic position of the genus in the produced tree in a robust way. Accession numbers of all sequences used herein are given in Table 1. Sequences from each targeted gene were separated in different files and multiple sequence alignments were performed using MAFFT v.7 (Katoh et al., 2002). MEGA v.6 (Kumar et al., 2008) was used to calculate genetic distances for each alignment. Relatively longer sequences with no overlapping fragments for the majority of the samples were trimmed prior to further data elaboration.

Given that ribosomal genes consist of multiple conserved and flanking hypervariable regions, related to their functional three-dimensional structure after gene expression, alignment might be challenging (Hancock and Vogler, 2000.). In order to test the sensitivity of produced alignments and remove possible poorly aligned regions for 18s and 28s genes, we used Gblocks v0.91b (Castresana, 2000) through the Gblocks server available at http://molevol.cmima.csic.es/castresana/Gblocks_server.html. The analysis was run allowing smaller final blocks, less strict flanking and gap positions. The positive effects of removing divergent and ambiguously-aligned blocks in phylogenies are discussed by Talavera and Castresana (Talavera and Castresana, 2007).

Phylogenetic analyses

The optimal nucleotide substitution model for each loci was selected according to Akaike's Information Criterion (AIC; Akaike, 1974) using jModeltest v.2.1.1 (Darriba et al., 2012). Phylogenetic reconstructions were conducted with BI and ML methods implemented in

MRBAYES v. 3.2.6 (Ronquist et al., 2012) and RAxML-NG web server (Kozlov et al., 2019) respectively.

The concatenated data set was fed as partition blocks to MrBayes. Bayesian Inference analysis was run with the selected model of nucleotide evolution for each gene, under the default settings for within-partition among-site rate variation, allowing rate heterogeneity between partitions. BI, applying Metropolis-coupled Markov Chain Monte Carlo algorithms, was set to run four independent times with eight chains per run for 20 million generations and a sampling frequency of 100. Stationarity and convergence among runs, were ensured by monitoring the average standard deviation of split frequencies of the four simultaneous and independent runs in MrBayes. Furthermore, likelihood values, as well as all other parameters estimated as indicators for the convergence among runs were monitored using Tracer v 1.5 (Rambaut and Drummond, 2007). From the sampled trees, 10% were discarded as the burn-in phase and a 50% majority-rule consensus tree was constructed from the remaining trees in MrBayes.

Maximum Likelihood trees were constructed under the same partitioning scheme and nucleotide substitution models. The reliability was tested by bootstrapping (Felsenstein, 1985) with 1,000 replicates.

Table 1: Species, locality of origin and GenBank accession numbers of individuals used in the molecular phylogenetic analyses.

Species	Family	Suborder	Section	Origin	Genes/ Acc. number			
					18s	28s	NAK	PEPCK
Ligia italica Fabricius, 1798	Ligiidae	Oniscidea	Diplocheta	Cyprus	MN171516	MN174838	MN234250	MN234312
Ligia oceanica Linnaeus, 1767	Ligiidae	Oniscidea	Diplocheta	Galicia (Spain)	AF255698	-	-	-
Ligia hawaiensis Dana, 1853	Ligiidae	Oniscidea	Diplocheta	Hawaii	-	KF546702	-	-
Ligia exotica Roux, 1828	Ligiidae	Oniscidea	Diplocheta	Kanagawa (Japan)	-	-	MG676443	-
Ligia exotica Roux, 1828	Ligiidae	Oniscidea	Diplocheta	China	-	-	-	KF002742
Ligidium ghigii Arcangeli, 1928	Ligiidae	Oniscidea	Diplocheta	Greece	MN171506	MN174818	MN234284	MN234303
Tauroligidium cf. *stygium* Borutzky, 1950	Ligiidae	Oniscidea	Diplocheta	Crimea	MN171509	MN174821	MN234255	MN234307
					MN171507	-	MN234256	MN234306
					-	MN174820	MN234270	MN234305
					-	MN174819	MN234271	MN234304
Typhloligidium coecum (Carl, 1904)	Ligiidae	Oniscidea	Diplocheta	Crimea	M171508	MN174822	-	MN234308
Typhloligidium coecum	Ligiidae	Oniscidea	Diplocheta	Caucasus	MN171510	MN174823	MN234251	MN234309
Helleria brevicornis Ebner, 1868	Tylidae	Oniscidea	Tylida	France	MN171518	MN174843	MN234285	MN234320
Tylos ponticus Grebnicki, 1874	Tylidae	Oniscidea	Tylida	Cyprus	MN171519	MN174844	MN234265	-

Mesoniscus alpicola (Heller, 1858)	Mesoniscidae	Oniscidea	Microcheta	Italy	MN171513	MN174829	MN234249	MN234321
Styloniscus magellanicus Dana, 1853	Styloniscidae	Oniscidea	Synocheta	Argentina	MN171512	MN174832	-	-
Androniscus roseus (C. Koch, 1838)	Trichoniscidae	Oniscidea	Synocheta	The Netherlands	MN171501	MN174824	MN234283	MN234313
Calconiscellus karawankianus (Verhoeff, 1908)	Trichoniscidae	Oniscidea	Synocheta	Croatia	-	MN174827	MN234277	MN234319
Caucasonethes sp.	Trichoniscidae	Oniscidea	Synocheta	Caucasus	-	MN174826	MN234268	MN234318
					-	MN174825	MN234269	MN234317
Tauronethes lebedinskyi Borutzky, 1949	Trichoniscidae	Oniscidea	Synocheta	Crimea	MN171505	MN174831	MN234272	MN234322
Trichoniscus provisorius Racovitza, 1908	Trichoniscidae	Oniscidea	Synocheta	Cyprus	MN171502	MN174834	MN234259	MN234314
					MN171503	MN174836	MN234253	MN234315
					MN171504	MN174835	MN234286	MN234316
Agnara madagascariensis (Budde-Lund, 1885)	Agnaridae	Oniscidea	Crinocheta	U.A.Emirates	MG887977	MG888003	MG887924	MN234325
Hemilepistus klugii (Brandt, 1833)	Agnaridae	Oniscidea	Crinocheta	Iran	MG887978	MG888011	MG887926	-
Hemilepistus schirasi Lincoln, 1970	Agnaridae	Oniscidea	Crinocheta	Iran	MG887979	MG888012	MG887927	-
Hemilepistus reaumurii (Milne-Edwards, 1840)	Agnaridae	Oniscidea	Crinocheta	Tunisia	MN171500	MN174828	MN234258	-

Protracheoniscus aff. *fossuliger* (Verhoeff, 1901)	Agnaridae	Oniscidea	Crinocheta	Greece	MN171494	MN174817	MN234281	MN234292
Armadillo officinalis Dumeril, 1816	Armadillidae	Oniscidea	Crinocheta	Cyprus	MN171498	MN174812	MN234252	-
Armadillidium vulgare (Latreille, 1804)	Armadillidiidae	Oniscidea	Crinocheta	Cyprus	MN171495	MN174837	-	MN234299
Cyphodillidium absoloni (Strouhal, 1934)	Armadillidiidae	Oniscidea	Crinocheta	Croatia	-	MN174814	MN234276	MN234295
Typhlarmadillidium sp.	Armadillidiidae	Oniscidea	Crinocheta	Croatia	-	MN174815	MN234273	MN234294
Cylisticus convexus (De Geer, 1778)	Cylisticidae	Oniscidea	Crinocheta	Greece	MN171493	MN174813	MN234280	MN234293
Oroniscus dalmaticus Strouhal, 1937	Oniscidae	Oniscidea	Crinocheta	Croatia		MN174816	MN234274	MN234297
Platyarthrus schoblii Budde-Lund, 1885	Platyarthridae	Oniscidea	Crinocheta	Cyprus	MN171492	MN174833	MN234254	MN234298
Trichorhina heterophthalma Lemos de Castro, 1964	Platyarthridae	Oniscidea	Crinocheta	The Netherlands (greenhouse)	MN171496	MN174845	MN234282	MN234300
Agabiformius excavatus Verhoeff, 1941	Porcellionidae	Oniscidea	Crinocheta	Cyprus	MG887969	MG888009	MG887921	-
Porcellio nasutus Strouhal, 1936	Porcellionidae	Oniscidea	Crinocheta	Cyprus	MG887980	MG887999	MG887911	-
Porcellionides cyprius (Strouhal, 1968)	Porcellionidae	Oniscidea	Crinocheta	Cyprus	MN171488	MN174808	MN234278	MN234287

Porcellionides pruinosus (Brandt, 1833)	Porcellionidae	Oniscidea	Crinocheta	Cyprus	MN171489	MN174809	MN234275	MN234288
Actaecia euchroa Dana, 1853	Scyphacidae	Oniscidea	Crinocheta	New Zealand	MG887985	MG888007	MG887930	MN234324
Levantoniscus makrisi Cardoso, Taiti and Sfenthourakis, 2015	Trachelipodidae	Oniscidea	Crinocheta	Cyprus	MN171490	MN174810	MN234260	MN234289
Levantoniscus bicostulatus Cardoso, Taiti and Sfenthourakis, 2015	Trachelipodidae	Oniscidea	Crinocheta	Cyprus	MN171491	MN174811	MN234257	MN234290
Trachelipus ratzeburgii (Brandt, 1833)	Trachelipodidae	Oniscidea	Crinocheta	Germany	MN171497	MN174830	MN234279	MN234291
Asellus aquaticus (Linnaeus, 1758).	Asellidae	Asellota	-	Greece	MN171511	MN174846	MN234267	MN234323
Colubotelson thomsoni Nicholls, 1944	Phreatoicidae	Phreatoicidea	-	Tasmania	AF255703	AF169711	-	-
Sphaeroma serratum (Fabricius, 1787)	Sphaeromatidae	Sphaeromatidea	-	Italy	MN171520	MN174842	MN234262	MN234301
					MN171517	MN174841	MN234261	MN234302
Idotea chelipes (Pallas, 1766)	Idoteidae	Valvifera	-	Italy	MN171515	MN174840	MN234263	MN234311
					MN171514	MN174839	MN234264	MN234310

RESULTS

Extracted DNA concentration was >15 ng/μl in all cases, with the A260/A280 purity rate over 1.5. Attempts to amplify and sequence all targeted loci were successful for almost all samples. The final compiled aligned dataset after Gblocks treatment consisted of 1,984 base pairs (bp). The initial alignment lengths and numbers of conserved, variable and parsimony-informative sites are shown in Table 2 for all sequenced loci separately. Among the tested models, the highest Akaike weight values, indicating the best fit to data, were exhibited by TIM2ef + I + G for 18s, TIM3 + G for 28s, TIM2 + I + G for NAK, and GTR + G for PEPCK.

Prior to calculation of genetic divergence, available sequences were grouped at the suborder level and those of Oniscidea were further grouped into the five known major subclades. *Ligia* specimens were grouped separately from the rest of the Diplocheta, as they appear to form a separate clade on the produced phylogenetic tree (Figure 5). Genetic distances between examined taxa appeared to be constantly higher for ribosomal genes compared to the protein-coding ones. Genetic variation ranged between 6.6-30.2% in the case of 18s, 33.3-71.6% for 28s, 16.7-30.6% for NAK and 19.3-29.5% for PEPCK. The minimum and maximum genetic divergence values were not constantly found between the same groups for all genetic markers. More specifically, the maximum genetic distance was found between Tylida-Crinocheta, Sphaeromatidae-Crinocheta, Asellota-Valvifera and Asellota-Crinocheta, whereas the minimum values were identified between Asellota-Phreatoicidea, Tylida-*Mesoniscus*, *Ligia*-Sphaeromatidae and Valvifera-'Diplocheta' (excluding *Ligia*) in the case of 18s, 28s, NAK and PEPCK genes, respectively. All within- and between-group p-distances are given in supplementary material (Appendix I).

The Bayesian Inference (BI) and Maximum Likelihood (ML) trees exhibited largely congruent topologies. Nevertheless, in some cases, high BI posterior probabilities did not coincide with high ML bootstrap values (>80). This can be attributed to the fact that, in contrast to BI, the ML method implemented in available software (e.g. RAxML, PhyML, IQ-TREE) perceives gaps (−) and missing data (given as N or? in DNA alignments) as unknown characters that do not provide additional information for the resolution of phylogenetic relationships. Two out of four targeted loci are coding rRNAs whose three-dimensional structure is dependent on highly conserved regions which are interrupted by variable regions accumulating mutations, including indels. These regions are not under strong evolutionary pressure and, hence, mutations

can explain the occurrence of gaps in final alignments. On the other hand, the BI approach takes into account insertion and deletion events that contain phylogenetically useful information. Therefore, only the BI tree is presented herein (Figure 5).

Table 2: Aligned bases length, before and after GBlocks treatment (for ribosomal genes), conserved, variable and parsimony-informative sites for all genes used in this study.

Gene	Alignment length (bp)		Conserved sites	Variable sites	Parsimony informative sites
	Before Gblocks Treatment	After Gblocks Treatment			
18s	1031	532	373	479	287
28s	1857	297	221	1,055	666
NAK	639	-	303	256	639
PEPCK	516	-	247	261	214

Holoverticata (*sensu* Schmidt 2008) is recovered as a well-supported clade, containing the traditionally recognised sub-clade structure: Crinocheta and Synocheta form two well-supported, monophyletic sister clades, and Microcheta is the intermediate clade of these and the more basal, monophyletic Tylida. Nevertheless, Diplocheta (hence, also Ligiidae) appear to be polyphyletic, with *Ligia* being the sister taxon of Valvifera + Sphaeromatidea, and the genera *Ligidium* Brandt, 1833, *Tauroligidium* Borutzky, 1950 and *Typhloligidium* Verhoeff, 1918, traditionally grouped in Ligiidae, forming a well-supported monophyletic group, as the sister clade of Holoverticata. The monophyly of Oniscidea as currently defined is questioned, and could be saved if *Ligia* is excluded from the taxon. The basal position of *Colubotelson* Nicholls, 1944 (Phreatoicidea) and *Asellus* Geoffroy, 1762 (Asellota), as well as the statistically supported retrieval of Valvifera and Sphaeromatidae within the 'Onisicdea' clade, indicates the closer relationship of terrestrial isopods with these two suborders. Phylogenetic relationships inside Crinocheta also show some interesting patterns with important implications for oniscidean taxonomy. Porcellionidae form a well-supported clade with Trachelipodidae and part of Agnaridae (as the latter appear to be polyphyletic), while Armadillidiidae, traditionally considered sister-group of the Porcellionidae, is grouped with representatives of other families (e.g., Cylisticidae and part of Agnaridae). Also, *Platyarthrus* Brandt, 1833 and *Trichorhina* Budde-Lund, 1908, presently included in the family Platyarthridae, do not seem to be related,

and the representative of the most diverse family Armadillidae appears in a more basal position within Crinocheta.

Within Synocheta, the monophyly of Trichoniscidae is not supported, as *Styloniscus* Dana, 1852, type-genus of Styloniscidae, seems to fall within the former. Moreover, no support for the monophyly of the subfamilies Trichoniscinae and Haplophthalminae could be found.

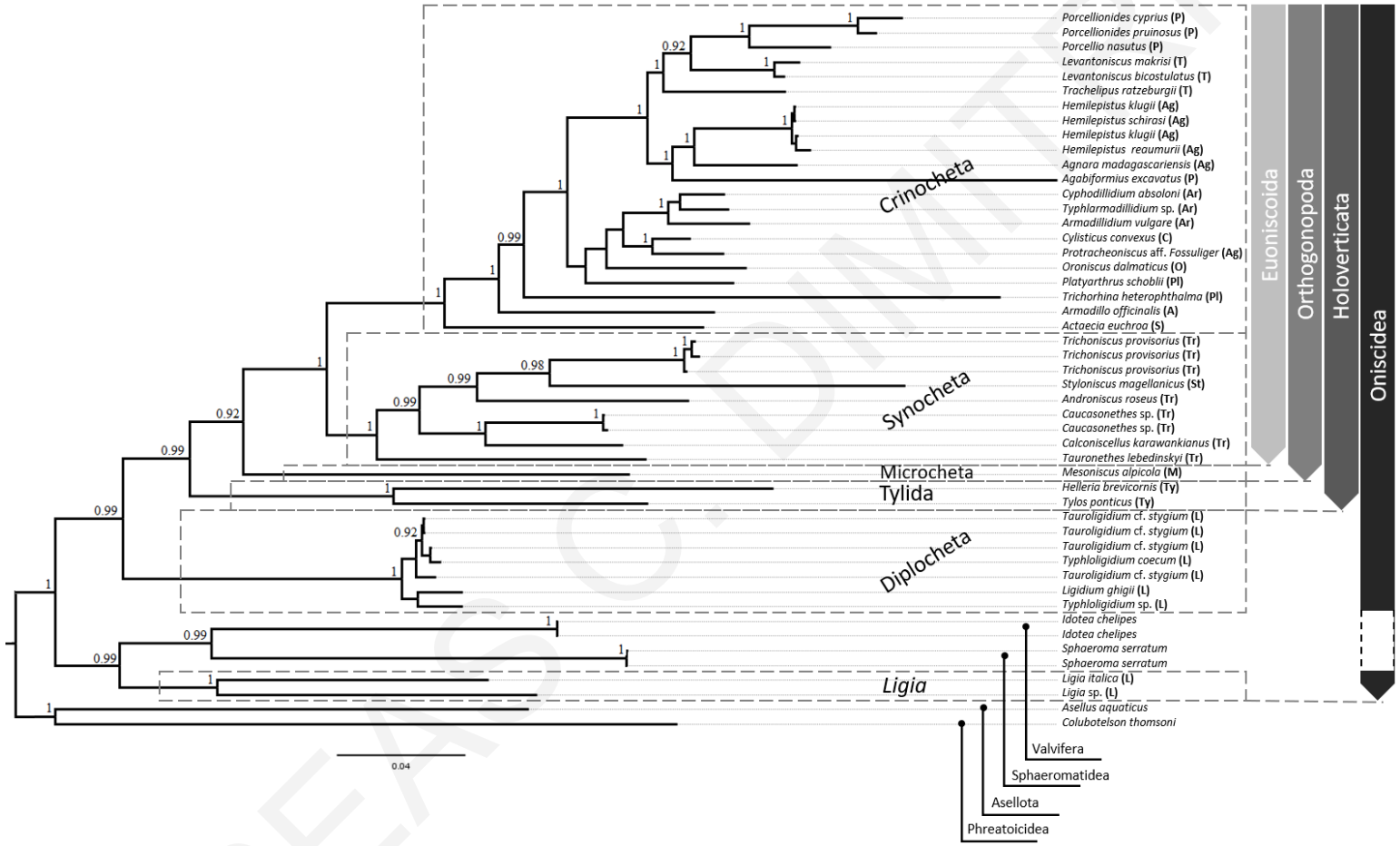

Figure 5. Fifty percent majority-rule consensus tree of the Bayesian Inference (BI) analysis constructed using 18s, 28s, NAK and PECK markers. Posterior probabilities (>90) are given above nodes. Letters within brackets at tip labels indicate the family of each specimen. L: Ligiidae, Ty: Tylidae, M: Mesoniscidae, Tr: Trichoniscidae, St: Styloniscidae, Pl: Platyarthridae, C: Cylisticidae, O: Oniscidae, S: Scyphacidae, Ag: Agnaridae, T: Trachelipodidae, P: Porcellionidae, Ar: Armadillidiidae, A: Armadillidae.

