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Diversification within an oceanic Mediterranean island: Insights from a terrestrial isopod

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ABSTRACT

Understanding intra-island patterns of evolutionary divergence, including cases of cryptic diversity, is a crucial step towards deciphering speciation processes. Cyprus is an oceanic island isolated for at least 5.3 Mya from surrounding continental regions, while it remains unclear whether it was ever connected to the mainland, even during the Messinian Salinity Crisis. The terrestrial isopod species Armadillo officinalis, that is widespread across the Mediterranean, offers the opportunity to explore intra-island divergence patterns that might exhibit geographical structure related also to the region's known paleogeography. Genome-wide ddRADseq, as well as Sanger sequencing for four mitochondrial and three nuclear loci data were generated for this purpose. In total, 71 populations from Cyprus, neighbouring continental sites, i.e., Israel, Lebanon and Turkey, and other Mediterranean regions, i.e. Greece, Italy, and Tunisia, were included in the analysis. Phylogenetic reconstructions and population structure analyses support the existence of at least six genetically discrete groups across the study area. Five of these distinct genetic clades occur on Cyprus, four of which are endemic to the island and one is widely distributed along the circum-Mediterranean countries. The sixth clade is distributed in Israel. The closest evolutionary relationship of endemic Cypriot populations is with those from Israel, while the evolutionary clade that is present in countries all around the Mediterranean is very shallow. Cladochronological analyses date the origin of the species on the island at ~ 6 Mya. Estimated f4 and D statistics as well as F_{ST} values indicate the genetic isolation between the populations sampled from Cyprus and surrounding continental areas, while there is evident gene flow among populations within the island. Species delimitation and population genetic metrics support the existence of three distinct taxonomic units across the study area, two of which occur on the island and correspond to the endemic clade and the widespread circum-Mediterranean one, respectively, while the third corresponds to Israel's clade. The islands' paleogeographic history and recent human activities seem to have shaped current patterns of genetic diversity in this group of species.

1. Introduction

In a relatively recent account of the prospects of island biodiversity studies, some of the most crucial under-explored subjects in urgent need of further research were identified (Warren et al., 2015). Among these are questions relating to clade differentiation and speciation patterns within islands, such as the role of arrival history in community assembly, of *in situ* evolution in ecosystem functioning, of gene flow in speciation, and why some lineages are richer than others. Such questions

are expected to be addressed using both phylogeographic and population genetic/-omic 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). So far, published work, focusing on spiders and plants from the Canary islands (Macías-Hernández et al., 2013; Puppo et al., 2016), birds from La Réunion (Gabrielli et al., 2020), land snails from the Galápagos (Phillips et al., 2020), insects from Hawaii (Hembry et al., 2021), as well

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as shrews and frogs from the Sunda Shelf islands (Demos et al., 2016; O'Connell et al., 2018), highlighted the role of ecology, geology, and island formation history in shaping patterns of genetic diversity through extinctions, bottlenecks, geographic isolation, and recolonizations.

Past studies on terrestrial isopods (Crustacea, Oniscidea) have revealed high genetic divergence at species or genus level, among individuals distributed at geographically close areas, including isolated islands and islets (Bidegaray-Batista et al., 2015; Kamilari et al., 2014; Klossa-Kilia et al., 2006; Parmakelis et al., 2008; Poulakakis and Sfenthourakis, 2008; Santamaria, 2019; Santamaria et al., 2013; Taiti et al., 2003). Nevertheless, none of the aforementioned studies has addressed diversification patterns within islands. The only actual reference on within-island diversification is given by Santamaria et al. (2013), where two distinct genetic lineages of *Ligia hawaiensis* are reported from the largest island of the Hawaiian archipelago.