DISCUSSION

This is the first time that nuclear protein-coding genes are used to resolve phylogenetic relationships among major groups of Oniscidea. The fact that this study is so far the only one that produced a fully resolved and robust molecular phylogeny of all five major oniscidean clades, proves the advantages of using these markers. NAK has been used before (Dimitriou et al., 2018) in terrestrial isopod phylogenetics, but at a lower taxonomic level. Of course, given the depth of phylogeny attempted herein, the use of mitochondrial genes, with their high mutation rates and, hence, saturation effects, is not appropriate (Philippe et al., 2011). Also, the use of untreated nuclear ribosomal genes sequences, such as of 18s and/or 28s, might have led to biased or insufficiently supported results, as they contain regions that evolve at very different rates. Gblocks treatment was recruited to overcome possible issues that may arise due to the properties of these regions. Herein, we managed to produce a robust and sufficiently inclusive phylogeny of terrestrial isopods using a more reliable data set of nuclear DNA markers. This phylogeny has important implications for oniscidean systematics, as it undermines the validity of several morphological characters traditionally used in terrestrial isopod taxonomy. The transition of isopods from the marine to the terrestrial environment might also need to be revisited in light of the new evidence.

A number of unique adaptations to terrestrial life have led authors to assume that Oniscidea underwent only one transition from marine to land (Broly et al., 2013; Hornung, 2011; Sfenthourakis and Taiti, 2015). However, the low number of studies using molecular data in the past failed to confirm the monophyly of Oniscidea (Lins et al., 2017; Michel-Salzat and Bouchon, 2000), but also failed to provide a consistent phylogenetic pattern (Mattern, 2003; Mattern and Schlegel, 2001). According to the results of our analysis, the monophyly of Oniscidea, as currently defined, is not supported, since the genus *Ligia*, generally considered as con-familiar with *Ligidium* and a small number of other related taxa, none of which exploit littoral environments, appears to be a closer relative of a group of marine isopods, such as the Valvifera and Sphaeromatidae. The monophyly of Oniscidea could be saved if *Ligia* is excluded. The assumed synapomorphies of 'Ligiidae', such as the residual maxillipedal segment at the back of the cephalon, are rather symplesiomorphies, as has been previously suspected. *Ligidium* and related genera of the polyphyletic family Ligiidae could be assigned to a new family (we propose Ligidiidae, from the most speciose genus *Ligidium*) that can be more safely defined by more reliable synapomorphies, such as the shape of the uropods with the endopod inserted distally compared to the exopod (cf. Figures 1B and 2B). The genus *Ligidioides* Wahrberg, 1922 (not included in our analysis)

has a uropod more similar to that of *Ligia*, i.e., with the insertions of the endopod and exopod at the same level (Wahrberg, 1922), and might remain in the family Ligiidae, but this has to be investigated by a future molecular analysis that also includes this genus. Lins et al. (2017) came to similar conclusions regarding the relationships of *Ligia* with marine taxa, but these authors did not include other Ligiidae in their analysis, so they could not discuss the monophyly of the family. A common evolutionary history of the mitochondrial genomes of *Ligia* and *Idotea* Fabricius, 1798 was highlighted also by Kilpert and Podsiadlowski (2006). The high genetic divergence between *Ligia* and *Ligidium* was also evident from their distant position in the phenetic tree presented by Michel-Salzat & Bouchon (2000). Our findings are in agreement with all of these studies, a fact that further corroborates our hypothesis.

In view of the new phylogeny, the critical question regarding the transition from the marine environment to land should be addressed by taking into account the ecology of species in the major clades and, most importantly, the fact that the relevant event(s) happened sometime in the middle or even lower Mesozoic (Broly et al., 2013), so that a large number of crucial forms might have been extinct without leaving any fossils of ancestral lineages. In fact, the oldest fossil Oniscidea are much younger and consist of highly derived forms (Broly et al., 2015), while coastal marine or amphibious forms of animals that do not have hard skeletons, shells or teeth, are rarely fossilized anyway.

Considering that: (a) the most basal clade (Diplocheta, excluding *Ligia*) consists of freshwater-related taxa, (b) the subsequent clade (Tylida) includes taxa mostly living along marine coasts (even though the genus *Helleria* is fully terrestrial), and with a divergent morphology compared to other Oniscidea (at least regarding the form of cephalon, the distinct epimera on most thoracic segments, and the unique type of respiratory structures on pleopods, not connected to those of other taxa, see Figure 3), and (c) Microcheta are fully terrestrial (albeit dependent on very high humidity) and they exhibit an overall morphology closer to that of the more derived Oniscidea (see Figure 4), one might consider revisiting scenarios regarding the transition of isopods form the marine environment to land. Even though most *Ligia* species are amphibious, there are some species that live inland (Schmalfuss, 1978; Taiti et al., 2003; Taiti and Howarth, 1996). This means that we might envision a similar but independent transition that led to the common ancestor of ‘Ligidiidae’, given that this group consists today of species mostly living in close connection to freshwater. On the other hand, Tylidae might represent another transition, since they exhibit many characters that are difficult to recreate via a plausible transformation series from Diplocheta-type characters (cf. Figures 1, 2 and 3). If this proves true, the next clade,

Microcheta, which is basal to all Orthogonopoda, connected to very humid, freshwater-related habitats and with a more differentiated morphology than Tylida in many characters (cf. Figures 3 and 4), would represent a third invasion to land, maybe using a freshwater path. Of course, this would undermine the actual monophyly of Oniscidea.

On the basis of current evidence, this is only a tentative hypothesis that has to be evaluated through careful elaboration of physiological traits and, hopefully, further fossil findings. Obviously, the very old origins of the Oniscidea (Broly et al., 2013), coupled with the difficulty of fossilization of these organisms, might have led to the permanent loss of crucial information from several basal clades representing possible direct ancestors of terrestrial forms. The phylogenetic reconstruction based on modern forms cannot recover such extinct clades, except in the case of some exceptional, but highly unlikely, fossils being found in the future.

The monophyly of Crinocheta and Synocheta seems to be unambiguous. The hypothesis by Tabacaru and Danielopol (1996) that Synocheta is a sister taxon with Mesoniscidae cannot be supported. The phylogenetic relationships inside the two major clades reveal that certain morphological characters that have been considered important in oniscidean taxonomy, such as the type and form of pleopodal lungs, the ornamentation of tergites or the shape of uropods, might not be very useful. In particular, Porcellionidae and Armadillidiidae, even though they seem to share a similar type of pleopodal lung, at least in comparison with that in Trachelipodidae, appear to belong to distant clades; the former related to Trachelipodidae and part of Agnaridae (the monophyly of which is not supported), and the latter to Cylisticidae and other families. This is in agreement with the recent findings by Dimitriou et al. (2018). In turn, Cylisticidae appears to be closer to Armadillidiidae, even though they have styliform uropods. Within Synocheta, the traditional distinction between Trichoniscinae and Haplophthalminae, based largely on the presence of ornamentation on tergites, does not seem to be supported since *Calconiscellus* Verhoeff, 1927, a member of Haplophthalminae, appears to be the sister-taxon of *Caucasonethes* Verhoeff, 1932 and nested within other genera of Trichoniscinae. Furthermore, the status of Styloniscidae as a separate family from Trichoniscidae is also undermined. More detailed analyses, using more extensive taxonomic sampling inside these clades, are necessary to clarify these issues. The closer relationship of terrestrial isopods with Valvifera and Sphaeromatidae than with Asellota or Phreatocidea, revealed by our analysis, agrees with the hypothesis of Brusca and Wilson (1991).

In conclusion, Oniscidea should not be considered monophyletic. Systematics in this very old group, which presents an amazing case of animal invasions to land, are in urgent need of extensive revision, taking into account robust molecular evidence. New techniques, such as whole genome sequencing, transcriptomics and ultra-conserved elements, should be applied to the whole range of terrestrial isopod taxa, in order to resolve the complete phylogenetic history of the group and shed light on crucial questions regarding the evolution of terrestriality in this taxon. Modern terrestrial isopoda is probably the only animal taxonomic group lower than Class that includes representatives of most steps of the transition from aquatic environments to almost all terrestrial environments, despite the presumed large number of extinct forms (Sfenthourakis and Hornung, 2018). Furthermore, considering the fact that these animals have evolved structures analogous to the complex organs of terrestrial vertebrates, such as lungs (pleopodal lungs) and the placenta (marsupial, egg-feeding 'cotelydons'; Sfenthourakis et al., 2020), a detailed phylogenetic reconstruction can provide invaluable information on many exciting aspects of evolutionary biology, but also physiology, behaviour, ecology, and several other fields.

CHAPTER 3

A molecular phylogeny of Porcellionidae (Isopoda, Oniscidea) reveals inconsistencies with present taxonomy

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A molecular phylogeny of Porcellionidae (Isopoda, Oniscidea) reveals inconsistencies with present taxonomy

ABSTRACT

Porcellionidae is one of the richest families of Oniscidea, globally distributed, but we still lack a comprehensive and robust phylogeny of the taxa that are assigned to it. Employing five genetic markers (two mitochondrial and three nuclear) we inferred phylogenetic relationships among the majority of Porcellionidae genera. Phylogenetic analyses conducted via Maximum Likelihood and Bayesian Inference resulted in similar tree topologies. The mtDNA genes cytochrome oxidase I (COI) and 16s rRNA (16s) were used for clade dating using previously published mutation rates. Our results provide evidence against the monophyly of both Porcellionidae and the largest genus of the family, *Porcellio*. These results are compared to previous published work based on morphological evidence. The genera *Leptotrichus* and *Brevurus* are not grouped with the rest of Porcellionidae whereas Agnaridae are grouped with part of Porcellionidae. *Armadillidium* and *Schizidium* (Armadillidiidae) occupy a basal position on the phylogenetic tree. Even though the African genera *Tura* and *Uramba* (distributed in East Africa) are grouped together, there is no general geographical pattern in other sub-clades. Additional taxonomic issues that arise in this work, such as the assignment of the recently described genus *Levantoniscus*, are also discussed. The status of Porcellionidae should be further revised and morphological characters traditionally used in Oniscidea taxonomy should be reconsidered in view of molecular evidence. The origin of the monophyletic clade within Porcellionidae, as indicated in the present work, is dated back to the Oligocene (~32 mya).

*Dimitriou, A. C., Taiti, S., Schmalfuss, H., & Sfenthourakis, S. (2018). A molecular phylogeny of Porcellionidae (Isopoda, Oniscidea) reveals inconsistencies with present taxonomy. *ZooKeys*, (801), 163.

INTRODUCTION

The Oniscidea family Porcellionidae is one of the richest in species, with 333 species, belonging to 19 genera, currently assigned to it (Sfenthourakis and Taiti, 2015). Family members are unable to conglobate, with the exception of the genus Atlantidium Arcangeli, 1936. There is remarkable morphological variation among Porcellionidae species and genera, especially in head structure, pleotelson, and body shape. Familial assignment of taxa is based mostly on the combination of two-character states, namely an antennal flagellum with two articles and the presence of monospiracular, covered lungs on the first two pairs of pleopods (Schmidt, 2003). However, certain authors, based on morphological and recent molecular work, suggest that these characters could be symplesiomorphies, as they are not exclusively found in Porcellionidae (Schmalfuss and Ferrara, 1978; Schmidt, 2003).

Different authors have found Porcellionidae to be closely related with Oniscidae, Trachelipodidae, Cylisticidae, Agnaridae or Armadillidiidae (Lins et al., 2017; Mattern, 2003; Michel-Salzat and Bouchon, 2000; Schmidt, 2008). Furthermore, monophyly of the most species-rich genera, *Porcellio* Latreille, 1804 and *Porcellionides* Miers, 1877, has been debated on the basis of both morphology (Schmalfuss, 1998, 1992; Vandel, 1962) and molecular evidence (Mattern, 2003; Michel-Salzat and Bouchon, 2000). More specifically, some *Porcellionides* species appear to be more closely related to the genus *Porcellio* (Mattern, 2003; Michel-Salzat and Bouchon, 2000) or even to the genus *Cylisticus* Schnitzler, 1853 that belongs to another family (Cylisticidae),than to other congeneric species (Michel-Salzat and Bouchon, 2000). Hence, also the monophyly of the family has been repeatedly questioned on the basis of both morphological and genetic data (Mattern and Schlegel, 2001; Michel-Salzat and Bouchon, 2000; Schmalfuss, 1989; Schmidt, 2008, 2003).

Members of Porcellionidae were originally reported from the circum-Mediterranean region, Atlantic islands, Arabian Peninsula and East Africa. Nowadays they are known from all over the world, being introduced into many regions by human activities (Schmidt, 2003). Porcellionidae are considered to be among the isopod species that are better adapted to terrestrial environments, and they can be found in a wide range of habitats, from tropical rainforests to deserts (Medini-Bouaziz et al., 2017; Schmidt, 2003)

The present study aims to a more detailed investigation of phylogenetic relationships among genera of Porcellionidae, using two mitochondrial and three nuclear genes that allow estimation of divergence times among extant taxa.

MATERIALS & METHODS

Sampling

Isopod specimens belonging to five Porcellionidae genera, one to Trachelipodidae (*Levantoniscus* Cardoso et al., 2015) and two to Armadillidiidae (*Armadillidium* Brandt, 1831 and *Schizidium* Verhoeff, 1901) were collected on Cyprus between 2014 and 2016. Additional specimens came from the collection of the Istituto per lo Studio degli Ecosistemi, deposited in the Museum of Natural History of the University of Florence, and from the personal collection of Helmut Schmalfuss. Members of the families Armadillidiidae, Agnaridae and Trachelipodidae that are assumed to be closely related to Porcellionidae were included in the analyses to test the monophyly of the latter, whilst specimens of the more distant families Scyphacidae (*Actaecia euchroa* Dana, 1853) and Philosciidae (*Chaetophiloscia elongata* Dollfus, 1884) were included as outgroups. More details about specimens used are given in Table 1.

We were not able to include specimens of five Porcellionidae genera, namely the monotypic *Congocellio* Arcangeli, 1950 and *Tropicocellio* Arcangeli, 1950, both distributed in the Democratic Republic of the Congo, *Dorypoditius* Verhoeff, 1942 from Mozambique, *Atlantidium* Arcangeli, 1936 form Madeira, and *Pondo* Barnard, 1937 from South Africa (Pondoland and Natal).

Molecular analyses

Fresh specimens were placed in 96% alcohol immediately after collection and stored at -20 °C. The majority of samples from museums and private collections had been preserved in 70% alcohol. Whole animals or legs of larger specimens were used for extraction of total genomic DNA using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. NanoDrop 2000/200c (Thermo Fisher Scientific Inc., USA) was used to determine the final concentration and purity (A260/A280nm absorption rate) of DNA extractions.

DNA extraction amplification and sequencing

The following mitochondrial and nuclear genetic loci were targeted using common PCR procedures: partial mitochondrial cytochrome *c* oxidase subunit 1 (COI), ribosomal 16S rRNA (16s), the nuclear, non-coding 18S ribosomal RNA (18s) and 28S ribosomal RNA (28s), and the protein coding Sodium-Potassium Pump (NAK). Mitochondrial COI and 16s genes were successfully amplified using the universal LCO1490/HCO2198 (Folmer et al.,

1994) and the widely used 16sar/16sbr and 16sar-intsf (Palumbi, 1996; Parmakelis et al., 2008) primers, respectively. The primer pairs 18sai/18sbi and 18Aimod/700R (Dreyer and Wagele, 2001; Raupach et al., 2009) were used for the amplification of 18s, and the 28sa/28sb pair (Whiting et al., 1997) was used successfully for all available samples. Finally, the protein coding NAK amplicons were targeted with NAK for-b/NAK rev 2 (Tsang et al., 2008) and the newly designed reverse primer NAK 638R: 5'- GGD RGR TCR ATC ATD GAC AT -3'.

All PCR reactions were performed in a Veriti thermal cycler (Applied Biosystems, USA) with the following common steps: a) initial denaturation for 5 min at 94 °C, followed by b) 5 cycles of 3 minutes equally separated at 94 °C/60 °C/72 °C, c) 5 cycles of 3 minutes equally separated at 94 °C/55 °C/72 °C, d) 10 cycles of 3 minutes equally separated at 94 °C/50 °C/72 °C, e) 10 cycles of 3 minutes equally separated at 94 °C/47 °C/72 °C, f) 10 cycles of 3 minutes equally separated at 94 °C/42 °C/72 °C, and g) a final extension step of 72 °C for 10 min. Beyond fresh specimens, this touchdown PCR approach with 50 cycles in total allowed us to successfully amplify genes from ill-preserved samples increasing specificity, sensitivity and yield, eliminating aspecific products (Korbie and Mattick, 2008).

The final reaction volume in all cases was 20 μL, and consisted of 0.1 μL of Kapa Taq DNA Polymerase (5U/μL), 1.2 μL of 25 mM MgCl2, 2 μL of Kapa PCR buffer A, 0.6 μL of 10 mM dNTP (Kapa) 0.6 μL of each primer (10 μM) and >10 ng of DNA template. PCR product purification was made using Qiaquick Purification Kit (Qiagen, Germany) under manufactures protocol instructions. Both DNA strands of purified products were sequenced at Macrogen facilities (Amsterdam, The Netherlands).

Table 1: Species, locality of origin, available sequence data from targeted genes, and Genbank accession numbers of individuals used in the molecular phylogenetic analyses.

Species *(code)*	*Locality*	*Genes*					Acc. No
		COI	16s	18s	28s	NAK	
Porcellionidae							
Proporcellio vulcanius (Verhoeff, 1908) (1)	Cyprus (Larnaca)	√	√		√	√	MG887933/MG887948/-/MG887988/MG887906
Agabiformius excavatus Verhoeff, 1941 (2)	Cyprus (Paphos)		√	√	√	√	-/MG887955/MG887969/MG888009/MG887921
A. excavatus (3)	Cyprus (Paphos)		√			√	-/MG887956/-/-/MG887922
Porcellio laevis Latreille, 1804 (4)	Cyprus (Lemesos)	√	√	√	√	√	MG887936/MG887957/MG887986/MG887993/MG887913
P. laevis (5)	Cyprus (Lemesos)	√	√	√	√	√	MG887937/MG887958/MG887987/MG887994/MG887914
Porcellionides pruinosus (Brandt, 1833) (6)	Cyprus (Larnaca)	√	√		√	√	MG887934/MG887949/-/MG888010/MG887907
P. pruinosus (7)	Cyprus (Larnaca)	√	√		√	√	MG887935/ MG887950/-/ MG887989/MG887908
Leptotrichus kosswigi Strouhal, 1960 (8)	Cyprus (Paphos)				√	√	-/-/-/MG888013/MG887915
L. kosswigi (9)	Cyprus (Paphos)		√	√	√	√	-/MG887963/MG887970/MG888014/MG887916
Porcellio nasutus Strouhal, 1936 (10)	Greece (Parnon)	√	√		√	√	MG887944/ MG887953/-/MG887998/MG887910
P. nasutus (11)	Greece (Parnon)		√	√	√	√	-/MG887954/MG887980/MG887999/MG887911
Tura sp. (12)	Kenya (Mombasa)	√	√	√	√	√	MG887946/ MG887966/MG887983/MG888001/MG887920
Caeroplastes porphyrivagus (Verhoeff, 1918) (13)	France (Toulon)	√		√	√		MG887932/-/MG887981/ MG887990/-
Uramba triangulifera Budde-Lund, 1910 (14)	Kenya (Aberdare National Park)		√		√	√	-/ MG887961/-/MG888002/MG887923
Thermocellio sp. (15)	Tanzania (Dar es Salaam)		√		√		-/ MG887962/-/MG887995/-
Lucasius pallidus (Budde-Lund, 1885) (16)	Italy (Sardinia)			√	√	√	-/-/MG887974/MG887992/MG887917
Mica tardus (Budde-Lund, 1885) (17)	Italy (Sardinia)		√		√		-/ MG887959/-/MG887996/-
Acaeroplastes melanurus melanurus (Budde-Lund, 1885) (18)	Italy (Sardinia)	√	√	√	√	√	MG887945/MG887960/MG887982/MG887991/MG887912
Soteriscus laouensis Taiti and Rossano, 2015 (19)	Morocco (Tirinesse)	√	√	√	√	√	MG887931/MG887964/MG887975/MG887997/MG887918
Brevurus masandaranus Schmalfuss, 1986 (20)	Iran				√	√	-/-/-/MG888008/MG887919
Porcellionides cilicius (Verhoeff, 1918) (21)	Cyprus (Nicosia)					√	-/-/-/-/MG887909
Trachelipodidae							

Levantoniscus bicostulatus Cardoso, Taiti and Sfenthourakis, 2015 (22)	Cyprus (Paphos)			√	√	√	-/-/MG887976 /MG888000/MG887928
Trachelipus aegaeus (Verhoeff, 1907) (26)	Greece (Naxos)	√	√	√		√	EF659961/KF891440/ MG887984 /-/MG887925
Agnaridae							
Hemilepistus klugii (Brandt, 1933 (23)	Iran (Isfahan)	√	√	√	√	√	MG887938/MG887951/ MG887978 /MG888011/MG887926
H. schirazi Lincoln, 1970 (24)	Iran (Shahreza)	√	√	√	√	√	MG887939/MG887952/ MG887979 /MG888012/MG887927
Agnara madagascariensis (Budde-Lund, 1885) (25)	U.A.E.			√	√	√	-/-/MG887977 /MG888003/MG887924
Armadillidiidae							
Armadillidium vulgare (Latrteille, 1904) (27)	Cyprus (Limassol)	√	√	√	√		KR424609/AJ419997/ MG887972/MG888006/-
Schizidium fissum (Budde-Lund, 1885) (28)	Cyprus (Paphos)			√	√		-/- /MG887973/MG888005/-
Philosciidae							
Chaetophiloscia elongata (Dollfus, 1884) (29)	Italy (Sardinia)	√	√	√	√	√	KJ668161/AJ388091/MG 887971/MG888004/- /MG887929
Scyphacidae							
Actaecia euchroa Dana, 1853 (30)	New Zealand	√	√	√	√	√	GQ302701/AJ388093/M G887985/MG888007/M G887930

Alignments and genetic divergence

Sequence chromatograms were manually edited and assembled with CodonCode Aligner (v. 3.7.1; CodonCode Corp., USA). Separate multiple alignments for each gene/data set were performed using MAFFT v.7 (Katoh et al., 2002). Our data were further enriched by a limited number of publicly available NCBI GenBank mtDNA sequences (Table 1). The final concatenated data set was partitioned by gene into five distinct data blocks. The optimal nucleotide substitution models were identified using PartitionFinder v.1.1.1 (Lanfear et al., 2012). Three independent runs in PartitionFinder were applied, using the greedy search algorithm with linked branch lengths in calculations of likelihood scores under the Bayesian Information Criterion (BIC). The difference between these three runs was the restriction of candidate models to only those that are implemented in MRBAYES v.3.2.6 (Ronquist et al., 2012), BEAST v. 2.3.0 (Bouckaert et al., 2014) or RAxML v. 8.1.21 (Stamatakis, 2014). Models that included both gamma distribution and invariable sites were neglected (Yang 2006).

Phylogenetic analyses

Construction of phylogenetic trees was conducted using Bayesian Inference (BI) and Maximum Likelihood (ML) methods. The analysis of BI was implemented in MRBAYES v. 3.2.6 (Ronquist et al. 2012) with four independent runs and eight chains per run for 3×10^7 generations, with a sampling frequency of 100. Consequently, the summaries of BI were based on 3×10^5 sampled trees from each run. The convergence and stationarity of each run was evaluated by monitoring the average standard deviation of split frequencies of the four simultaneous and independent runs in MRBAYES, and further by inspection of generation versus log probability of the data plot viewed in TRACER v.1.5.0 (Rambaut and Drummond, 2007). The -ln value reached stationarity well before pre-requested 10^7 generations. From the sampled trees, 25% were discarded as burn-in phase. Therefore a majority rule consensus tree relied on 300,004 trees and posterior probabilities were calculated as the percentage of samples recovering any particular clade (Huelsenbeck and Ronquist, 2001).

RAxML (v. 8.1.21) (Stamatakis, 2014) was recruited for Maximum Likelihood analyses which were conducted using the RAxMLGUI v.1.5 platform (Silvestro and Michalak, 2012). The GTR+G model of evolution was used for the estimation of parameters for each partition. The optimum ML tree was selected after 500 iterations and the reliability of the branches was assessed by 1,000 thorough bootstrap replicates (Felsenstein, 1985).

Clock calibration and divergence time estimation

Molecular dating of clades was inferred using BEAST v. 2.3.0 (Bouckaert et al., 2014), The appropriate model of nucleotide substitution, as indicated by PartitionFinder under the BIC criterion was implemented for each marker in our partitioned analysis. Due to the absence of reliable geological or fossil data related to taxa included in our analyses, time of divergence was calibrated based on available gene-specific substitution rates. More specifically, the substitution rates of the mitochondrial genes 16s and COI were used as reported from previous studies for isopods (Held, 2001; Kamilari et al., 2014; Poulakakis and Sfenthourakis, 2008). Clock rate was set at 0.0007 (substitutions per site per Myr) for 16s and 0.0082 (min rate 0.0078, max 0.0086) for COI.

Four independent runs were performed for 100 million generations, each sampling every 5,000th generation. An uncorrelated lognormal relaxed clock under a Yule tree prior and the default options for all other prior and operator settings, were used in each case. Trace plots were inspected in order to compare the divergence estimates across runs and ensure the convergence of Markov Chain Monte Carlo chains using TRACER v. 1.5 (Rambaut and

Drummond, 2007). Resulting log files were combined, after removing 10% as burn-in, using LOGCOMBINER v.2.3.0 (Bouckaert et al., 2014). A maximum clade credibility tree exhibiting the means of node heights was constructed with TREEANNOTATOR v.2.3.0 (Bouckaert et al., 2014).

RESULTS

At least four out of five targeted genes were successfully amplified and sequenced for the great majority of available individuals, with final DNA extraction yield over 20 ng/μl and A260/A280 purity rate over 1.5. Since some important samples were old (collected more than two decades ago, mainly from Africa) or ill-preserved for a long time (i.e., in 70% alcohol) we didn't manage to retrieve sequences from all targeted genes. However, specimens not represented by all gene fragments were also included in the analyses. The final concatenated alignment obtained consisted of 3,841 base pairs (bp). More details about the aligned sequences length, conserved, variable and parsimony-informative sites for each gene are given in Table 2.

Table 2: Aligned bases length, conserved, variable and parsimony-informative sites for each gene used in the present analysis.

Gene	Alignment length (bp)	Conserved sites	Variable sites	Parsimony informative sites
COI	655	214	434	302
16s	454	151	277	211
18s	863	417	332	177
28s	1167	314	827	567
NAK	702	512	188	109

Available sequences were separated in different groups at the genus level except for *Porcellio* species which were treated as different groups due to the alleged non-monophyly of the genus. Between groups p-genetic distances for each gene are given in supplementary material (Appendix II). The best-fit nucleotide substitution models for each partition/gene selected under the BIC criterion were (for both MRBAYES and BEAST) the HKY+G+X, HKY+G+X, TRNEF+G, TRN+G, TRN+G+X and GTR+G+X for COI, 16s, 18s, 28s and NAK genes, respectively. The selected model under --raxml commandline option at PartionFinder was the GTR+G (-ln =26511.0556641) for all genes.

Maximum Likelihood and Bayesian Inference analyses (implemented both in BEAST and MRBAYES) resulted into phylogenetic trees with similar, well-supported topologies. Given the congruence among the results of the two methods, only the Bayesian tree is presented herein (Figure 1). The ML tree is given in Appendix II (Figure S1). The separate analysis of different gene markers showed that the concatenated tree topology is mainly determined by nuclear genes. Missing data, and possibly also the depth of the phylogeny, led to largely unresolved trees for mtDNA markers. Nevertheless, these were

used mainly to estimate node dates based on published mutation rates. The poor mtDNA-based resolution did not affect the final tree, given that the tree based solely on nuclear genes (see Appendix II) has identical topology.

Our results provide evidence against the monophyly of both the family Porcellionidae and the genus *Porcellio*. *Brevurus* appears to belong to a supported distant clade, external to that formed by the remaining Porcellionidae+Trachelipodidae+Agnaridae. *Leptotrichus* is an external branch to Agnaridae + part of Porcellionidae. Monophyly of Agnaridae is supported. *Levantoniscus* forms the sister clade of all monophyletic Porcellionidae. Finally, Armadillidiidae branches early in the tree, not showing any close relationship to Porcellionidae.

The African genera *Tura* and *Uramba* are sister taxa sharing a common ancestor at around 22.2 mya (95% HPD 12.1 - 33.5 mya) and are grouped with *Agabiformius*. On the other hand, *Thermocellio,* also distributed in Kenya and the neighboring Tanzania, appears to be more closely related to *Porcellio laevis*, native to Europe and North Africa. Another African/Atlantic genus, *Soteriscus*, forms a well-supported clade with *Lucasius* and *Mica* that are distributed in Africa and on some Mediterranean islands. The Mediterranean genera *Acaeroplastes*, *Caeroplastes*, *Porcellionides* and *Proporcellio*, together with part of *Porcellio,* are grouped in the most derived clade that diverged at around 27 mya.

The genus *Porcellio* as currently perceived is represented in two well-supported separate clades. *P. laevis* groups with *Thermocellio* while *P. nasutus* with *Acaeroplastes* in a clade also including *Caeroplastes*.

Genetic distances between Porcellionidae genera (or species in the case of the non-monophyletic *Porcellio*) varied significantly among genes. The range of variation per gene is: COI: 16.9-50.3 %; 16s: 16.9-36.5 %; 18s:3.6-28.5 %; 28s:0.4-44.2%; NAK: 2.3-9.1%. The p-distances between *Trachelipus* and *Agnara* for NAK, and *P. laevis* and *Lucasius* for 18s, could be artifacts due to the comparatively shorter sequence length in *Agnara* and *P. laevis*, respectively (see Appendix II).

It is worth noticing also that minimum and maximum distances are not exhibited by the same taxa for all genes. More specifically, highest / lowest genetic divergence is found between the following groups: *Tura* - *Porcellio nasutus* / *Soteriscus* - *Leptotrichus* (16s), *Porcellio laevis* - *Lucasius* / *Proporcellio* - *Porcellionides* (COI), *Agabiformius* - *Porcellio nasutus* / *Caeroplastes* - *Acaeroplastes* (18s), *Brevurus* - *Thermocellio* / *Porcellio laevis* -

Thermocelio (28s) and *Uramba* - *Brevurus* / *Proporcellio* - *Porcellionides* (NAK). The allegedly congeneric *Porcellio* species never exhibit a minimum genetic distance.

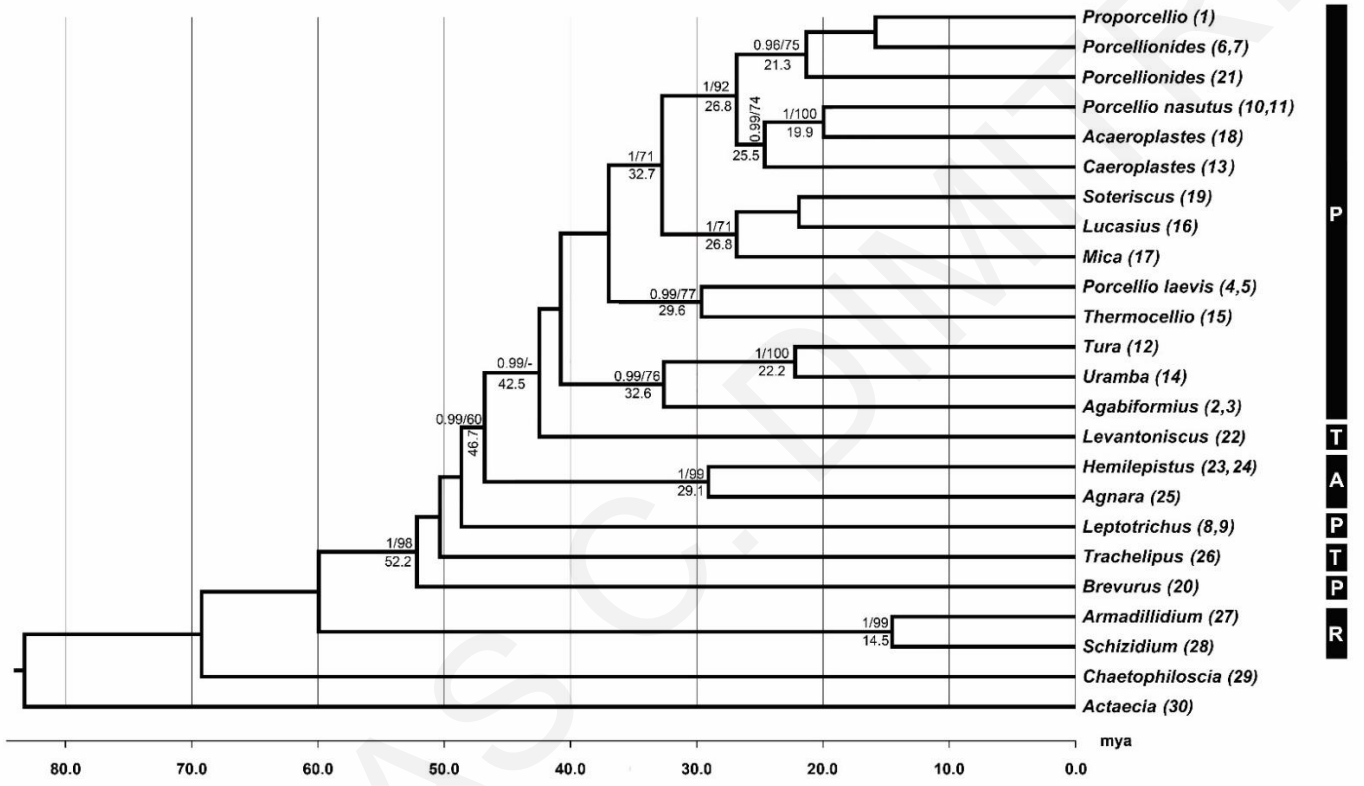

Figure 1. Dated phylogram based on concatenated data set including five genes (COI, 16s, 18s, 28s, NAK), generated using a relaxed lognormal clock in BEAST. BI posterior probabilities (>0.9) and ML bootstrap values (>60) are presented above the nodes. Estimated mean divergence time is given below the nodes only where nodes are statistically supported or the topology was identical between BI, ML and BEAST analyses. Subclades including individuals from more than one species have been collapsed to genus level, since all (except *Porcellio*) were monophyletic. Abbreviations: P. Porcellionidae, T. Trachelipodidae, A. Agnaridae, R. Armadillidiidae. Numbers in parentheses after each taxon name refer to numbering of taxa in Table 1.

DISCUSSION

This is the first comprehensive study aiming to resolve phylogenetic relationships among Porcellionidae genera using a multi-locus approach, thus increasing reliability of results. Our findings undermine the monophyly of both the family Porcellionidae and the genus *Porcellio*, in line with suggestions by previous authors (Mattern, 2003; Michel-Salzat and Bouchon, 2000; Schmalfuss, 1989; Schmidt, 2003, 2008).

The extremely high genetic distances, which reached up to 50.3 in mtDNA and 44.2 in nDNA, are confirming the vast divergence among taxa within Porcellionidae. Observed inconsistencies of group distances among different genes highlight the usefulness of the multi-locus approach followed herein for a reliable phylogenetic reconstruction of the taxa examined.

In view of the herein estimated phylogeny, a monophyletic Porcellionidae should exclude *Brevurus* and *Leptotrichus*. Moreover, the supposedly subtle morphological differences between *Leptotrichus* and *Agabiformius* that had led to a presumed sister-group relationship between these genera, are misleading, since they are found to be very distant (Schmalfuss, 2000; Verhoeff, 1908). *Brevurus* has been proposed as a possible synonym of *Porcellium* Dahl, 1916 (a genus of Trachelipodidae) (Khisametdinova and Schmalfuss, 2012.), an hypothesis that cannot be evaluated in view of our results.

The genus *Levantoniscus*, tentatively assigned to Trachelipodidae (Cardoso et al., 2015), has been found to be closer to the monophyletic subgroup of Porcellionidae. Given that the genus appears as the sister clade of all remaining monophyletic Porcellionidae, we cannot propose the assignment of this taxon into the same family, given that no known morphological characters can be used as synapomorphies of such a taxon. The characters considered as autapomorphies of *Levantoniscus* by Cardoso et al. (2015) could as well define a separate new family. A more inclusive phylogeny is required before we can decide on its familial status, given also the lack of robust synapomorphies defining Trachelipodidae, a family in need of a sound revision.

As indicated by the tree topology, Porcellionidae is more closely related to Trachelipodidae and Agnaridae rather than Armadillidiidae. A similar result has been found by Lins et al. (2017), even though these authors had included only two species in two genera (*Porcellio* and *Porcellionides*) of Porcellionidae in their analysis. It is evident that morphological characters traditionally used in Oniscidea systematics, such as the structure

of pleopodal lungs, the number of flagellar segments and the head structure, do not seem to provide adequate evidence that support a robust taxonomy, at least not in all cases.

In conclusion, the monophyly of Porcellionidae as currently perceived cannot be supported by molecular evidence. Of course, we still need to identify phenotypic synapomorphies defining the family, since the characters used so far cannot be considered as valid. In addition, the genus *Porcellio* needs to be revised, as it appears to be polyphyletic, comprising of at least two separate groups.

The monophyletic subgroup of Porcellionidae seems to have an African origin, diverging at the end of the Palaeogene (Oligocene) and then differentiating further during the Miocene. Based on the cladochronology estimated herein, more basal cladogenetic events, leading to the branching of other related families, happened in the Eocene. This chronology is compatible with the very old (Mesozoic) origin of Oniscidea suggested by (Broly et al., 2015, 2013).

CHAPTER 4

Intra-island diversification of a terrestrial isopod species on an oceanic Mediterranean island reveals cryptic speciation and multiple colonizations

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Intra-island diversification of a terrestrial isopod species on an oceanic Mediterranean island reveals cryptic speciation and multiple colonizations

ABSTRACT

Cyprus is an oceanic island that has been isolated for at least 5.3 Mya from surrounding continental regions, while it is still doubtful whether it had been connected to mainland even during the Messinian Salinity Crisis. *Armadillo officinalis*, is a terrestrial isopod distributed throughout the Mediterranean and all over the island, without showing any evidence for significant divergence either in morphology or in genetic structure. In order to explore possible intra-island divergence of this species, genome-wide ddRAD, as well as Sanger sequencing data for four mitochondrial and three nuclear loci were generated. The final dataset includes individuals from 71 populations from Cyprus, neighbouring continental regions, and other Mediterranean countries. Phylogenetic reconstructions and population structure analyses reveals the presence of five distinct genetic lineages/clusters within Cyprus from which four are endemic to the island. Observed genetic divergence is not reflected in the morphology of the species despite the occurrence of a distinct color morph in some Cypriot populations. The closest evolutionary relationship of Cypriot populations is with individuals from Israel, while a shallow evolutionary clade is present in countries around the Mediterranean. Cladochronological analyses date the origin of the species on the island at around ~6 Mya. Species delimitation and phylogenetic analysis support the existence of at least five discrete lineages across the study area. Our results highlight a combination of the islands' paleogeographic history with human effects in determining current patterns of genetic diversity in this putative super-species.

INTRODUCTION

In a recent account of the prospects of island biology studies 50 years after the probably most seminal ecological theory, MacArthur-Wilson equilibrium theory of island biogeography (ETIB), Warren et al. (2015) identified some of the most crucial under-explored subjects in urgent need of further research. Among these are questions related to clade differentiation and speciation patterns within islands, such as the role of arrival history in community assembly, the role of *in situ* evolution in ecosystem functioning, the role of gene flow in speciation, and why some lineages are richer than others. Such questions are expected to be addressed by using both phylogeographic and population genomic/genetic data (Gillespie, 2016). Despite the proliferation of phylogeographic and phylogenetic studies in the past few decades, research on evolutionary dynamics within isolated islands has not kept pace with larger-scale inter-island studies (Shaw and Gillespie, 2016). Regarding Oniscidea in particular, already published works identified high genetic divergence at species or genus level among individuals distributed at geographically close areas including isolated islands and islets (Kamilari et al., 2014; Klossa-Kilia et al., 2006; Parmakelis et al., 2008; Poulakakis and Sfenthourakis, 2008). Nevertheless, diversification patterns within an island have not been addressed so far.