Cyprus, located at the eastern Mediterranean Sea basin, lies within a global biodiversity hotspot (Marchese, 2015; Myers et al., 2000). The island has been isolated for at least 5.3 Ma from surrounding continental regions, to which it has probably never been connected, making it one of the very few, and by far the largest, oceanic islands of the Mediterranean Sea (Constantinou and Panavides, 2013). The island has taken its modern configuration quite recently (mid- to late Pleistocene) through the establishment of a land-bridge connection (Mesaoria plain) between the two formerly isolated paleoislands that today make the two main mountain ranges (Troodos at the central-western part, and Pentadaktylos at the north-northeastern part of the island, respectively; Fig. 1). These mountains first emerged from the sea surface approximately 15-20 Mya and remained separate for most of their geological past. Therefore, we might expect modern populations to retain some signal of past isolation between these two former paleoislands, even if obscured to some extent by biotic interchanges and population admixture. Published phylogenies on various Cypriot taxa support close phylogeographic relationships of the island with adjacent eastern Mediterranean regions (Israel, Lebanon, Syria, and southeast Turkey), which suggest these regions were the source of origin of several taxa currently found on the island (Kotsakiozi et al., 2018; Lymberakis et al., 2007; Poulakakis et al., 2013, 2005; Sfenthourakis et al., 2017).

The terrestrial isopod Armadillo officinalis Duméril, 1816 (Crustacea,

Isopoda, Oniscidea, Armadillidae) is widely distributed across the Mediterranean Sea and the coasts of the western Black Sea (Schmalfuss, 2003). In fact, it is among the most commonly found Oniscidea species in the Mediterranean biome. It is regarded characteristic of Mediterranean-type ecosystems, having been called the 'animal equivalent of the olive tree' (Boyko et al., 2019; Schmalfuss, 1983, 1996). It is a well-defined taxon with low morphological variation (Schmalfuss, 1996) that inhabits areas with a variety of substrates (sandy, silty-clayey or rocky) and vegetation types (Messina et al., 2014). It 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 studies (Agodi et al., 2015) and in bioacoustics (Cividini and Montesanto, 2020; Cividini et al., 2020).

The present study aims to explore population structure within Cyprus, using also populations of Armadillo officinalis from several Mediterranean areas for comparison. Given the limited dispersal ability of terrestrial isopods, as well as the landscape heterogeneity, the complex geological history, and the long isolation of Cyprus, we could expect high genetic diversification between mainland and island populations. Possible patterns of genetic diversification among different Cypriot populations, especially those from localities with different habitat characteristics that can be considered as terrestrial 'habitat islands', will be evaluated in view also of the long existence of Cyprus in the form of two paleoislands. Time-calibrated phylogenies are employed to evaluate the timing of population diversification within Cyprus, as well as the role of human activities and island's paleogeography in shaping present patterns. In particular, we aim to test whether: (i) the paleogeography of the island (i.e. its long existence as two paleoislands) is reflected in patterns of genetic divergence, (ii) the Messinian Salinity Crisis (MSC, ~ 6-5.3 Mya; Krijgsman et al., 1999) has facilitated the arrival of the species on the island, and (iii) the north-eastern part of the eastern Mediterranean coasts acted as the source of the species' introduction to the island, given that it was the closest part to the mainland during sea level subsides in the Pleistocene and the most probable area where connections might have been established during the MSC.



Fig. 1. Armadillo officinalis populations collected from Cyprus. More details about location codes are given in Table S1.

2. Materials and methods

2.1. Sampling

At least three individuals per population of the targeted species were collected from 54 populations distributed all over Cyprus (Fig. 1). Further sampling was made in Greece, from where 18 individuals from eight different populations were collected. Collected material was placed in >96% ethanol and stored at -20 °C until further processing. The final dataset included also 26 specimens from five populations from Turkey and two from Israel, while Sanger data from Italy and Tunisia were retrieved from NCBI GenBank (Table S1).

Total genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's proposed protocol. Retrieved DNA quality and quantity was assessed through agarose gel electrophoresis (TAE 1.5%, Ethidium Bromide stained), as well as by fluorescence measurements in Qubit 4 fluorometer (InvitrogenTM, Thermo Fisher Scientific, Waltham, USA), respectively.