Cyprus, located in the eastern Mediterranean Sea basin lies in one of the global biodiversity hotspots (Myers et al., 2000). It has been isolated for at least 5.3 Ma from surrounding continental regions and probably was never connected with any of these, making it one of the very few, and by far the largest, oceanic islands in the Mediterranean (Constantinou and Panagides, 2013).

The island has taken its modern form recently (mid- to late Pleistocene) through the establishment of a land-bridge connection (Mesaoria plain) between the two formerly isolated islands that today make the two main mountain ranges (Troodos at the central-western part, and Pentadaktylos at the north-northeastern part; Figure 1). These mountains first emerged from the sea surface several million years ago (ca. 20-15), and remained separate for most of their geological past. Therefore, we might expect to find in modern populations some signal of this past isolation and/or biotic interchanges and population admixture between these former islands.

The terrestrial isopod species *Armadillo officinalis* Duméril, 1816 is distributed along the Mediterranean and the western Black Sea coasts (Schmalfuss, 2003). In fact it is among the most commonly found Oniscidea species, a taxon characteristic of

Mediterranean-type ecosystems, the 'animal equivalent of the olive tree' (Boyko et al., 2019; Schmalfuss, 1983, 1996). Morphologically, it is a well-defined taxon with low morphological variation (Schmalfuss, 1996). *A. officinalis* is one of the two species of the genus found so far in Cyprus (Schmalfuss 1996; Sfenthourakis pers. comm). It inhabits areas with a variety of substrates (sandy, silty-clayey or rocky) and vegetation (Messina et al., 2014), and is considered a xeric species exhibiting adaptations that limit water loss, such as a thick tegument, a tight closure into a ball when conglobating, nocturnal habits and a relatively long duration of the moult cycle compared to other terrestrial isopods (Montesanto and Cividini, 2018). Recently, the species has been used as a model organism in ecotoxicological and bioacoustic research studies (Agodi, 2015; Cividini et al., 2020).

The present study aims to identify whether there is geographic structure among conspecific populations of *A. officinalis* within Cyprus, also using populations from neighbouring countries for comparison. Given the limited dispersal ability of terrestrial isopods and the landscape heterogeneity, complex geological history and long isolation of Cyprus, we could expect high genetic diversification between mainland and island populations, as well as among populations from different localities within Cyprus, especially those that can be considered as terrestrial habitat "islands". Time calibrated phylogenies will allow us to evaluate the role of human activities and the island's paleogeography in determining present patterns. Our hypotheses are that: i) the paleogeography of the island (two distinct islands recently connected) is reflected in patterns of genetic divergence, ii) the Messinian Salinity Crisis (~ 6 - 5.3 Mya), facilitated arrival of the species on the island, as supported by studies on other taxa (Poulakakis et al., 2013; Sfenthourakis et al., 2017), iii) the north-central part of the eastern Mediterranean coasts is the source of introduction of the species to the island, and iv) *in situ* differentiation is driven mainly by geographic and habitat isolation within the island.

MATERIALS & METHODS

Sampling

At least three individuals of the targeted species were collected from 54 populations distributed all over Cyprus (Figure 1). Sampling effort was extended to Greece from where we collected individuals from eight different populations. Collected material was placed in >96% alcohol and stored at -20°C until further elaboration. The final dataset includes also samples from five populations from Turkey and Israel, kindly sent to us by colleagues, while data from individuals distributed in Italy (acc. no FN824106 - FN824110) and Tunisia (acc. no AJ388094) were retrieved from NCBI GenBank (Table S1; Appendix III).

Whole genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's proposed protocol. Retrieved DNA quality and quantity were assessed using agarose gel electrophoresis (TAE 1.5%), the NanoDrop 2000/200c (Thermo Fisher Scientific Inc., USA) and Qubit 4 Fluorometer (Invitrogen™, Thermo Fisher Scientific, Waltham, USA)

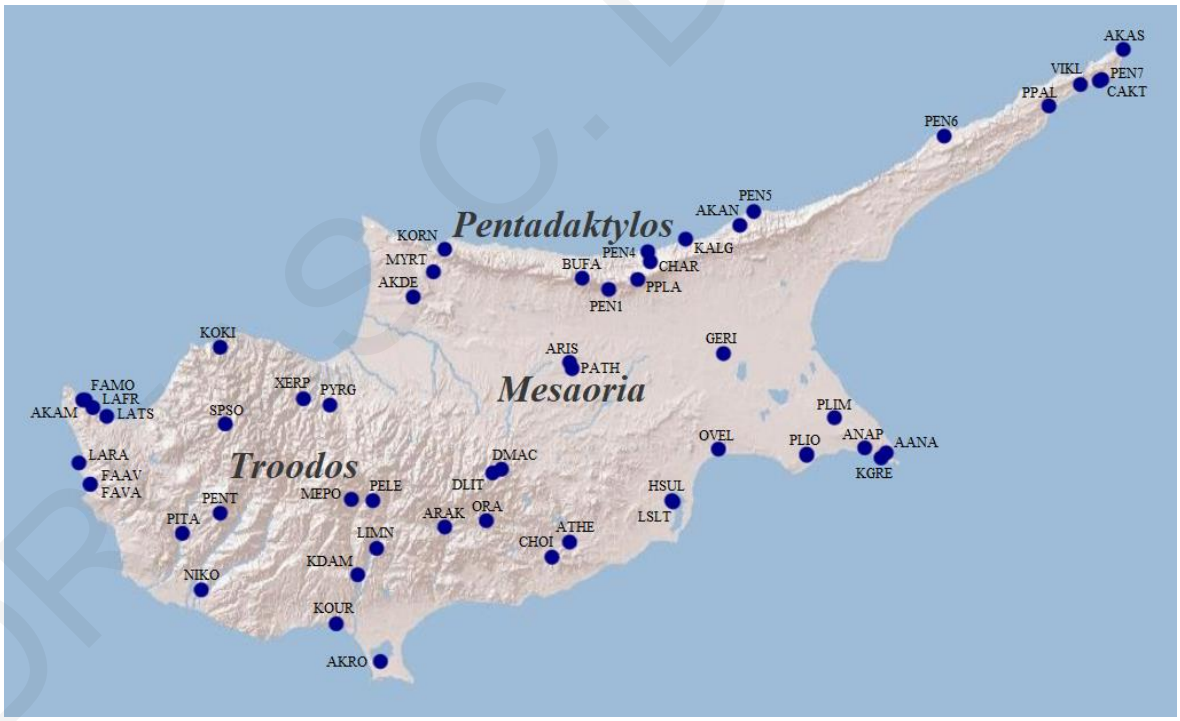

Figure 1. *A. officinallis* populations collected from Cyprus. More details about location codes are given in Table S1 (Appendix III).

Targeted loci with Sanger

Seven genetic loci, four mitochondrial and three nuclear, were amplified using common PCR procedures. Namely, the mitochondrial Cytochrome c oxidase subunit I (COI), Cytochome b (Cytb), 12s ribosomal RNA (12s) and 16S ribosomal RNA (16s) genes were targeted from three or more individuals of each population. Primer pairs LCO1490: 5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3' / IsoCoiRint: 5'- GCY CCY GCY AAW ACA GGK ARD GA -3' (Folmer et al., 1994; Koutmos, 2008) , CB3F: 5'- GAG GAG CAA CTG TAA TTA CTA A -3' (Barraclough et al., 1999) / CB4R: 5'- AAA AGA AAR TAT CAT TCA GGT TGA AT -3', 12SCRF: 5'- GAG AGT GAC GGG CGA TAT GT -3' / 12SCRR: 5'- AAA CCA GGA TTA GAT ACC CTA TTA T -3' (Hanner and Fugate, 1997) and newly designed 16sTRF: 5'- CTG ACT GTG CTA AGG TAG CA -3' / 16sTRH: 5'- CGG TYT GAA CTC AGA TCA YGT GA -3' were used for this purpose. Thermocycling conditions were adapted from Dimitriou et al. (2018). A subset of individuals representing all divergent genetic clades, as indicated by bioinformatics analysis, were selected for sequencing the more conserved nuclear genes. More specifically, the nuclear genetic markers 18s and 28s rRNA genes, and the protein-coding Sodium-Potassium Pump (NAK) were amplified following already published protocols (Dimitriou, 2018; Dimitriou et al., 2019). PCR products were purified and sent for sequencing at Macrogen facilities (Amsterdam, The Netherlands). All sequences generated within the framework of this study will be deposited in GenBank before publication.

Genomic library preparation

In total, 168 individuals from 24 populations were selected for the preparation of the genomic libraries. The ddRAD libraries were constructed following the protocol described by Peterson et al. (2012) with some minor modifications described in Lanier et al. (2015). The initial input amount of DNA was 300ng and EcoRI and MseI restriction enzymes were used to digest genomic DNA. Illumina sequencing adapters as well as a unique barcode was ligated to each specimen. Including attached oligos 375 to 475 bp long fragments were size-selected using Pippin Prep (Sage Science, Beverly, Massachusetts, USA) and then amplified via polymerase chain reaction (PCR) using the iProof High-Fidelity DNA Polymerase (Bio-Rad). Each library was sequenced in a separate HiSeqX lane (Illumina, San Diego, CA, USA; 150bp pair end reads) at Macrogen NGS facilities in South Korea (Seoul, South Korea).

Data processing

Sanger sequencing results were delivered as chromatograms and the authenticity of all PCR products was tested by the application of the Blast algorithm. Assemblages were generated using CodonCode Aligner (v. 3.7.1; CodonCode Corp., USA) and edits were made where necessary. Publicly available sequences of the confamiliar genus *Spherillo* Dana,1853 were retrieved and included in our analyses to serve as outgroup. More specifically, 16s and COI sequences of *S. dorsalis* and *S. obscurus* were used for this purpose.

Multiple sequence alignments for each gene were performed online using the MAFFT v.7 webserver (https://mafft.cbrc.jp/alignment/server/) following the Q-INS-I strategy for 18s, 28s, 16s, and 12s genes, as proposed for rRNA genes with secondary structure (Katoh et al., 2002). Produced alignments were fed to jMODELTEST v.2.1.1 (Darriba et al., 2012) for the selection of the best DNA substitution model according to the BIC Criterion.

Generated sequences divergence (p-distance) between and within predefined groups considering, i) geographic distribution, ii) statistical support on nodes of the constructed phylogenetic trees, and iii) mitochondrial species delimitation results, were calculated using MEGA v.6 (Kumar et al., 2008). Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenetic reconstructions were performed in MRBAYES v. 3.2.6 and RAxML-NG, respectively (Kozlov et al., 2019; Ronquist et al., 2012). BI analysis was run four independent times with eight chains per run for 10 million generations and 5 million generations in cases of Sanger and ddRAD data, respectively. Convergence among runs was monitored with TRACER v. 1.5 (Rambaut and Drummond, 2007). The reliability of ML results was evaluated by bootstrapping (Felsenstein, 1985).

The ML phylogenetic tree produced by the mtDNA dataset was fed to mPTP web server (available through: https://species.h-its.org/) where species delimitation analysis was performed (Kapli et al., 2017). Taking into account the species delimitation, preliminary results and the statistically well-supported monophyletic clades by BI and ML analyses, a subset of individuals representative of the taxon's genetic divergence were selected. The aforementioned nuclear genetic loci were sequenced for this subset and further analyses were performed on this dataset.

Raw Illumina reads were demultiplexed based on the unique sequence barcode used for each sample using iPyrad v 0.9.62 (Eaton and Overcast, 2020). Demultiplexed data were further processed setting the mindepth option to 6 and the clust_threshold to 0.9.

Additional data filtering aiming to compile a more phylogenetically meaningful dataset excluding a considerable amount of missing data as described by (Psonis et al., 2021) was applied. More precisely the filtering was run setting min_var to -1 and the min_taxa option to 23 aiming to retained loci with at least 23 unique sequences. Unlinked SNPs, with the fewest missing characters for each locus, were selected for the generation of the data supermatrix.

Divergence time estimation, Species tree and Species delimitation

Cladochronological estimations and species tree analysis were conducted using StarBEAST2 v 2.6.3 (Ogilvie et al., 2017). Regarding species tree analysis, specimens were divided into groups based on the aforementioned criteria. Molecular dating was calibrated using already published substitution rates for 16s and COI genes, estimated for other isopod taxa (Held, 2001; Kamilari et al., 2014; Poulakakis and Sfenthourakis, 2008). Analysis was let to run for 100 million generations four independent times, sampling a tree every 5000th generation. Convergence between runs was secured by plotting the log probability of the data in TRACER v 1.5 (Rambaut and Drummond, 2007). Generated log files were combined with LOGCOMBINER v 2.6. after discarding the first 10% of the produced trees, and a maximum clade credibility tree exhibiting the means of node heights was constructed using TREEANOTATOR v.2.6.3 (Ogilvie et al., 2017).

Species delimitation analysis based on all seven amplified genes was conducted in BPP v 4.1.3 (Flouri et al., 2018). Implemented analysis within the multispecies coalescent framework was run setting the number of samples (nsample) to 10^5 and burnin at 10%. The effect of θs and τ_0 priors on our results was evaluated by testing different combinations of these parameters, as suggested by the software developers.

SNP based species delimitation and species tree analyses were also conducted with SNAPP in BEAST2 v 2.6.3. Prior to species tree analysis, species delimitation/path sampling analysis was performed aiming to identify the best possible taxon sets for the dataset in hand. The analysis was run nine times, splitting available sequences to 24, 18, 12, 8, 6, 5, 4, 3 and 2 population sets, considering geographical and produced phylogenetic patterns. Path sampling was run with 24 steps for 100,000 MCMC iterations while pre-burnin was set to 10,000. The best option fitting our data was based on the calculated marginal L estimate (MLE) and Bayes Factors (BF). The expected divergence prior (theta) was set according to available sanger data while the speciation rate prior λ (Lambda) was calculated using the

Python script "yule.py" (http://www.phyletica.com). Species tree analysis was let to run for for 5 million generations performing 2 independent runs.

Population structure analyses

Filtered ddRAD data were used for the construction of a SNPs supermatrix that was fed to STRUCTURE v.2.3.4, aiming to identify patterns of population structure using a Bayesian clustering approach. Ten replicates of the analysis were run for K varying from 1 to 10 for 500,000 generations setting burnin to 100,000 on UCY Cluster using Structure threader (Pina-Martins et al., 2017). The K that best fits our data was determined monitoring the estimated mean Ln likelihood and the ΔK Evanno method (Evanno et al., 2005) employed in STRUCTURE HARVESTER (Earl and von Holdt, 2012). The consistency of the results between runs was tested with CLUMPAK (Kopelman et al., 2015). Based on the membership coefficient values (Q≥ 90%) admixed individuals were removed and new datasets comprising initially inferred clusters were created for a second hierarchical structuring.

Aiming to further evaluate the robustness of our results using an alternative approach, we also performed Discriminant Analysis of Principal Components (DAPC) which is a non-model based method not depended on the assumption of Hardy-Weinberg equilibrium. The analysis was implemented using R package Adegenet v.2.0.0 (Jombart and Ahmed, 2011). The optimal number of clusters was identified running K-means comparing clustering solutions using Bayesian Information Criterion (BIC).

Finally, the pairwise population fixation index (F_{ST}) was estimated using R package Hierfstat (Goudet, 2005). The WC model (Weir and Cockerham, 1984) was selected for the calculation of F_{ST}s while the statistical significance of the results was assessed through 100 bootstrap replicates.

RESULTS

Generated datasets

High quality genomic DNA was isolated from all available specimens with the final extraction yield exceeding 25 ng/μl and A260/A280 purity rate over 1.5 in all cases. Targeted loci were successfully amplified and sequenced for the great majority of specimens. The final concatenated aligned dataset of all Sanger sequenced loci consisted of 1,945 bp excluding, and 3,896 bp including nuclear genes. Regarding ddRAD sequencing, in total more than 1.23 billion reads were produced from both Illumina lanes used for all 168 individuals included in our analyses. Excluding individuals with prohibitively high missing data an average of 4,121,935 reads per sample were retained. Among the 115,512 identified loci, 11,687 were non variable. After filtering the final concatenated dataset consisted of 1,026,968 sites while the SNPs supermatrix consisted of 3,685 unliked base pairs.

Genetic divergence - Phylogenetic analyses

Genetic distances between predefined groups varied for each gene as follows: 12s: 4.47-10.72 %, 16s: 3.90-11.13 %, COI: 5.56-11.33 %, Cytb: 6.32-13.86 %, 18s:0.67-2.54 %, 28s: 0.92-2.28 %, NAK: 0.00-0.79 %. With the exception of COI, where the maximum genetic distance was observed between GR and ISR groups, in all other cases the highest values were calculated between GR and Cypriot groups (Figure 2). On the other hand, excluding the 28s gene, where the minimum genetic distance was between GR and CY5, for all other loci the lowest values were exhibited among clades from Cyprus (Tables S2, S3, S4, S5).

BI and ML phylogenetic reconstructions based on the mitochondrial genome resulted into identical topologies concerning the statistically well-supported clades (Figure 2). Groupings of populations at the produced trees based on both types of datasets revealed a pattern of geographic differentiation. More specifically, five statistically well-supported monophyletic clades seem to be present on the island (Figures 2, 4). These divergent genetic lineages are distributed, i) at the southern part of Troodos, ii) across the Pentadaktylos range, iii) along the Mesaoria plain, iv) at the western part of Troodos range and v) all over the island and surrounding continental areas (Figure 3). In total, three genetically "misplaced" individuals were found, one belonging to CY1 clade at the western part of the island and two individuals belonging to CY2 clade at the south-easter part of the island instead of the north. Individuals of the widespread CY5 clade are forming a shallow statistically well-supported clade representing populations from Cyprus, Israel, Turkey, Greece, Italy and Tunisia

(Figure 3). It is worth noticing that none of the other clades found on Cyprus showed genetic similarity with any of the 17 non-Cypriot populations included in analyses based either on Sanger or on ddRAD data. On the other hand, although Sanger data indicate the existence of two distinct genetic lineages, one occurring only in Israel and one in Greece and Turkey, the same result is not supported by the genomic data. In fact, the individuals that formed the GR clade are grouped with the rest individuals representing the "Mediterranean" CY5 clade (Figure 4). Both datasets though indicate a closer phylogenetic placement of the population from Israel with the Cypriot lineages than the CY5 clade.

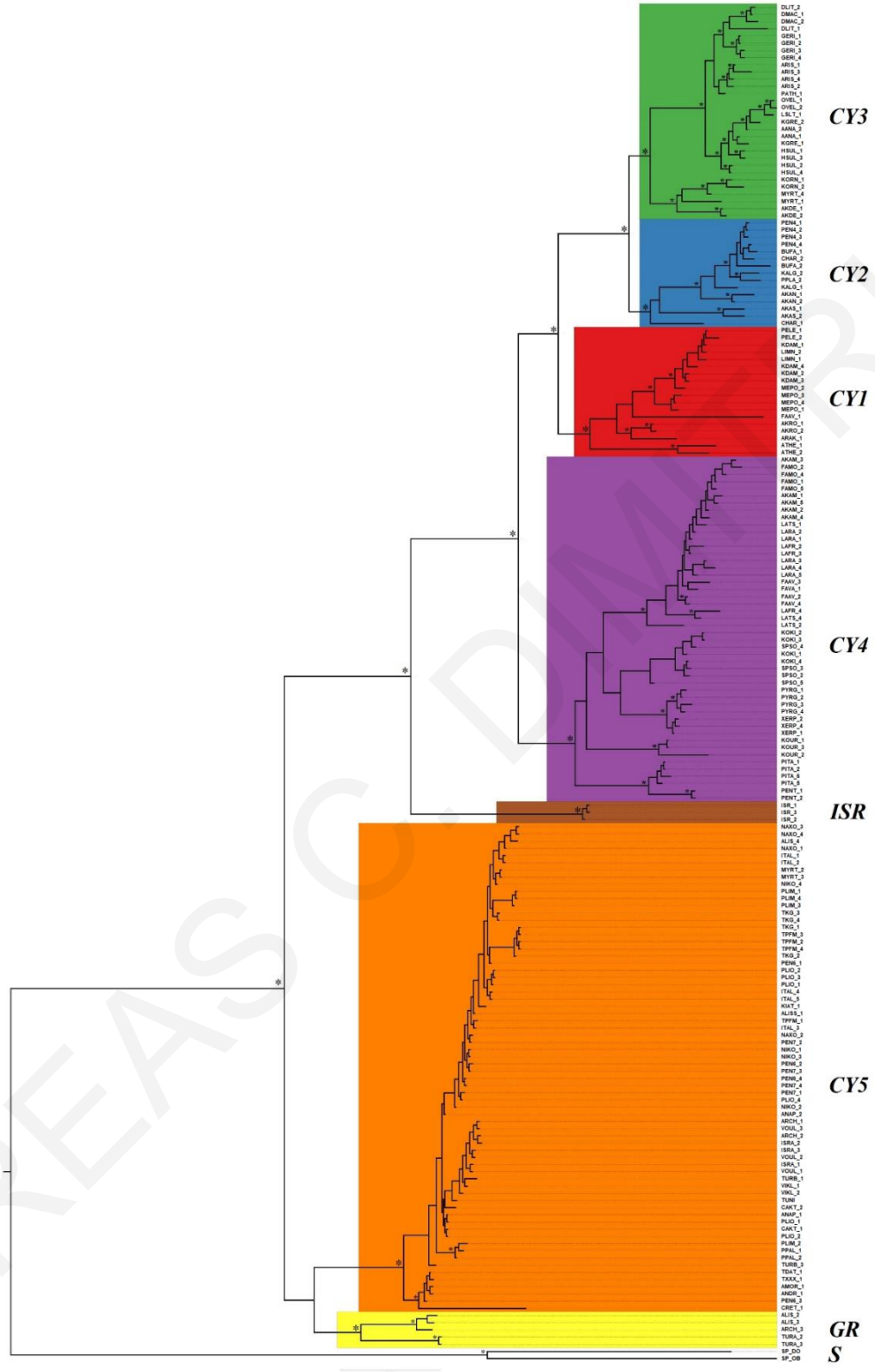

Figure 2. 50% majority-rule consensus tree from the Bayesian Inference (BI) analysis constructed using COI, 16s, 12s and Cytb markers. S tars on the nodes indicate identical topology between BI and ML analyses, with bootstrap values >80 and posterior probabilities >0.9. Colours correspond to localities presented in Figure 3.

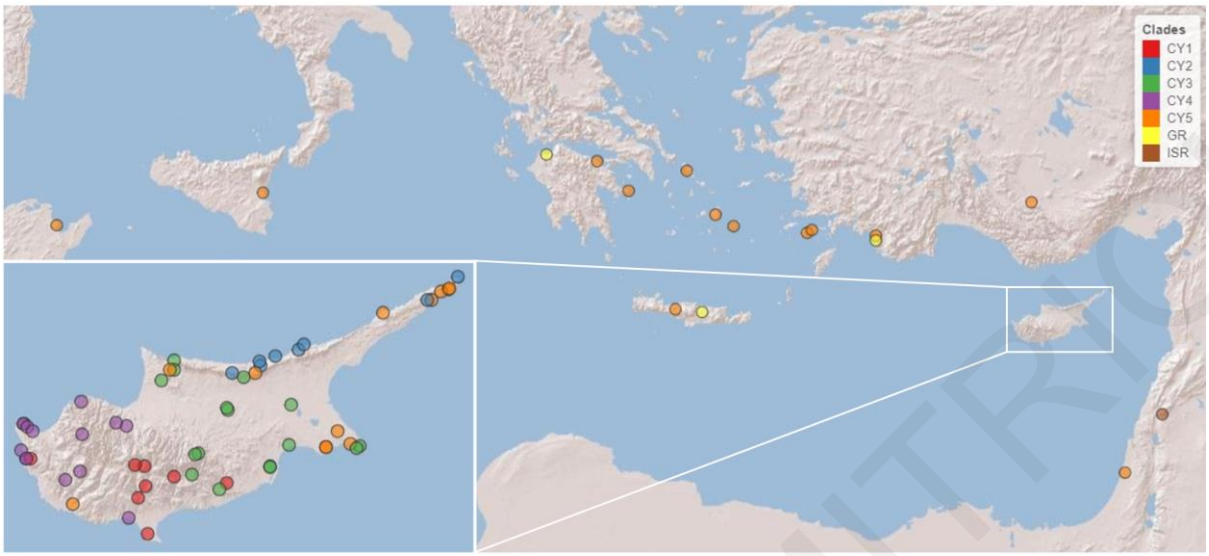

Figure 3. Map showing the geographic origin of all specimens used. Different colours correspond to genetic lineages revealed by phylogenetic analyses (Figure 2).

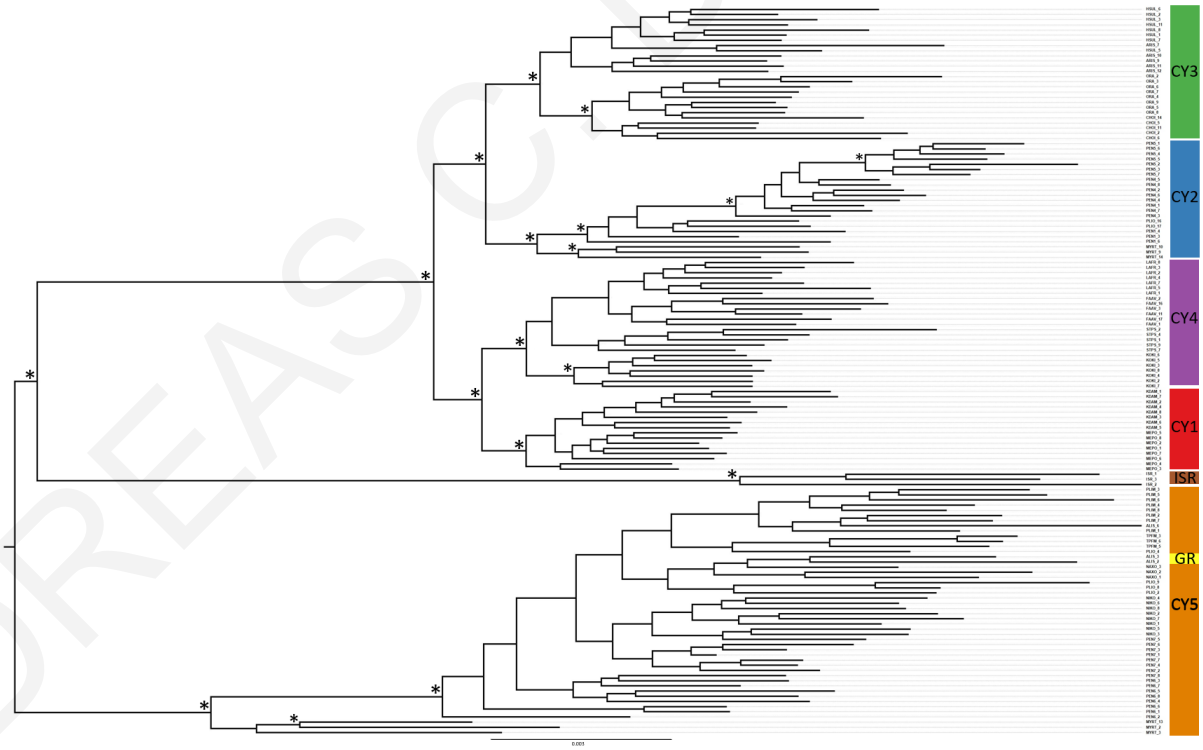

Figure 4. Maximum likelihood phylogenetic tree (ML) based on the concatenated ddRADseq dataset. Asterisks on the nodes represent bootstrap values >98 and BI posterior probability equal to 1.

Cladochronology and Species Delimitation

Based on Sanger data, cladochronological analysis dates the origin of the taxon on the island at ~6 Mya (95% HPD: 4.13-8.14) while the Cypriot populations (CY1 - CY4) sharing a common ancestor with the individuals from Israel, at ~15.7 Mya (95% HPD: 10.98-19.64). The widely distributed in the circum-Mediterranean area clade CY5 appears to be more closely related to the GR clade found in Greece and Turkey, and has diverged at ~7 Mya (Figure 5). Regarding species delimitation, the majority of analyses under different tested priors supported the existence of five genetic *A. officinalis* lineages (posterior probabilities >95). One of these lineages is representing CY1, CY2 and CY3 clades, whereas the other clades (CY4, CY5, GR, ISR) are representing distinct lineages. Alternatively, setting the priors to $\theta \sim IG(21, 0.2)$, $\tau_0 \sim IG(3, 0.004)/\theta \sim IG(3, 0.002)$, $\tau_0 \sim IG(21, 0.004)$, or $\theta \sim IG(3, 0.002)$, $\tau_0 \sim IG(3, 0.0004)$ or $\theta \sim IG(21, 0.02)$, $\tau_0 \sim IG(3, 0.004)$ the analysis comes up with seven (CY1, CY2, CY3, CY4, CY5, ISR, GR), three (CY1CY2CY3 CY4 CY5GRISR) and a single delimited species, respectively.

Taking into account the impact of missing data on the path sampling, only 15% of missing data were allowed to be present at the SNAPP input file. For this purpose the initial dataset was filtered using poppr R package (Kamvar et al., 2014). Marginal Likelihood Estimates (MLE) and Bayes Factor (BF) favored the three "species" scenario (Table S6; Appendix III) where the clades CY5, ISR and CY1 - CY4 were given as separate units. Based on this delimitation the constructed species tree supported the closer relationship of the lineages found on Cyprus with the CY5 clade.

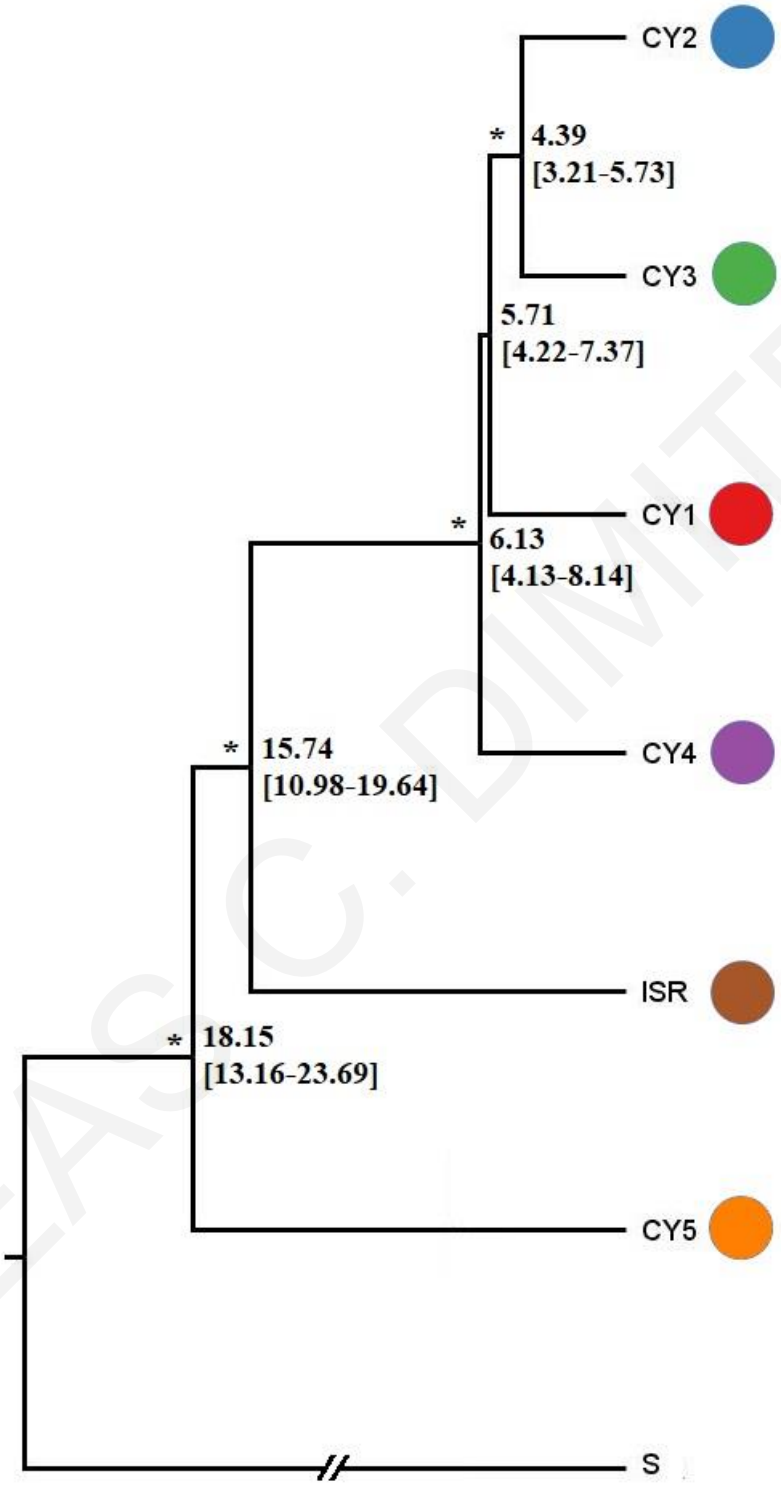

Figure 5. Dated species tree based on the concatenated data set including seven genes (COI, 16s, 12s, Cytb, 18s, 28s, NAK), generated using a relaxed lognormal clock in BEAST. Stars on the nodes indicate strong statistical support (posterior probability >0.9).

Population structure

After the completion of the first hierarchical level of clustering analysis implemented in Structure, examined individuals were, in every repetition, assigned in two groups (best K =2). Only eight individuals (5.5%) exhibited a mixed ancestry profile from which seven are coming from two populations, Israel (ISR) and Myrtou (MYRT). It is worth noticing that one of the two clusters included only individuals collected from Cyprus while at the second cluster populations from Cyprus, Greece and Turkey are grouped together (Figure 6).

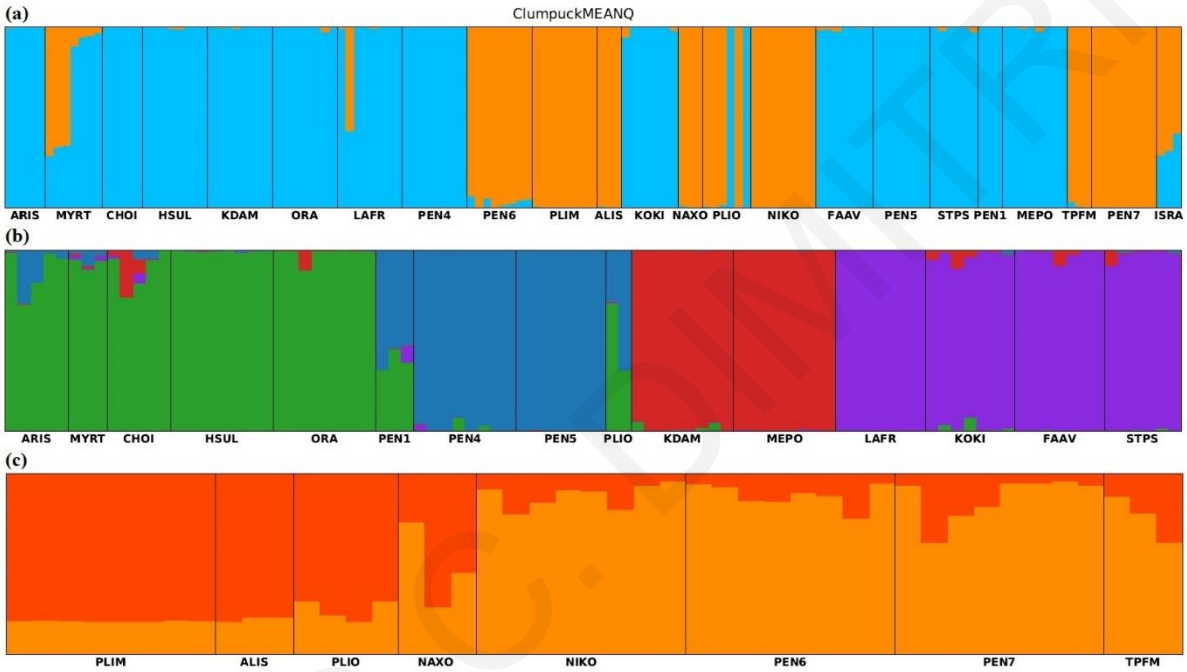

Figure 6. Population structure after two hierarchical STRUCTURE analysis levels using genome-wide SNPs. Each vertical line is a different individual, while different colours represent the estimated Q values corresponding to the assignment probabilities of each individual to putative population clusters. The fist hierarchical clustering (a) divided the dataset in two separate clusters. These two cluster were further analysed showing further sub-structuring, b: blue cluster, c:orange cluster.

All individuals with a membership coefficient <0.9 were excluded and two new subsets, one for each cluster, were created before proceeding with a second level of hierarchical structuring. Additional population structure was detected in both cases. More precisely, within the cluster including only specimens distributed on Cyprus, according to the optimal K, individuals were assigned to four geographically distinct regions which correspond to the statistically well-supported phylogenetic clades CY1 - CY4. On the other hand, in the case of clade CY5, individuals are separated further in two groups, with the

assignment coefficients of the cluster including individuals from PLIM, ALISS, PLIO and NAXO not exceeding 82% (Figure 6).

The population structuring was additionally explored using DAPC by ascertaining the relationship between individual genotypes. According to the k-means method, a k value of 3 (corresponds to the lowest BIC value) offers the optimal clustering solution, minimizing the within group, and at the same time maximizing the between group variance. The DAPC plot supported the separation between the phylogenetically inferred clades CY5, CY2 and the rest of the "Cypriot" clades (CY1, CY3, CY4) grouped with Israel (Figure 7a). Further sub-structuring between these four clades was investigated using the same method. According to the K-means algorithm, the selected k for this new subset was 2 and the new discriminant analysis plot presents the examined individuals in two distinct groups where only samples from ISR population are included in one of these groups (Figure7b). A third level of analysis using only the remaining "Cypriot" clades, discriminates the CY3 clade from CY4 and CY1 which belong to the same group (Figure 7c). The posterior probability for the great majority of individuals was 1 or >0.9 while the very few individuals with high percentage of missing data were "misplaced", according to the produced phylogenetic patterns.