2.2. Sanger data

2.2.1. Targeted loci

Seven genetic loci, four mitochondrial and three nuclear, were targeted and successfully amplified using PCR. The mitochondrial Cytochrome c oxidase subunit I (COI), Cytochome b (Cyt b), 12S ribosomal RNA (12S) and 16S ribosomal RNA (16S) gene fragments were targeted from three or more individuals of each population. Primer pairs used to amplify aforementioned loci are given in Table S2. Thermocycling conditions were adapted from Dimitriou et al. (2018). A subset of individuals representing all divergent mitochondrial clades, as indicated by phylogenetic and species delimitation analyses, were selected for sequencing the more conserved nuclear genes. In particular, the mitochondrial phylogenetic patterns were assessed using mPTP (available at: https://species.h-its.org/) in order to select a subset of individuals successfully capturing the taxon's genetic divergence for sequencing of nuclear genetic loci. More specifically, the nuclear genetic markers 18S, 28S rRNA genes, and the protein-coding Sodium-Potassium Pump (NaK) were amplified following previously published protocols (Dimitriou et al., 2018; Dimitriou et al., 2019). PCR products were purified with Qiaquick Purification Kit (Qiagen, Germany) following the manufacturer's instructions, and sequenced using BigDye Terminator cycle sequencing chemistry (v.3.1) on an ABI3730XL automated sequencer at Macrogen facilities (Amsterdam, The Netherlands). Both strands of the PCR products were sequenced using the primer sets used in the PCR assays.

2.2.2. Alignment, nucleotide substitution model selection and genetic distances

Sanger sequencing results were delivered as chromatograms and the authenticity of retrieved sequences was tested by comparing to BLAST results using NCBI's default settings. All generated sequences exhibit high similarity to already published data as well as among each other. Sequences were assembled and edited in CodonCode Aligner (v. 3.7.1; CodonCode Corp., USA) and edits were made where necessary. Previously published sequences (16S and COI) of the confamiliar genus *Spherillo* Dana,1853 (*S. dorsalis* and *S. obscurus*) were retrieved and included in our analyses to serve as outgroups.

Multiple sequence alignments for each gene were performed online using the MAFFT v.7 webserver (https://mafft.cbrc.jp/alignmen t/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.

In order to evaluate genetic divergence, groups of populations were determined based on: (i) geographic distribution, (ii) statistical support on nodes of the constructed phylogenetic trees, and (iii) mitochondrial species delimitation results (see below). Kimura 2-parameter mean genetic distances (Kimura, 1980) between and within predefined groups were calculated using MEGA v.11 (Tamura et al., 2021).

2.2.3. Phylogenetic analyses

Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenetic reconstructions were performed in MRBAYES v. 3.2.6 and RAxML-NG v. 1.0.2, respectively, (Kozlov et al., 2019; Ronquist et al., 2012) by applying for each gene the best fitting model of evolution indicated by jMODELTEST v.2.1.1 (Table S3). Both analyses were run separately for the mtDNA the nuDNA and the concatenated dataset including all seven sequenced loci. In all cases BI analysis was run four independent times with eight chains per run for 10 million generations. Convergence among runs was monitored with TRACER v. 1.5 (Rambaut and Drummond, 2007). The statistical support of ML results was evaluated with 1000 bootstraps (Felsenstein, 1985).

2.2.4. Divergence time estimation, species tree and species delimitation

Cladochronological estimations and species tree analysis were conducted based on all seven amplified genes, using StarBEAST2 v 2.6.3 (Ogilvie et al., 2017). Regarding species tree analysis, specimens were divided into groups based on the results of mtDNA phylogeny. 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 5,000th generation. Convergence between runs was evaluated 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 TREEAN-NOTATOR v.2.6.3 (Ogilvie et al., 2017).

The nuDNA and the concatenated Sanger datasets were used for species delimitation analysis conducted in BPP v 4.1.3. Mitochondrial genes were treated as a single locus, as suggested by the software developers (Flouri et al., 2018). Implemented analysis within the multispecies coalescent framework was run setting the number of samples (nsample) to 300,000 and burnin at 10%. The effect of θ s and τ_0 priors on our results was evaluated by testing different combinations of these parameters, as suggested by the software developers.

2.3. ddRAD data

2.3.1. Genomic library preparation

Based on the mtDNA phylogenetic reconstruction, 168 individuals from 24 populations in Cyprus were selected, representing all identified genetic clades, for preparing genomic libraries. 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 300 ng, and EcoRI and MseI restriction enzymes were used to digest genomic DNA. Illumina sequencing adapters as well as a unique barcode were ligated to each specimen. Including attached oligos, 375–475 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). Prepared libraries were sequenced on a HiSeqX platform (Illumina, San Diego, CA, USA; 150 bp paired-end reads) at Macrogen NGS facilities (Seoul, South Korea).

2.3.2. Data processing

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 by setting the mindepth option to 6 and the clust_threshold to 0.9 while leaving all the other parameters at default settings. 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 = -1, min_info = -1 and min_taxa option to 23 aiming to retain loci with at least 23 unique sequences. The resulting list of loci was concatenated in a supermatrix while unlinked SNPs with the fewest missing characters for each locus were selected for generating individual genotypes.

2.3.3. Phylogenetic reconstructions

The filtered dataset was fed to MRBAYES v. 3.2.6 for two independent runs of four chains each for 2 million generations with 10% burn-in and sampling every 1000th generation. Convergence among runs was evaluated in TRACER v.1.5. In addition, maximum likelihood reconstructions were performed with RAxML-NG v.1.0.2 under the GTR model, as suggested for concatenated loci representing whole genomes (Leaché et al., 2015) and evaluated using 500 bootstrap replicates.

2.3.4. Species delimitation on SNPs data

SNPs-based species delimitation and species tree analyses were conducted with SNAPP in BEAST2 v 2.6.3. Prior to species tree analysis, species delimitation through the use of Bayes factors with marginal likelihood estimates via path sampling analysis was performed aiming to identify the most suitable taxon sets. All sampled populations were initially treated as different "species" and then gradually lumping them, alternative species delimitation models reflecting: (i) the geographic distribution of samples and (ii) the phylogenenetic patterns detected in this study were tested (Leaché et al., 2014). The analysis was run nine times, splitting available sequences to 24, 18, 12, 8, 6, 5, 4, 3 and 2 population sets, considering both geographical and phylogenetic patterns. Aiming to limit the amount of missing data in the final dataset that could lead to spurious SNAPP function and results, the initial dataset was filtered using poppr R package (Kamvar et al., 2014). Path sampling was run with 24 steps for 10⁵ MCMC iterations while pre-burnin was set to 10,000. The best option fitting our data was based on the Bayes Factors (BF) of the calculated marginal likelihood estimates (MLE). 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) using tree height and number of tips as inputs. Species tree analysis ran for 5 million generations performing two independent runs.

2.3.5. Population structure and gene flow analyses

Filtered ddRAD data were used for constructing an unlinked SNPs supermatrix which was fed to STRUCTURE v.2.3.4, aiming to identify patterns of population structure using this model-based Bayesian clustering approach. Ten replicates of the analysis were run for each K that varied from 1 to 10. Runs were conducted for 500,000 generations, setting burn-in to 100,000 using STRUCTURE THREADER (Pina-Martins et al., 2017). The K that best fits our data was determined by monitoring the estimated mean Ln likelihood and by the Δ K Evanno method (Evanno et al., 2005), as employed in STRUCTURE HARVESTER (Earl and vonHoldt, 2012). Consistency among runs was evaluated with CLUMPAK (Kopelman et al., 2015). Samples allocation to the inferred clusters was based on membership coefficient values (Q \geq 0.9). Individuals with Q \leq 0.9 were considered admixed and were removed from further analyses with STRUCTURE in a hierarchical structuring analysis mode.

Furthermore, Discriminant Analysis of Principal Components (DAPC), as implemented in R package adegenet v2.0.0 (Jombart and Ahmed, 2011 was employed as an alternative non-model-based method, not dependent on the assumption of Hardy-Weinberg equilibrium, to analyse our data. The optimal number of clusters was identified by running K-means comparing clustering solutions using Bayesian Information Criterion (BIC).

Furthermore, the same dataset was used for the pairwise population fixation index (F_{ST}) calculation in the R package Hierfstat (Goudet,

2005). The WC model (Weir and Cockerham, 1984) was selected for calculating F_{ST} s, while the statistical significance of our results was assessed through 100 bootstrap replicates. The f4 statistic (Reich et al., 2009) and the respective z-scores were estimated with TREEMIX (Pickrell and Pritchard, 2012) via a block jackknife resampling of 10 SNPs, aiming to detect any signal of gene flow between predefined phylogenetic clades. Finally, for the same purpose, the D statistic was calculated for the same population groups with Dsuite software (Malinsky et al., 2021).