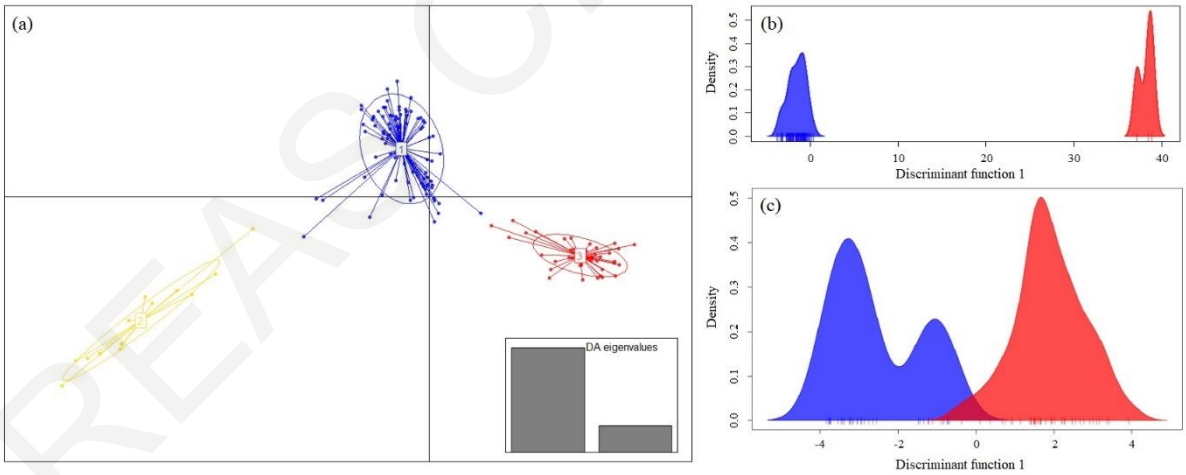

Figure 7. DAPC scatterplot of all individuals based on two principal components. a) Group 1 includes individuals assigned to the CY1, CY3, CY4, and ISR clades according to phylogenetic analyses. Individuals from the CY2 clade are forming the group 2 while the rest of the individuals belonging to CY5 clade are forming a separate group; b) Withing group 1 analysis discriminates the populations from Cyprus and Israel; c) The remaining Cypriot populations are further separated in two groups corresponding to the CY3 and CY4 - CY1 clades.

Estimated pairwise F_{ST} values between the six main *Armadillo* lineages varied from 0.10 to 0.45 between CY1 - CY4 and CY3 - ISR / CY2 - ISR clades, respectively (Table 1). Fixation indexes for all populations used, as well as bootstrap upper and lower limits, are given in Tables S7, S8 and S9.

Table 1: Estimated F_{ST} values between the major identified genetic clades according to Weir and Cockerham (1984)

Clade	CY1	CY2	CY3	CY4	CY5	ISR
CY1	--					
CY2	0.20	--				
CY3	0.14	0.18	--			
CY4	0.10	0.18	0.18	--		
CY5	0.38	0.40	0.41	0.39	--	
ISR	0.43	0.45	0.45	0.44	0.41	--

DISCUSSION

Conducted phylogenetic analyses based on Sanger data revealed the existence of six distinct genetic lineages of the focal species, five of which occur in Cyprus and two at the surrounding continental areas. With the exception of the GR clade as a distinct lineage, the same diversification pattern was retrieved using Sanger or genome-wide ddRAD data, highlighting the robustness of our results. These findings are not reflected in the morphology of the species, as it exhibits limited morphological variation across its distribution (Schmalfuss, 1996). The agreement between the two types of data in such a divergent taxon indicate that the combination of amplified genes could lead to reliable results in cases where genomic approaches are not applicable, at least in the case of Oniscidea. Calculated genetic distances fall within the limits of already published data from other Oniscidea species concerning the same loci. More precisely, the genetic distances among populations of *Ligidium beieri* reach up to 7.4%, 7.3% and 15.6% in the case of 12s, 16s and COI, respectively (Klossa-Kilia et al., 2006) while in *Trachelipus aegaeus* the maximum distance for 16s was 20.3% and for COI 19% (Kamilari et al., 2014). Much lower conspecific genetic distances were observed in *Armadillidium pelagicum* in Tunisia, where sequence divergence reached up to 2.1% (Charfi-Cheikhrouha, 2003). At the same time, the maximum calculated distances among *A. officinalis* lineages exceed the minimum sequence divergence observed among well-defined *Ligidium* species in case of 16s and COI (Klossa-Kilia et al., 2006).

Under the light of the estimated phylogeny, one of the revealed lineages is spreading across the study area and possibly the whole species distribution, as sampling sites extend from central-west to eastern Mediterranean coasts. This widespread CY5 clade, which is the genetically most common lineage across the study area, seems to be dispersed by humans. This statement is supported by the small genetic distances within this clade, hence the shallow unresolved phylogenetic relationships of the grouped entities. Furthermore, the absence of any geographic pattern within the island or across the clade's distribution enhances this hypothesis. Human activities seem to preserve an ongoing gene flow between populations spreading across the Mediterranean coasts and individuals probably introduced into new regions. Cyprus could be an example of such a case, where at least two distinct introductions took place in the past, as inferred by the population structure patterns (Figure 6c).

In contrast, regarding within Cyprus diversification, the rest of the lineages exhibit clear geographic patterns within the island, with CY2 being restricted to the Pentadaktylos

mountain region while lineages CY4 and CY1 being distributed at the western and the southern part of the Troodos range respectively. A well-supported distinct genetic clade (CY3) occurs between the two main land masses at Mesaoria plain. A more thorough view of phylogenetic relationships within Cyprus shows that the closer relationship between Measoria and Pentadaktylos clades indicate that the plain that extends today between the two main mountains of the island is inhabited by populations originating from the northern part. Moreover, since only one individual of the CY1 clade was found at the western part, and two of CY2 at the south-eastern part, we could assume that these were transferred by humans, as one was found at the entrance of the Akamas gorge where a famous natural trail begins, and the other close to a touristic impacted area. The divergence of the GR clade seems to be supported only by mitochondrial data, as the ddRAD phylogenetic analyses form a statistically well-supported monophyletic group that includes individuals from both the GR and the CY5 clades. This could make us think that GR is a recently divergent clade where, in contrast to mtDNA, the diversity has not yet been reflected at the slowly evolving nuclear genome. Among the two populations sampled from Israel, one belongs to the CY5 clade while the other is comprising a separate clade. The close evolutionary relationship of the later Israel clade with the four lineages found only on Cyprus, suggests that this area could be the source of introduction of the species. Various studies focusing on different taxa came to similar conclusions, supporting a Near-East origin of various taxa currently occurring on the island (e.g. Poulakakis et al., 2013; Sfenthourakis et al., 2017).

In line with generated phylogenies, inferred population structure based on SNP data employing STRUCTURE analyses, identified the same distinct genetic units. The genetic isolation between the populations occurring only on Cyprus and the rest is becoming obvious after the first hieratical clustering, where only a small percentage of individuals appeared to be admixed. One of these individuals was collected from the north-western part of the island while the rest are coming from two populations, those of MYRT and ISR. The geographical position of Myrtou at the edge of the western part of Pentadaktylos, between the geographical limits of CY2 and CY3 clades, seems to be a zone where the two lineages "hybridize" and hence admixed individuals were identified. This statement is also supported by the fact that individuals collected from this area were phylogenetically assigned to CY2, CY3 and CY5 clades. On the other hand, the mixed genetic profile of individuals from Israel indicates the closer relationship between Cyprus than the rest of the examined continental populations, which makes sense if we take into account the aforementioned phylogenetic patterns, the geographic proximity, and the known paleogeographic history of the island.

Although it is still debated whether Cyprus was ever connected to neighbouring mainland (Syria or southern Anatolia), the subsidence of the sea level during the Messinian Salinity Crisis (MSC) facilitated the arrival of many taxa on the island (Constantinou and Panayides, 2013; Hadjisterkotis et al., 2000; Plötner et al., 2010). According to the cladochronological analysis, the divergence time of populations found only on Cyprus corresponds to the MSC, when the island was either connected with Anatolia through a landbridge or more accessible via stepping stones. This scenario is also supported by the closer evolutionary relationship of the CY1 - CY4 populations with the extant population from Israel rather than Greece or Turkey. The populations from Cyprus and Israel, excluding the CY5 clade, share a common ancestor at ~15.74 Mya. We speculate that a more recent ancestor could be found between the populations from Cyprus and populations distributed closer to the area where the island was possibly connected to the mainland. These findings are also confirmed by DAPC analysis where individuals from Cyprus and Israel, excluding CY2 clade, are grouped together. Representatives of the "Mediterranean" CY5 clade and CY2 clade are assigned in two different groups. The same analysis focusing on the Cypriot-Israel group separates at the first level the population from Israel from Cypriot and at the second level clade CY3 with C4 - CY1. The possible genetic differentiation between the latter two clades seems to be too low for the sensitivity of this method. The sister clade relationship of these two geographically neighbouring clades is also supported by the genomic-based phylogenetic trees.

Based on Sanger data, according to the dominant scenario of the species delimitation analysis, the existence of three entities (CY5, CY1CY2CY3, CY4) is supported on Cyprus, two of which are "endemic" to the island, while genetic divergence of individuals from ISR indicate that this clade should also be considered as a different taxonomic unit. On the other hand, species delimitation using SNAPP suggested the existence of three distinct taxonomic units from which one occurs on Cyprus, one on Israel, and the third is widespread all over the study area. Combining the two results of these two types of data we could assume that we have at least two taxa on Cyprus, one of them endemic to the island.

The genetic isolation between these three taxa is also indicated by the high F_{ST} values, ranging from 0.38 - 0.45 between these groups. Furthermore, within Cypriot lineages F_{ST}s reaching up to 0.2 support the existence of gene flow barriers between populations occurring on the island.

Cladochronological analyses coupled with phylogenetic and population genetic patterns, indicate multiple colonisations to the island by *A. officinalis*. It seems that the first

populations of the taxon were established on the island during the MSC, facilitated by the paleogeography of the area at the time. Nevertheless, the presence of the CY5 clade, common along circum-Mediterranean coasts, as well as the absence of any within island geographic pattern, indicate a more recent, human mediated introduction of this group.

In order to evaluate within-island genetic diversification, we should take into account, i) the absence of gene flow between island and mainland populations, ii) habitat heterogeneity, iii) the complex geological history of the island, and iv) the long continuous presence of humans for more than 10,000 years. Considering the aforementioned factors plus the relatively small size of Cyprus, we could say that it is a speciation hotspot, at least for species with low overseas dispersal ability.

CHAPTER 5

Synopsis

Synopsis

Within the framework of this thesis a collection of isopod specimens from around the globe, as well as a thorough collection of samples from many sites within Cyprus, compiled a comprehensive dataset that allowed the investigation of phylogenetic relationships within Oniscidea at different taxonomic levels, as well as among the focal taxon and other Isopoda groups. Among the innovations of this study are: i) the inclusion of all five major Oniscidea clades in a single molecular phylogenetic analysis, ii) the use of a multi-locus approach to resolve a phylogeny at the family level, iii) the use of highly conserved protein-coding loci to resolve deep phylogenetic relationships within Oniscidea, iv) the thorough examination of a species genetic divergence within the narrow geographical limits of an oceanic island such as Cyprus, and v) the application of ddRAD protocol to gather NGS data for terrestrial isopod phylogenetic and population genetics purposes.

Generated datasets were enriched with publicly available data from previews studies where possible, and results were evaluated taking into consideration previously published studies. The main findings of my thesis could be summarized as follows:

1) Results indicate the closer relationship of Oniscidea with Valvifera and Sphaeromatidae than with Asellota or Phreatoicidea.

2) The close evolutionary relationship of the amphibian genus *Ligia* with the marine taxa Valvifera and Sphaeromatidea is questioning the monophyly of Oniscidea, as well as of Diplocheta.

3) The newly established family Ligidiidae, including *Ligidium* and related genera, is the more ancestral clade among Oniscidea.

4) The sister clade relationship of the more apical clades Crinocheta and Synocheta, as well as their monophyly is unambiguous.

5) Within Synocheta, the traditional distinction between Trichoniscinae and Haplophthalminae, based largely on the presence of ornamentation on tergites, does not seem to be supported.

6) The current taxonomic status of Styloniscidae as a separate family from Trichoniscidae is questioned.

7) The monophyly of Porcellionidae, one of the richest families of Oniscidea that is distributed globally, as well as of certain genera within the family, is undermined.

8) Porcellionidae monophyly could be saved if the genera *Leptotrichus* and *Brevurus* are excluded.

9) The monophyletic Porcellionidae, in the sense suggested herein, has an African origin that dates back to the Oligocene (~32 Mya).

10) Porcellioniodae is more closely related to Trachelipodidae and Agnaridae rather than Armadillidiidae.

11) Even though Armadillidiidae seems to share a similar type of pleopodal lungs with Porcellionidae, molecular data support its closer relationship with Cylisticidae.

12) Extremely high genetic distances (p-distance: 50.3 in mtDNA and 44.2 in nDNA) within established taxa highlight the vast divergence among taxonomically closely related groups (genera within Porcellionidae)

13) The existence of five genetically divergent lineages of *Armadillo officinalis* on Cyprus proved that morphological similarity might be misleading regarding the evolutionary relationships between conspecific individuals even if they occur within narrow geographic limits.

14) The widespread CY5 clade of *A. officinalis* seems to be transported by humans, in view of the shallow, unresolved phylogenetic relationships within the group, the small within-group genetic distances, and the absence of any geographical structure.

15) Aiming to comprehend the within-Cyprus genetic diversification, we should take into account: i) the absence of gene flow between island and mainland populations, ii) habitat heterogeneity, iii) the complex geological history and the long isolation of the island, and iv) the long continuous presence of humans for more than 10,000 years on the island.

16) Produced patterns of genetic diversity indicate that the geological history of Cyprus had a great impact on biodiversity patterns.

17) The long isolation, the complex geological history, habitat heterogeneity, and the long presence of humans on the island of Cyprus, render it a speciation hotspot, at least for species with low overseas dispersal ability.

18) The estimated divergence time of all *A. officinalis* populations, representing genetic lineages found only on the island, correspond to the MSC, when Cyprus could have been connected to Anatolia through a land bridge or at least be much closer to the latter with stepping-stone islands in-between. This scenario is also supported by the closer evolutionary relationship of the CY1 - CY4 populations with the extant population from Israel.

19) Phylogenetic relationships among '*A. officinalis'* populations within Cyprus support a closer relationship of Measoria with Pentadaktylos clade, indicating that individuals from the northern part of the island inhabited the main plain of the island between the two mountains.

20) In line with previously published studies, focusing on various taxa, the results support that the source of origin of *A. offcinalis* on Cyprus is the region surrounding the north-eastern part of the Mediterranean Sea.

21) At least one endemic and probably cryptic species within the presumably morphologically invariant '*A. officinalis*' group has been identified.

22) Morphological and genetic data should be combined before taxonomic revisions could be proposed.

23) The validity of several morphological characters traditionally used in terrestrial isopod taxonomy needs to be re-evaluated.

24) A more extensive taxonomic sampling is needed to adequately portray the evolutionary history of Oniscidea.

25) Current oniscidean taxonomy should be reconsidered under the light of new molecular data and available tools for gathering genetic data.

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APPENDIX I

Percentage sequence divergence (p-distance) among the main Oniscidea clades and other isopod suborders

Table S1: Percentage sequence divergence (p-distance) among the main Oniscidea clades and other isopod suborders for 18s.

	Group	1	2	3	4	5	6	7	8	9	10
1	**Crinocheta**	13.59									
2	**Synocheta**	12.76	4.42								
3	**Microcheta**	15.68	10.96	n/c							
4	**Tylida**	30.22	24.74	28.23	43.40						
5	**Diplocheta***	14.75	8.66	10.70	25.66	1.25					
6	**Sphaeromatidea**	18.25	15.28	16.29	29.33	16.30	0.00				
7	***Ligia***	16.36	8.78	12.86	30.00	10.57	16.48	11.67			
8	**Valvifera**	15.07	8.42	13.23	25.89	8.48	16.73	8.80	0.00		
9	**Phreatoicidea**	14.69	9.10	11.84	26.00	8.38	14.99	9.54	9.11	n/c	
10	**Asellota**	14.31	9.33	8.90	24.97	8.35	14.53	8.78	8.20	6.62	n/c

* Not including *Ligia*, treated separately based on its position in the phylogenetic tree.

Table S2: Percentage sequence divergence (p-distance) among the main Oniscidea clades and other isopod suborders for 28s.

	Group	1	2	3	4	5	6	7	8	9	10
1	**Crinocheta**	46.21									
2	**Synocheta**	43.28	31.57								
3	**Microcheta**	43.08	38.12	n/c							
4	**Tylida**	42.85	36.71	32.27	33.78						
5	**Diplocheta***	50.19	48.32	45.11	42.87	22.44					
6	**Sphaeromatidea**	53.35	50.99	48.07	46.59	43.55	0.00				
7	***Ligia***	52.18	49.99	47.02	42.42	48.75	50.31	60.34			
8	**Valvifera**	51.37	49.98	44.13	45.33	37.14	46.38	50.55	0.00		
9	**Phreatoicidea**	66.70	65.24	64.93	66.61	66.47	70.60	68.82	66.25	n/c	
10	**Asellota**	68.25	68.93	71.57	68.38	68.55	64.40	71.50	67.54	71.10	n/c

* Not including *Ligia*, treated separately based on its position in the phylogenetic tree.

Table S3: Percentage sequence divergence (p-distance) among the main Oniscidea clades and other isopod suborders for NAK.

	Group	1	2	3	4	5	6	7	8	9
1	**Crinocheta**	9.52								
2	**Synocheta**	17.88	12.78							
3	**Microcheta**	20.13	19.29	n/c						
4	**Tylida**	19.95	19.09	20.09	16.06					
5	**Diplocheta***	22.23	20.08	19.82	20.24	0.62				
6	**Sphaeromatidea**	19.14	18.80	18.69	18.80	17.76	0.00			
7	***Ligia***	21.02	18.81	19.28	20.04	21.07	16.71	15.58		
8	**Valvifera**	26.89	0.22	0.22	0.22	0.22	0.22	0.22	0.22	
9	**Asellota**	26.23	26.92	26.78	26.40	30.57	27.75	27.71	30.64	n/c

* Not including *Ligia*, treated separately based on its position in the phylogenetic tree.

Table S4: Percentage sequence divergence (p-distance) among the main Oniscidea clades and other isopod suborders for PEPCK.

	Group	1	2	3	4	5	6	7	8	9
1	**Crinocheta**	9.10								
2	**Synocheta**	20.56	15.08							
3	**Microcheta**	23.82	22.62	n/c						
4	**Tylida**	23.09	22.84	24.67	n/c					
5	**Diplocheta***	21.75	22.98	22.44	20.98	2.24				
6	**Sphaeromatidea**	22.43	24.51	22.86	25.82	22.41	0.00			
7	***Ligia***	22.29	23.32	23.44	23.73	22.71	21.75	18.76		
8	**Valvifera**	22.32	24.68	24.23	22.35	19.31	20.04	20.11	0.00	
9	**Asellota**	29.46	28.80	28.50	28.76	25.25	27.44	26.16	23.75	n/c

* Not including *Ligia*, treated separately based on its position in the phylogenetic tree.

APPENDIX II.

Percentage sequence divergence among the main clades of Porcellionidae and Maximum Likelihood phylogenetic tree.

Table S1: Percentage sequence divergence (p-distance) among the main clades of Porcellionidae for 16s. Each genus comprised a different group, except for *Porcellio* whose species were treated as different groups because they don't form a monophyletic group.

Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. ***Acaeroplastes***																
2. ***Agabiformius***	22.3															
3. ***Leptotrichus***	21.2	22.6														
4. ***Mica***	23.9	21.7	22.5													
5. ***Porcellionides***	23.4	27.3	21.8	24.7												
6. ***Porcellio laevis***	21.5	18.7	21.7	17.1	23.8											
7. ***Porcellio nasutus***	25.1	23.1	26.4	23.4	23.6	20.6										
8. ***Proporcellio***	21.1	23.9	21.9	24.1	18.5	21.1	23.0									
9. ***Soteriscus***	20.8	22.1	16.9	21.2	23.2	21.4	25.1	23.5								
10. ***Thermocellio***	25.9	22.8	24.5	23.5	27.7	23.3	27.1	25.0	25.4							
11. ***Tura***	29.6	30.1	33.5	29.8	31.6	28.9	36.5	28.3	31.9	31.3						
12. ***Uramba***	29.2	26.3	27.1	25.9	28.2	25.2	29.7	28.8	27.0	19.9	31.1					
13. ***Armadillidium***	20.7	24.3	21.0	25.0	26.5	22.6	25.2	21.6	22.5	23.4	29.6	26.6				
14. ***Hemilepistus***	21.8	23.0	20.3	24.7	22.6	21.6	23.2	20.6	20.9	26.6	31.2	26.2	22.3			
15. ***Trachelipus***	25.2	26.5	23.1	23.4	28.2	25.0	27.2	22.4	23.0	28.8	33.0	29.3	23.0	23.0		
16. ***Chaetophiloscia***	30.1	30.5	32.1	30.3	33.6	30.7	32.5	34.6	29.3	34.3	35.3	34.6	26.4	31.5	32.4	
17. ***Actaecia***	32.8	32.9	32.4	29.3	29.6	30.1	33.2	29.9	31.5	32.5	37.6	35.0	35.6	30.8	31.5	37.9

Table S2: Percentage sequence divergence (p-distance) among the main clades of Porcellionidae for COI. Each genus comprised a different group, except for *Porcellio* whose species were treated as different groups because they don't form a monophyletic group.