3. Results

3.1. Sequencing results

Targeted loci were successfully amplified and sequenced for the majority of specimens. The final concatenated aligned dataset of all Sanger sequenced loci consisted of 1,945 bp of mtDNA and 1,951 bp of nuDNA gene fragments (in a total of 3,896 bp). Regarding ddRAD sequencing, >1.23 billion reads were produced for all 168 individuals. An average of 4,121,935 reads per sample were retained, excluding individuals with prohibitively high missing data. Among the 115,512 identified loci, 11,687 were non variable with an average of 7,755 loci for each sample. After filtering, the final concatenated dataset consisted of 1,026,968 sites, while the SNPs supermatrix consisted of 3,685 unlinked SNPs (59.4% missing data) from an equal number of loci.

3.2. Genetic divergence – phylogenetic analyses

BI and ML phylogenetic reconstructions based on the mtDNA and the concatenated Sanger dataset resulted in similar topologies with statistically well-supported clades (Fig. 2, Fig. S1). Groupings of populations at the produced trees based on both Sanger (mtDNA, mtDNA/nuDNA) and ddRad datasets revealed a pattern of geographic differentiation. More specifically, five statistically well-supported clades are present on Cyprus and six or seven across the study area according to ddRAD or Sanger mtDNA data, respectively (Figs. 2-4). Within Cyprus, identified clades are distributed: (i) at the southern part of Troodos Mt. (southern paleoisland; CY1 clade), (ii) across Pentadaktylos Mt. range (northern paleoisland; CY2 clade), (iii) along the Mesaoria plain (area uplifted in late Pleistocene connecting the two paleoislands; CY3 clade), (iv) at the western part of the Troodos Mt. range (CY4 clade), and v) all over the island and other circum-Mediterranean areas (OFF clade - named after A. officinalis as it corresponds to the nominal species according to our results herein - see Discussion; Fig. 3). The phylogeny based on mtDNA data divide this latter clade in two subclades (MED and GRT), even though individuals from the same populations (ALIS, ARCH; Table 1) are grouped in different clades. One more clade (ISR) consists of individuals collected from Israel.

It has to be noted that during sampling we identified a color variation in two populations, namely one from the central part of the island (Nicosia, ARIS) and one from a site at the southern part (near Larnaka, HSUL). Individuals from these populations exhibited lighter coloration and white epimera, while all other populations consisted of the common uniformly dark grayish colored animals. This variation has not been recorded before from any other part of the species' distribution. Beyond the observed white colouration of the epimera in some populations, no morphological differences were detected among sampled specimens.

All individuals with white epimera are grouped together with specimens with the common coloration in the CY3 clade. In total, three genetically "misplaced" individuals, i.e., not in accordance to observed geographic patterns, were identified based on mtDNA and ddRAD data: one belonging to the CY1 clade but found at the western part of the island (Fig. 2), and two individuals belonging to CY2 clade but found at the south-eastern part (Fig. 4). The widespread OFF (MED + GRT) clade is shallow, and includes individuals from Cyprus, Israel, Turkey, Greece, Italy, and Tunisia (Fig. 3). Regarding the nuDNA dataset, individuals



Fig. 2. 50% majority-rule consensus tree from the Bayesian Inference (BI) analysis constructed using COI, 16S, 12S and Cytb markers. Stars on the nodes indicate identical topology between BI and ML analyses, with bootstrap values >80 and posterior probabilities >0.9. Colours correspond to identified clades and this correspondence has been used in all subsequent figures.

belonging to the CY1-CY4 clades form one well-supported group (Fig. S2). It is worth noticing that, based solely on Sanger nuDNA data or solely on ddRAD data, one of the aforementioned misplaced individuals collected from the western part of the island is placed in its 'correct' clade (CY4). In all cases, ddRAD and Sanger datasets indicate a close phylogenetic relationship of the Israeli population (ISR clade) with Cypriot clades CY1–CY4, rather than with the OFF clade distributed all over the Mediterranean (Figs. 2 and 4).

Sanger mtDNA data indicate the existence of two distinct genetic subclades one occurring solely in Greece and Turkey (GRT clade), while this result is not supported by Sanger nuDNA or ddRAD data in which individuals forming the GRT clade are grouped with the rest of the individuals in the OFF clade (Fig. 4).

Genetic distances between predefined groups did not vary significantly among mtDNA or among nuDNA genes, but exhibited some variation within each gene (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%, and NaK: 0.00–0.79%). The maximum genetic distance was observed between the GRT subclade and the Cypriot group (Fig. 2), with the exception of COI, where maximum genetic distance was estimated between the GRT and ISR groups. The lowest genetic distances were observed among clades from Cyprus (Tables S2–S5), with the exception of 28S where minimum genetic distance was estimated between the OFF subclades GRT and MED (Fig. 5).

3.3. Cladochronology and species delimitation

Based on the concatenated Sanger dataset, the origin of the populations from Cyprus dates back to \sim 6 Mya (95% HPD: 4.13–8.14), with the Cypriot clades (CY1–CY4) sharing a common ancestor with ISR at \sim 15.7 Mya (95% HPD: 10.98–19.64).

Regarding species delimitation based on the concatenated (mtDNA



Fig. 3. Geographic origin of specimens. Different colours correspond to genetic lineages revealed by phylogenetic analyses (Fig. 2).



Fig. 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.

+ nuDNA) and the nuDNA Sanger datasets, the analyses under different priors/models supported the existence of one to seven distinct *A. officinalis* lineages (posterior probability > 0.90) in both datasets (see Tables S7, S8). In the case of the concatenated dataset, the presence of five distinct lineages was supported under three different models [posterior probabilities > 0.98]. One of these lineages includes the CY1, CY2 and CY3 clades, whereas the other clades/subclades (CY4, MED, GRT and ISR) remain distinct.

of missing data were allowed at the SNAPP input file (Leaché et al., 2014). MLEs and BF favoured the three "species" scenario (Table S10), where clades OFF, ISR, and CY1–CY4 were given as separate units. Based on this delimitation, the constructed species tree supported the closer relationship of the Cypriot clade with the OFF clade.

3.4. Population structure

Considering the impact of missing data on path sampling, only 15%

After the completion of the first hierarchical level of clustering

Table 1

Estimated F_{ST} values among the major identified genetic clades according to Weir and Cockerham (1984).

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



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

analysis, *A. officinalis* individuals were assigned to the same two clusters in all replicate runs for K = 2. The first cluster included only individuals collected from Cyprus and corresponds to clades CY1, CY2, CY3, and CY4, while the second consisted of populations from Cyprus, Greece and Turkey belonging to OFF clade (Fig. 6). Only eight individuals (5.5%) exhibited a mixed ancestry profile, one found at the north-western part of the island (LAFR population, CY4), while the rest originate from two populations, those from Israel (ISR) and Myrtou (CY2, CY3, OFF) in Cyprus.

Additional population structure was detected at the second level of hierarchical clustering in both cases. Within the cluster consisting of specimens only from Cyprus, individuals were assigned to four geographically distinct regions corresponding to the statistically wellsupported phylogenetic clades CY1-CY4. Individuals from OFF clade were further separated in two groups not exhibiting any geographical pattern. Assignment coefficients of the latter cluster including individuals from Cyprus (PLIM, PLIO) and Greece (ALISS, NAXO) did not exceed 82% (Fig. 6).

Population structuring explored through DAPC by ascertaining the relationship between individual genotypes yielded K = 3, according to the K-means method (corresponding to the lowest BIC value). DAPC supported the separation among the following phylogenetically inferred clades: (i) OFF (cluster 1), (ii) CY2 (cluster 2), and (iii) the rest of the "Cypriot" clades (CY1, CY3, CY4) grouped with Israel (cluster 3) (Fig. 7a). Further sub-structuring within cluster 3 was investigated using the same method. According to K-means algorithm, the selected K for this new subset was 2 and the new discriminant analysis separates the examined individuals into two distinct groups: Israeli populations, and the three Cypriot clades (CY1, CY3, CY4; Fig. 7b). A third level of analysis in the last group with the three "Cypriot" clades, discriminates CY3 clade from the clades CY4 and CY1 with the assignment posterior probabilities being in the great majority of individuals >0.90 (Fig. 7c).

The pairwise F_{ST} values between the six main *Armadillo* clades supported by the ddRAD phylogenies, varied from 0.10 to 0.45 between CY1–CY4 and CY3–ISR/CY2–ISR clades, respectively (Table 1). Fixation indexes for all populations groups, as well as bootstrap upper and lower limits, are presented in Tables S10–S12. The f4 and D statistics support the existence of gene flow among "Cypriot" (CY1–CY4) clades, while no gene flow between this group and ISR or OFF clades was found (Tables 2 and 3).