Group	1	2	3	4	5	6	7	8	9	10	11	12	13
1. ***Acaeroplastes***													
2. ***Caeroplastes***	28.9												
3. ***Lucasius***	44.9	42.2											
4. ***Proporcellio***	23.4	17.2	41.5										
5. ***Porcellionides***	20.1	23.6	45.6	16.9									
6. ***Porcellio laevis***	22.6	33.4	50.3	28.5	18.2								
7. ***Porcellio nasutus***	19.5	28.3	46.8	23.8	23.3	20.9							
8. ***Soteriscus***	24.9	19.1	39.8	17.9	21.4	29.5	26.5						
9. ***Tura***	23.7	33.5	49.6	24.4	22.7	23.3	24.4	27.3					
10. ***Armadillidium***	22.6	32.8	50.8	28.5	19.5	19.2	21.4	30.8	23				
11. ***Hemilepistus***	21.2	33.4	50.0	29	18.6	19.9	21.9	30.5	23.2	18.9			
12. ***Trachelipus***	21.1	28.0	47.0	21.4	19.9	20.8	20.1	24.8	23.8	19.2	19.3		
13. ***Chaetophiloscia***	21.9	38.2	51.4	32.1	21.4	20.7	21.1	33.3	23.9	20.2	19.8	21.8	
14. ***Actaecia***	21.1	26.1	47.0	24.4	22.0	22.2	22.8	24.3	24.3	21.2	20.6	20.5	19.6

Table S3: Percentage sequence divergence (p-distance) among the main clades of Porcellionidae for 18s. Each genus comprised a different group, except for *Porcellio* whose species were treated as different groups because they don't form a monophyletic group.

Group	1	2	3	4	5	6	7	8	9	10	11	12	13	13	14	15
1. ***Acaeroplastes***																
2. ***Agabiformius***	25.4															
3. ***Caeroplastes***	3.6	26.0														
4. ***Leptotrichus***	13.5	25.6	14.4													
5. ***Lucasius***	10.9	25.4	11.2	12.1												
6. ***Porcellio laevis***	9.7	3.4	5.4	7.5	2.4											
7. ***Porcellio nasutus***	3.6	28.5	4.1	14.6	11.2	7.5										
8. ***Soteriscus***	11.9	25.3	11.4	11.7	3.9	2.5	11.8									
9. ***Tura***	13.9	22.6	14.9	11.6	14.5	5.6	17.0	13.0								
10. ***Agnara***	14.4	26.8	14.8	14.1	13.6	3.7	15.3	12.8	13.9							
11. ***Hemilepistus***	16.0	25.2	16.2	13.4	14.7	5.5	16.9	14.7	15.0	8.2						
12. ***Armadillidium***	11.6	25.0	12.5	10.8	9.5	8.5	11.8	9.8	11.3	12.9	12.1					
13. ***Schizidium***	12.9	25.4	13.9	10.5	10.1	8.5	13.3	10.4	11.0	13.6	12.6	2.0				
14. ***Levantoniscus***	7.1	22.3	7.4	8.4	5.9	0.9	7.6	7.4	8.6	8.9	8.8	7.1	6.9			
15. ***Trachelipus***	17.4	31.0	17.0	13.0	16.6	12.7	19.8	16.4	19.2	15.6	16.8	11.8	10.9	12.0		
16. ***Chaetophiloscia***	12.7	24.7	13.8	9.6	10.3	8.5	13.4	11.3	10.6	13.0	12.2	4.8	3.7	5.9	10.4	
17. ***Actaecia***	18.2	28.5	19.0	13.9	16.4	8.5	21.6	16.7	18.5	16.3	16.4	7.9	9.7	9.5	19.1	8.0

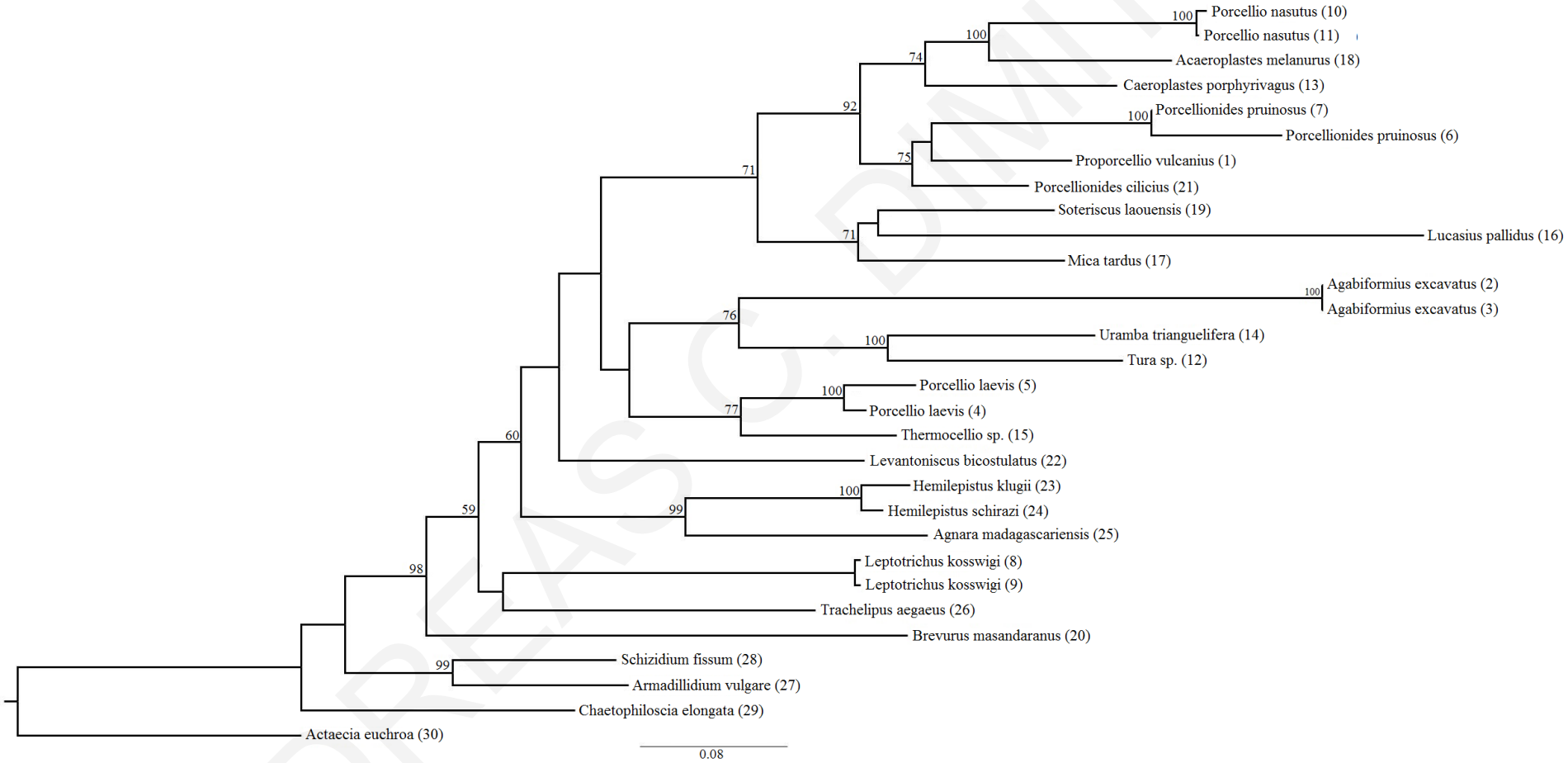
Table S4: Percentage sequence divergence (p-distance) among the main clades of Porcellionidae for 28s. Each genus comprised a different group, except for *Porcellio* whose species were treated as different groups because they don't form a monophyletic group.

Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. ***Acaeroplastes***																					
2. ***Agabiformius***	28.9																				
3. ***Brevurus***	32.3	34.1																			
4. ***Caeroplastes***	14.0	30.0	29.7																		
5. ***Leptotrichus***	33.8	41.5	36.7	30.1																	
6. ***Lucasius***	20.4	20.6	29.5	28.2	30.1																
7. ***Mica***	20.6	21.3	31.0	28.1	29.5	17.5															
8. ***Porcellionides***	22.2	24.0	23.4	26.4	32.2	24.6	25.5														
9. ***Porcellio laevis***	20.1	20.7	30.5	26.3	28.6	19.1	17.6	26.0													
10. ***Porcellio nasutus***	17.7	35.9	35.8	17.2	41.0	24.1	22.9	24.4	19.9												
11. ***Proporcellio***	12.9	17.0	20.3	17.4	25.3	20.5	20.5	19.6	20.4	17.0											
12. ***Soteriscus***	22.8	21.0	32.2	30.2	30.9	16.9	17.3	25.5	22.8	26.7	20.6										
13. ***Thermocellio***	22.0	16.7	44.2	21.4	30.4	21.5	17.9	26.9	0.4	23.5	19.8	28.4									
14. ***Tura***	31.8	29.1	33.0	30.6	36.9	17.6	21.3	24.3	20.1	34.9	19.5	20.7	16.9								
15. ***Uramba***	32.8	32.9	34.8	31.6	41.2	18.9	22.0	26.0	21.5	38.8	19.5	21.8	18.3	17.2							
16. ***Armadillidium***	33.0	36.8	35.6	36.4	38.9	30.4	35.2	28.4	35.4	34.6	25.6	34.9	50.0	33.6	35.3						
17. ***Schizidium***	31.8	34.5	34.6	27.2	37.2	25.7	27.5	25.8	26.5	33.1	20.7	28.0	25.9	30.6	33.9	25.4					
18. ***Agnara***	23.6	24.5	31.1	24.6	31.3	25.0	27.4	26.1	27.1	26.4	20.2	27.7	28.5	23.3	27.2	34.3	29.2				
19. ***Hemilepistus***	33.1	41.5	38.6	28.2	42.1	26.2	25.4	27.2	23.5	38.6	20.5	27.5	26.5	35.6	38.1	36.7	36.5	22.4			
20. ***Levantoniscus***	24.5	29.1	30.3	25.1	33.1	23.0	23.2	23.4	22.8	28.2	17.5	25.5	25.6	26.1	28.9	30.8	28.0	25.4	31.2		
21. ***Chaetophiloscia***	27.5	32.9	35.1	24.7	32.9	22.8	22.4	25.7	21.5	31.2	21.3	23.9	19.5	32.7	33.3	31.5	24.7	23.6	35.2	29.6	
22. ***Actaecia***	33.8	38.3	39.0	36.9	40.7	28.1	28.2	29.7	29.8	38.8	23.5	32.8	46.4	39.9	42.4	31.5	32.7	30.3	41.1	34.3	33.6

Table S5: Percentage sequence divergence (p-distance) among the main clades of Porcellionidae for NAK. Each genus comprised a different group, except for *Porcellio* whose species were treated as different groups because they don't form a monophyletic group.

Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *Acaeroplastes*																	
2. *Agabiformius*	7.4																
3. *Brevurus*	6.6	7.0															
4. *Leptotrichus*	6.6	6.6	6.0														
5. *Lucasius*	5.8	6.2	6.2	5.1													
6. *Porcellionides*	5.1	6.0	5.9	6.0	5.1												
7. *Porcellio nasutus*	3.4	7.2	7.2	6.4	4.8	3.8											
8. *Porcellio laevis*	5.1	6.0	6.4	5.5	4.3	4.9	4.5										
9. *Proporcellio*	4.5	6.2	6.6	5.7	4.7	2.6	3.4	4.5									
10. *Soteriscus*	6.0	7.7	6.0	6.6	5.2	5.1	5.6	4.6	5.5								
11. *Tura*	7.3	6.6	6.8	7.8	7.1	6.2	7.7	7.2	7.2	6.2							
12. *Uramba*	7.0	6.1	9.1	8.1	6.9	7.0	7.2	6.9	6.9	8.3	4.8						
13. *Agnara*	7.1	5.6	n/c	3.0	5.1	4.6	3.9	6.7	4.4	5.9	4.9	5.1					
14. *Hemilepistus*	6.2	7.1	4.9	6.8	5.8	4.6	4.9	6.3	5.0	4.8	5.7	7.8	3.4				
15. *Levantoniscus*	8.1	8.2	8.7	8.3	7.3	7.1	7.0	7.1	6.7	8.5	9.4	9.0	6.5	7.5			
16. *Trachelipus*	6.5	7.1	5.9	5.7	5.3	4.4	4.4	6.6	4.5	4.8	6.6	7.8	2.0	3.6	6.5		
17. *Chaetophiloscia*	11.1	10.9	10.0	10.0	7.9	10.3	9.4	10.7	9.1	10.5	11.1	12.2	8.9	9.4	11.4	8.7	
18.*Actaecia*	20.6	18.3	22.7	20.4	18.0	19.2	19.3	19.2	18.6	19.4	18.8	20.3	12.3	16.4	20.3	18.7	17.3

Figure S1. Maximum likelihood phylogenetic tree constructed using 5 genes (COI, 16s, 18s, 28s, NAK). Only the bootstrap values above 50 are given on the nodes.



APPENDIX III

Sequence divergence, ML phylogenetic tree, Path sampling models and estimated F_{ST}s.

Table S1: List of populations (in Lab code alphabetical order) included in our analyses with their corresponding origin details and clade that they belong according to our results.

Lab code	Locality - Region	Country	Coordinates		Clade
			latitude	longitude	
AANA	Agioi Anargyroi	Cyprus	34.976550	34.071480	CY3
AKAM	Akamas	Cyprus	35.069650	32.326730	CY4
AKAN	Akanthou	Cyprus	35.382880	33.753020	CY2
AKAS	Akrotiri Kastros	Cyprus	35.694620	34.587430	CY2
AKDE	Agios Georgios	Cyprus	35.254280	33.042640	CY3
AKRO	Akrotiri	Cyprus	34.601150	32.969170	CY1
ANAP	Agia Napa	Cyprus	34.985180	34.023290	CY5
ARAK	Arakapas	Cyprus	34.844170	33.109590	CY1
ATHE	Agios Theodoros	Cyprus	34.816440	33.380650	CY1
BUFA	Bufavento	Cyprus	35.287500	33.409440	CY2
CAKT	Chrisi Akti	Cyprus	35.638980	34.534230	CY5
CHAR	Charkeia	Cyprus	35.317150	33.556770	CY2
DLIT	Dasos Lithrodonda	Cyprus	34.946150	33.233830	CY3
DMAC	Dasos Machera	Cyprus	34.939410	33.215340	CY3
FAMO	Fontana Amorosa	Cyprus	35.071130	32.322380	CY4
FAVA	Faraggi Avaka	Cyprus	34.920570	32.337970	CY4
GERI	Geri	Cyprus	35.153410	33.716950	CY3
KALG	Kalogrea	Cyprus	35.357500	33.633600	CY2
KGRE	Kavo Greco	Cyprus	34.966210	34.059290	CY3
KORN	Kornos	Cyprus	35.339320	33.108810	CY3
KOUR	Kourio	Cyprus	34.669850	32.873770	CY4
LAFR	Loutra Afroditis	Cyprus	35.057080	32.344720	CY4
LARA	Lara	Cyprus	34.958050	32.312670	CY4
LATS	Latsi	Cyprus	35.040300	32.373720	CY4
LIMN	Limnatis	Cyprus	34.805150	32.960940	CY1
LSLT	Larnaka Salt Lake	Cyprus	34.889720	33.604520	CY3
OVEL	Oveliskos	Cyprus	34.982730	33.705720	CY3
PATH	Parko Athalassas	Cyprus	35.127770	33.388510	CY3
PELE	Pelendri	Cyprus	34.889790	32.954440	CY1
PENT	Pentalia	Cyprus	34.867940	32.621100	CY4
PEN5	Akanthou	Cyprus	35.406802	33.783598	CY2
PITA	Pitargou	Cyprus	34.831900	32.539380	CY4
PLIO	Potamos Liopetriou	Cyprus	34.972880	33.898630	CY2, CY5
PPAL	Panagia Paleochoritissa	Cyprus	35.593720	34.425980	CY2, CY5
PPLA	Panagia Plataniotissa	Cyprus	35.286280	33.531220	CY5
PYRG	Pyrgolofos	Cyprus	35.061780	32.860500	CY4
SPSO	Stavros tis Psokas	Cyprus	35.026730	32.630980	CY4
VIKL	Vikla	Cyprus	35.632740	34.494750	CY5
XERP	Xeros Potamos	Cyprus	35.073100	32.803000	CY4
MEPO	Mesa Potamos	Cyprus	34.893278	32.905972	CY1
KDAM	Kouris Dam	Cyprus	34.756778	32.921694	CY1

KOKI	Kokkina	Cyprus	35.164167	32.621556	CY4
NIKO	Nikokleia	Cyprus	34.730278	32.581111	CY5
PLIM	Paralimni	Cyprus	35.038833	33.958667	CY5
PEN4	Charkeia	Cyprus	35.334887	33.551487	CY2
FAAV	Faraggi Avaka	Cyprus	34.920583	32.337778	CY1, CY4
MYRT	Myrtou	Cyprus	35.299528	33.086694	CY2, CY3, CY5
HSUL	Hala Sultan teke	Cyprus	34.887694	33.607111	CY3
ARIS	Aglantzia	Cyprus	35.139000	33.381556	CY3
PEN6	Gialousa	Cyprus	35.542240	34.196695	CY5
PEN7	Chrisi Akti	Cyprus	35.642295	34.540233	CY5
PEN1	Kythrea	Cyprus	35.2680000	33.467440	CY2, CY3
CHOI	Choirokitia	Cyprus	34.7900300	33.342500	CY3
ORA	ORA	Cyprus	34.8554700	33.200440	CY3
ITAL	Catania	Italy	37.448675	15.062155	CY5
TUNI	Tunis	Tunisia	36.847299	10.303970	CY5
KIAT	Kiato	Greece	38.009023	22.746144	CY5
VOUL	Galatas	Greece	37.483280	23.470270	CY5
ALISS	Alissos	Greece	38.137806	21.588000	GR
NAXO	Naxos	Greece	37.045028	25.465861	CY5
AMOR	Amorgos	Greece	36.822196	25.890410	CY5
ANDR	Andros	Greece	37.844286	24.799504	CY5
ARCH	Archanes	Greece	35.234760	25.152340	CY5
CRET	Rethymno	Greece	35.273510	24.534796	CY5
ISR	Majdal Shams	Israel	33.284278	35.755194	ISR
ISRA	Unknown	Israel	32.162604	34.886430	CY5
TKG	Konya Guneysinir	Turkey	37.266083	32.729583	CY5
TPFM	Petlangic fethiye mugla	Turkey	36.658444	29.152611	CY5
TURA	Ölüdeniz	Turkey	36.557000	29.159580	GR
TXXX	Mesudiye	Turkey	36.712146	27.571033	CY5
TDAT	Reşadiye	Turkey	36.748461	27.682044	CY5

Table S2: Percentage sequence divergence (p-distance) among the identified clades for 12s (below diagonal) and Cytb (above diagonal). Within-group distances for both genes are given at the diagonal (12s/Cytb). Nc stands for no calculation where no more than one sequences were available within a group.