4. Discussion

Phylogenetic analyses based on Sanger mitochondrial data revealed the existence of seven distinct genetic clades of Armadillo officinalis. Four of them occur solely on Cyprus, one on Cyprus and other circum-Mediterranean regions, one in Greece and Turkey, and one in Israel. An almost identical diversification pattern was retrieved using genomewide ddRAD data, with the exception of the widespread OFF clade that includes populations from both the GRT and the MED clades, which are not retrieved as distinct. These findings are not reflected in the morphology of the species that exhibits limited morphological variation throughout its distribution (Schmalfuss, 1996). A distinct colour morph found in some Cypriot populations (individuals had whitish epimera) does not match any genetically identified group. Genetic distances among clades and groups of clades fall within the limits of published data from other Oniscidea species for 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). In Trachelipus aegaeus, the maximum distance was 20.3% for 16S and 19% for COI (Kamilari et al., 2014). Nevertheless, according to these studies, the aforementioned taxa might correspond to species groups rather than single species. Much lower conspecific genetic distances were observed in Armadillidium pelagicum from Tunisia, where sequence divergence reached up to 2.1% in the case of 16S (Charfi-Cheikhrouha, 2003). On the other hand, the maximum calculated distances among A. officinalis lineages exceeded the minimum sequence divergence observed among well-defined Ligidium species in the case of 16S and COI (Klossa-Kilia et al., 2006). Therefore, in the absence of robust taxonomic resolution, genetic distances might be misleading since it is not clear whether they refer to species or higher/ lower taxonomic levels.

At the same time, as shown by recently published work, especially in the case of Oniscidea, taxonomic assignment at the species level should not be based solely on morphology, but the evaluation of molecular data is needed for the identification, delimitation and description of species (Zimmermann et al., 2015; 2018a, 2018b).

One of the clades retrieved by our phylogenetic analyses of all datasets is distributed across the central-eastern Mediterranean. This widespread and genetically divergent clade seems to have been



Fig. 6. Population structure after two hierarchical STRUCTURE analysis levels using genome-wide SNPs. Each vertical line corresponds to a different individual, while different colours represent the estimated Q values corresponding to the assignment probabilities of each individual to putative population clusters. The first hierarchical clustering (a) divided the dataset into two separate clusters. These two clusters were further analysed showing further sub-structuring, b: blue cluster, c: orange cluster. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



Fig. 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 (blue colour). Individuals from the CY2 clade are forming the group 2 (yellow colour) while the rest of the individuals belonging to OFF clade are forming a separate group (red colour); (b) Within group 1, analysis discriminates the populations from Cyprus (blue colour) and Israel (red colour); (c) The remaining Cypriot populations are further separated in two groups corresponding to the CY3 (red colour) and CY4–CY1 clades (blue colour). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dispersed by humans, at least partially, as suggested by the small genetic distances and shallow unresolved phylogenetic relationships among the groups comprising it. Furthermore, the absence of any geographic structure in this clade within Cyprus or across the clade's distribution, further corroborates this hypothesis. Human activities seem to preserve an ongoing gene flow among populations spread across Mediterranean coasts by transferring individuals via various materials (such as soil and agricultural products).

Excluding this widespread (OFF) clade, the other clades exhibit distinct geographic patterns within Cyprus, with the CY2 clade restricted to Pentadaktylos Mt. (north-northeastern paleoisland), and the clades CY4 and CY1 restricted at the western and the southern parts of Troodos Mt. (western paleoisland), respectively. A well-supported distinct genetic clade (CY3) occurs at the recently uplifted Mesaoria

Table 2

Observed f4-statistics for the main phylogenetic clades given as "populations" to the analysis.

Population groups				f4 statistic	Standard error	Z score	
CY1	CY4	OFF	ISR	-0.00035	0.0003	-1.1558	
CY2	CY3	OFF	ISR	-0.00015	0.00092	-0.1615	
CY1	CY2	CY3	CY4	-0.002	0.00079	-2.5445	
CY3	CY1	CY4	CY2	-0.0029	0.00069	-4.3233	
CY2	CY3	CY4	CY1	0.00099	0.00048	2.0736	

plain, between these two main landmasses. A more thorough view of the phylogenetic relationships within Cyprus shows that this plain that recently connected the two main mountains (paleoislands) is inhabited by populations originating from the northern part of the island. Moreover, since only two individuals of the CY2 clade were found at the southeastern part of Cyprus instead of the northern one, we could assume that these were transferred by humans, as they were collected at a site heavily impacted by touristic activities.