Group	CY1	CY2	CY3	CY4	ISR	CY5	GR
CY1	2.25/4.60	9.25	8.55	8.01	10.34	13.22	13.10
CY2	6.56	1.79/3.15	6.32	9.53	12.63	13.50	13.76
CY3	7.31	4.47	1.70/3.00	8.74	11.37	12.62	13.86
CY4	6.92	8.50	7.87	3.08/3.61	9.80	13.41	12.82
ISR	10.58	9.80	9.63	9.31	0.32/nc	13.82	10.61
CY5	9.70	9.34	9.00	9.42	7.10	0.37/0.97	7.18
GR	10.72	10.15	9.94	10.01	6.86	5.06	0.00/nc

Table S3: Percentage sequence divergence (p-distance) among the identified clades for 16s (below diagonal) and COI (above diagonal). Within-group distances for both genes are given at the diagonal (16s/COI). Nc stands for no calculation where no more than one sequences were available within a group.

Group	CY1	CY2	CY3	CY4	ISR	CY5	GR	Outgroup
CY1	2.29/2.78	7.08	7.33	7.04	9.63	10.60	10.94	22.10
CY2	6.03	2.74/2.52	5.57	8.09	10.17	10.41	11.05	21.47
CY3	3.90	4.42	2.54/3.50	8.29	10.58	9.64	9.99	21.76
CY4	4.50	6.70	5.55	2.82/3.86	9.99	10.20	10.48	22.99
ISR	6.17	9.09	7.67	7.73	0.00/0.00	10.88	11.33	22.35
CY5	8.81	9.32	9.22	9.97	9.16	0.79/0.91	7.88	21.41
GR	8.54	11.13	9.61	11.11	7.14	4.83	0.25/4.08	22.18
Outgroup	23.17	23.13	22.95	23.67	23.08	21.95	20.82	7.51/17.89

Table S4: Percentage sequence divergence (p-distance) among the identified clades for 18s (below diagonal) and 28s (above diagonal). Within-group distances for both genes are given at the diagonal (18s/28s). Nc stands for no calculation where no more than one sequences were available within a group.

Group	CY1	CY2	CY3	CY4	ISR	CY5	GR
CY1	0.84/1/12	1.02	1.05	1.10	1.45	1.58	1.84
CY2	0.67	nc/0.71	1.20	1.12	1.15	1.40	1.58
CY3	0.76	0.75	0.75/0.72	1.12	1.97	1.72	2.28
CY4	0.68	0.82	0.78	0.43/1.02	1.33	1.37	1.77
ISR	2.17	2.05	1.93	2.05	nc/nc	1.15	1.24
CY5	2.38	2.39	2.29	2.18	1.86	1.05/0.67	0.92
GR	2.32	2.02	2.43	2.54	2.03	1.28	nc/nc

Table S5: Percentage sequence divergence (p-distance) among the identified clades for NAK. Within-group distances for both genes are given at the diagonal. Nc stands for no calculation where no more than one sequences were available within a group.

Group	CY1	CY2	CY3	CY4	ISR	CY5	GR
CY1	0.00						
CY2	0.00	0.00					
CY3	0.00	0.00	0.00				
CY4	0.00	0.00	0.00	0.00			
ISR	0.57	0.57	0.57	0.57	nc		
CY5	0.63	0.63	0.63	0.63	0.79	0.63	
GR	0.38	0.38	0.38	0.38	0.57	0.32	nc

Figure S1. Maximum likelihood phylogenetic tree constructed using 4 genes (COI, 16s, 12s, Cytb). Only the bootstrap values above 80 are shown as stars on the nodes.

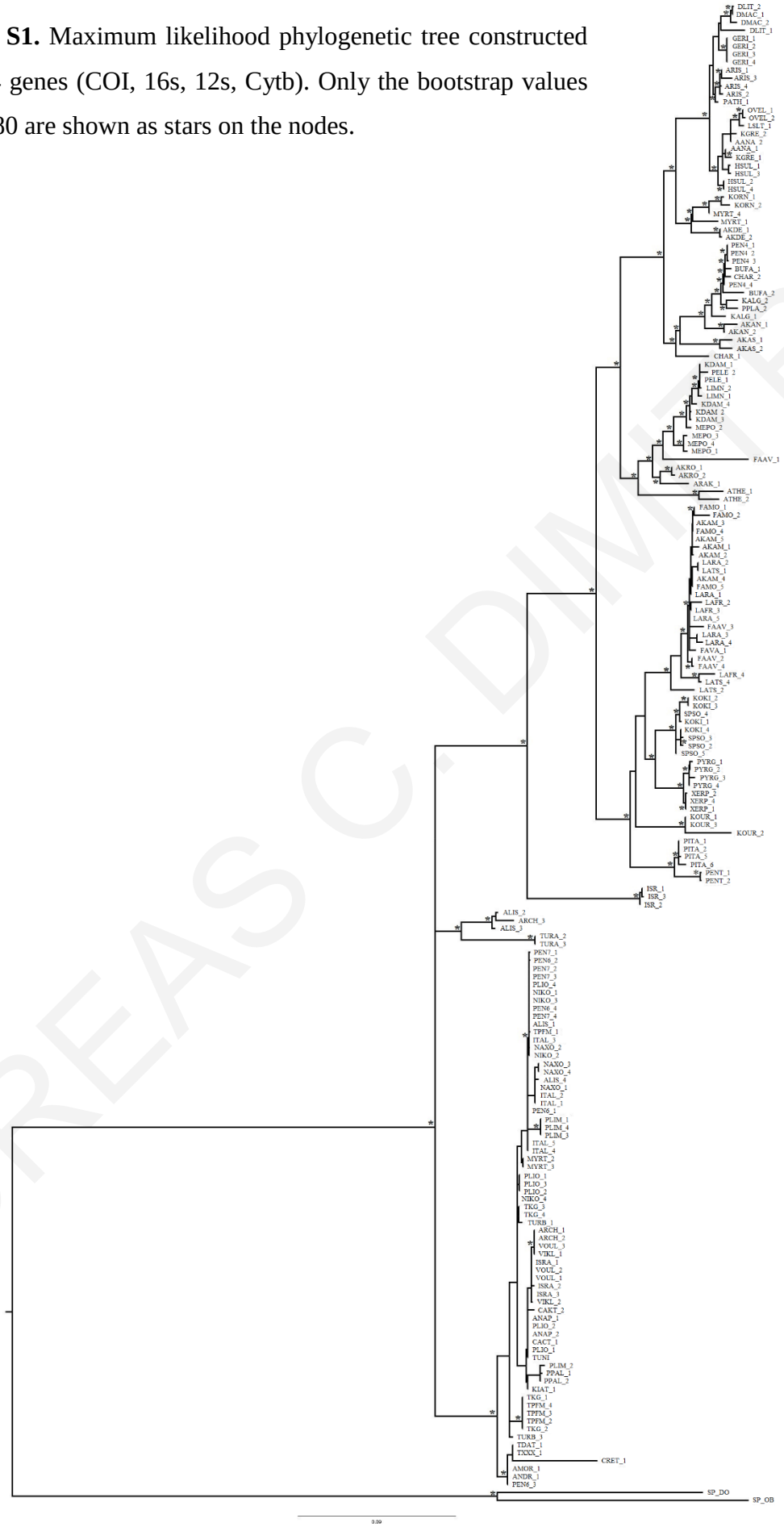


Table S6: SNAPP path sampling results for the nine tested species delimitation schemes. Individuals from MYRT population were treated as two "species" since phylogenetically split between two distinct genetic clades. MLE: Marginal likelihood estimate, BF: Bayes factor.

Model/Pops	Population allocation	MLE	BF	Rank
24	All populations as separate species	-194.194	--	2
18	NIKO, PLIMN-PLIO, ALIS, NAXO, TPFM, PEN7, PEN6, PEN4-PEN5, PEN1, KOKI, MYRTA, MYRTB, STPS, FAAV-LAFR, KDAM-MP, HSUL-ARI, CHOI-ORA, ISR	-278.019	-167.651	3
12	NIKO-PLIMN-PLIO- ALIS-NAXO-TPFM-PEN7, PEN6, PEN4-PEN5, PEN1, KOKI-STPS, MYRTA, MYRTB, FAAV-LAFR, KDAM-MP, HSUL-ARI, CHOI-ORA, ISR	-360.378	-332.368	4
8	NIKO-PLIMN-PLIO- ALIS-NAXO-TPFM-PEN7 PEN6-MYRTA, PEN4-PEN5-PEN1-MYRTB, KOKI-STPS, FAAV-LAFR, KDAM-MP, HSUL-ARI, CHOI-ORA, ISR	-372.912	-357.436	6
6	NIKO-PLIMN-PLIO- ALIS-NAXO-TPFM-PEN7 PEN6-MYRTA, PEN4-PEN5-PEN1-MYRTB, KOKI-STPS-FAAV-LAFR, KDAM-MP, HSUL-ARI-CHOI-ORA, ISR	-371.564	-354.740	5
5	NIKO-PLIMN-PLIO- ALIS-NAXO-TPFM-PEN7 PEN6-MYRTA, PEN4-PEN5-PEN1-MYRTB, KOKI-STPS-FAAV-LAFR-KDAM-MP, HSUL-ARI-CHOI-ORA, ISR	-375.328	-362.270	8
4	NIKO-PLIMN-PLIO- ALIS-NAXO-TPFM-PEN7 PEN6-MYRTA, PEN4-PEN5-PEN1-MYRTB HSUL-ARI-CHOI-ORA, KOKI-STPS-FAAV-LAFR-KDAM-MP, ISR	-373.473	-358.560	7
3	NIKO-PLIMN-PLIO- ALIS-NAXO-TPFM-PEN7 PEN6-MYRTA, PEN4-PEN5-PEN1-MYRTB HSUL-ARI-CHOI-ORA-KOKI-STPS-FAAV-LAFR-KDAM-MP, ISR	-133.976	120.436	1
2	NIKO-PLIMN-PLIO- ALIS-NAXO-TPFM-PEN7 PEN6-MYRTA, PEN4-PEN5-PEN1-MYRTB HSUL-ARI-CHOI-ORA-KOKI-STPS-FAAV-LAFR-KDAM-MP-ISR	-407.189	-425.990	9

Table S7: Estimated F_{ST}s between the major identified genetic clades after 100 bootstraps according to Weir and Cockerham (1984). Upper limit values are given above the diagonal and lower limit values below the diagonal.

Clade	CY1	CY2	CY3	CY4	CY5	ISR
CY1	-	0.22	0.17	0.12	0.42	0.47
CY2	0.16	-	0.22	0.22	0.42	0.49
CY3	0.11	0.14	-	0.21	0.44	0.50
CY4	0.07	0.15	0.14	-	0.42	0.48
CY5	0.34	0.36	0.37	0.35	-	0.45
ISR	0.40	0.41	0.42	0.41	0.36	-

Table S8: Estimated F_{ST}s between examined populations according to Weir and Cockerham (1984).

Pop	ARIS	MYRT	CHOI	HSUL	KDAM	ORA	LAFR	PEN4	PEN6	PLIM	ALIS	KOKI	NAXO	PLIO	NIKO	FAAV	PEN5	STPS	MEPO	PEN1	ISR	TPFM	PEN7
ARIS	--																						
MYRT	0.09	--																					
CHOI	0.09	0.08	--																				
HSUL	0.08	0.14	0.11	--																			
KDAM	0.18	0.18	0.13	0.17	--																		
ORA	0.13	0.17	0.03	0.12	0.13	--																	
LAFR	0.25	0.19	0.23	0.27	0.16	0.25	--																
PEN4	0.14	0.17	0.25	0.23	0.24	0.26	0.28	--															
PEN6	0.35	0.26	0.37	0.40	0.38	0.41	0.39	0.43	--														
PLIM	0.44	0.32	0.45	0.47	0.45	0.47	0.46	0.52	0.14	--													
ALIS	0.35	0.22	0.35	0.39	0.34	0.38	0.41	0.43	0.09	0.13	--												
KOKI	0.16	0.16	0.16	0.22	0.12	0.20	0.15	0.25	0.39	0.47	0.37	--											
NAXO	0.37	0.24	0.36	0.43	0.38	0.41	0.41	0.44	0.07	0.19	0.07	0.38	--										
PLIO	0.25	0.16	0.24	0.30	0.30	0.34	0.31	0.31	0.06	0.16	0.04	0.30	0.05	--									
NIKO	0.41	0.33	0.43	0.45	0.41	0.44	0.42	0.48	0.15	0.12	0.13	0.41	0.12	0.15	--								
FAAV	0.17	0.12	0.21	0.21	0.08	0.19	0.08	0.22	0.32	0.39	0.33	0.10	0.32	0.19	0.35	--							
PEN5	0.24	0.21	0.30	0.29	0.24	0.30	0.31	0.04	0.40	0.50	0.44	0.27	0.47	0.31	0.48	0.25	--						
STPS	0.12	0.06	0.11	0.15	0.08	0.15	0.11	0.14	0.22	0.35	0.29	0.07	0.30	0.12	0.29	0.09	0.21	--					
MEPO	0.19	0.18	0.15	0.21	0.04	0.16	0.20	0.24	0.38	0.46	0.36	0.11	0.40	0.29	0.43	0.14	0.27	0.08	--				
PEN1	0.06	0.01	0.12	0.11	0.15	0.15	0.17	0.14	0.27	0.42	0.33	0.15	0.30	0.13	0.34	0.13	0.15	0.14	0.12	--			
ISR	0.39	0.36	0.36	0.45	0.42	0.44	0.45	0.44	0.44	0.49	0.41	0.42	0.40	0.34	0.49	0.33	0.46	0.28	0.40	0.26	--		
TPFM	0.28	0.16	0.29	0.29	0.25	0.31	0.29	0.32	0.04	0.11	0.07	0.26	0.08	0.06	0.10	0.25	0.36	0.31	0.29	0.29	0.30	--	
PEN7	0.33	0.21	0.33	0.35	0.33	0.35	0.34	0.42	0.02	0.10	0.07	0.36	0.08	0.04	0.01	0.30	0.42	0.25	0.35	0.38	0.37	0.07	--

Table S9: Estimated F_{ST}s between examined populations after 100 bootstraps according to Weir and Cockerham (1984). Upper limit values are given above the diagonal lower limit values below the diagonal.

Pop	ARIS	MYRT	CHOI	HSUL	KDAM	ORA	LAFR	PEN4	PEN6	PLIM	ALIS	KOKI	NAXO	PLIO	NIKO	FAAV	PEN5	STPS	MEPO	PEN1	ISR	TPFM	PEN7
ARIS	--	0.14	0.14	0.13	0.24	0.18	0.30	0.20	0.40	0.49	0.41	0.19	0.42	0.28	0.45	0.23	0.30	0.19	0.26	0.12	0.45	0.33	0.38
MYRT	0.05	--	0.14	0.20	0.22	0.21	0.24	0.22	0.31	0.37	0.27	0.20	0.29	0.21	0.38	0.17	0.25	0.10	0.23	0.06	0.40	0.21	0.26
CHOI	0.04	0.03	--	0.17	0.19	0.10	0.30	0.31	0.44	0.50	0.42	0.20	0.44	0.30	0.47	0.28	0.37	0.18	0.21	0.19	0.42	0.36	0.38
HSUL	0.04	0.09	0.07	--	0.22	0.18	0.32	0.28	0.45	0.51	0.45	0.27	0.48	0.34	0.50	0.28	0.35	0.20	0.26	0.18	0.50	0.34	0.40
KDAM	0.14	0.14	0.08	0.12	--	0.18	0.21	0.29	0.42	0.50	0.39	0.15	0.43	0.33	0.45	0.13	0.31	0.11	0.08	0.20	0.46	0.29	0.37
ORA	0.09	0.12	0.03	0.08	0.09	--	0.31	0.30	0.45	0.52	0.44	0.24	0.47	0.38	0.48	0.25	0.36	0.19	0.22	0.22	0.49	0.36	0.40
LAFR	0.20	0.15	0.18	0.22	0.10	0.19	--	0.33	0.44	0.51	0.46	0.20	0.47	0.36	0.46	0.13	0.37	0.16	0.25	0.24	0.51	0.34	0.39
PEN4	0.09	0.13	0.19	0.18	0.20	0.21	0.24	--	0.47	0.56	0.48	0.30	0.50	0.35	0.52	0.27	0.09	0.19	0.30	0.20	0.50	0.38	0.47
PEN6	0.29	0.22	0.31	0.34	0.33	0.35	0.34	0.38	--	0.19	0.14	0.43	0.13	0.10	0.19	0.37	0.45	0.28	0.43	0.34	0.50	0.08	0.05
PLIM	0.39	0.27	0.40	0.41	0.41	0.43	0.41	0.45	0.09	--	0.18	0.52	0.25	0.20	0.17	0.45	0.56	0.42	0.53	0.48	0.55	0.16	0.15
ALIS	0.29	0.17	0.27	0.33	0.29	0.33	0.35	0.39	0.03	0.08	--	0.42	0.13	0.10	0.21	0.39	0.50	0.36	0.41	0.41	0.47	0.12	0.12
KOKI	0.11	0.12	0.11	0.17	0.08	0.15	0.10	0.20	0.35	0.43	0.31	--	0.43	0.35	0.46	0.14	0.32	0.12	0.15	0.21	0.47	0.31	0.41
NAXO	0.31	0.19	0.28	0.37	0.31	0.35	0.33	0.39	0.03	0.13	0.00	0.32	--	0.10	0.18	0.38	0.53	0.37	0.46	0.37	0.46	0.14	0.13
PLIO	0.19	0.11	0.18	0.24	0.25	0.27	0.27	0.25	0.02	0.11	0.01	0.25	0.01	--	0.19	0.24	0.36	0.16	0.35	0.19	0.40	0.09	0.08
NIKO	0.36	0.29	0.37	0.40	0.36	0.39	0.37	0.43	0.10	0.07	0.06	0.37	0.07	0.11	--	0.40	0.52	0.34	0.48	0.39	0.53	0.13	0.05
FAAV	0.12	0.08	0.15	0.16	0.04	0.13	0.03	0.17	0.26	0.35	0.25	0.04	0.26	0.15	0.30	--	0.30	0.14	0.20	0.21	0.41	0.31	0.36
PEN5	0.18	0.15	0.25	0.22	0.20	0.26	0.26	-0.01	0.35	0.45	0.39	0.22	0.41	0.26	0.43	0.20	--	0.27	0.32	0.23	0.51	0.42	0.48
STPS	0.06	0.02	0.06	0.10	0.03	0.10	0.04	0.08	0.17	0.30	0.21	0.01	0.24	0.08	0.23	0.02	0.14	--	0.13	0.20	0.35	0.38	0.31
MEPO	0.14	0.14	0.10	0.16	0.01	0.12	0.16	0.19	0.33	0.42	0.30	0.06	0.33	0.25	0.38	0.09	0.21	0.04	--	0.16	0.45	0.35	0.40
PEN1	0.01	-0.04	0.06	0.06	0.09	0.08	0.12	0.06	0.23	0.36	0.26	0.09	0.24	0.08	0.28	0.07	0.08	0.06	0.05	--	0.32	0.37	0.44
ISR	0.34	0.32	0.31	0.41	0.38	0.40	0.40	0.39	0.40	0.45	0.36	0.36	0.34	0.30	0.44	0.26	0.41	0.23	0.35	0.18	--	0.35	0.43
TPFM	0.22	0.12	0.21	0.22	0.18	0.25	0.22	0.25	0.00	0.05	0.00	0.19	0.00	0.01	0.03	0.18	0.29	0.23	0.22	0.23	0.23	--	0.13
PEN7	0.26	0.16	0.26	0.28	0.27	0.29	0.26	0.36	-0.01	0.05	0.00	0.30	0.04	0.00	-0.03	0.23	0.37	0.18	0.29	0.31	0.30	0.00	--