The divergence of the Greece + Turkey (GRT) subclade seems to be supported only by mtDNA data, as in ddRAD and Sanger nuDNA trees a statistically well-supported monophyletic group is formed including individuals from both the GRT and MED subclades. This may suggest that GRT is a recently divergent clade where diversity has not yet been reflected in the slowly evolving nuclear genome. Furthermore, incongruent patterns between phylogenies based on mtDNA and nuDNA datasets could be explained by incomplete lineage sorting. Among the two populations sampled from Israel, one belongs to the OFF clade, while the other forms a distinct clade (ISR). The close evolutionary relationship between ISR and the four lineages found only on Cyprus suggests that this area might be close to the original source of introduction of A. officinalis to the island. Past studies have come to similar conclusions, supporting a Near-East origin of various taxa currently occurring on Cyprus (e.g., Poulakakis et al., 2013; Sfenthourakis et al., 2017).

In line with generated phylogenies, inferred population structure based on SNP data identified the same distinct genetic units. Genetic isolation of clades represented solely on Cyprus (i.e., excluding those in the OFF clade) from the rest is becoming obvious after the first hierarchical clustering, where only a very 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 found at a site in the northern part of the island (Myrtou, MYRT). Individuals from Israel (ISR) populations are also admixed. The geographical position of Myrtou at the edge of the western part of the Pentadaktylos Mt., between the geographical limits of the CY2 and CY3 clades, seems to indicate a zone of hybridization between the two lineages. This statement is also supported by the fact that individuals collected from this area were phylogenetically assigned either to CY2, or the CY3 and the OFF clades. On the contrary, the mixed genetic profile of individuals from Israel indicates a closer relationship between Cyprus and Israel than with other continental/Mediterranean populations. This relationship is wellsupported by the aforementioned phylogenetic patterns, geographic proximity, and known paleogeographic history of Cyprus.

Although it is still debatable whether Cyprus was ever connected to neighbouring mainland (Syria or south-eastern Anatolia), the subsidence of the sea level during the MSC facilitated the arrival of several 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 connected with, or being closer to Anatolia either through a land bridge or via a series of intermediate islets used as stepping-stones. This scenario is also supported by the closer evolutionary relationship between CY1-CY4 populations with the extant population from Israel (being geographically closer to the possible connection/proximity area of Cyprus with the mainland) rather than with those from Greece or Turkey. Populations from Cyprus and Israel, excluding the OFF clade, share a common ancestor at $\sim \! 15.74$ Mya. We speculate that a somewhat more recent ancestor could be found distributed at an area where Cyprus was possibly connected or been closer to the mainland (i.e., somewhere around Syria/SE Turkey). The closer evolutionary relationship of Cypriot clades with ISR, rather than with OFF, is also supported by DAPC, where individuals from Cyprus and Israel are grouped together. The same analysis focusing on the Cypriot-Israel group, separates the ISR population from that of Cyprus at the first level, and at the second level the clade CY3 from CY4-CY1. The possible genetic differentiation between the latter two phylogenetic clades seems to be too low for the sensitivity of this method. The sister clade relationship of these two (CY4-CY1) geographically neighbouring clades is also supported by the genomic-based phylogenetic trees.

Species delimitation using BPP results in either seven, five or one species (Tables S8 and S9). This suggests that Sanger data cannot provide a robust resolution of taxonomic affinities among clades in this case. On the other hand, SNAPP, based on the much richer ddRAD dataset, as well as phylogenetic results, suggest the existence of three distinct taxonomic units: one occurring in Cyprus, one in Israel, and one distributed across the whole study area. Hence, we assume that at least two Armadillo species exist on Cyprus, one of them being endemic to the island and the other the widespread in circum-Mediterranean countries, while one more species occurs in Irsael. Genetic isolation among these three clades is also indicated by the high F_{ST} (0.38–0.45) and f4 values which were shown not to be statistically different from zero. Furthermore, FSTS vary from 0.1 to 0.2, and f4 as well as D statistics are different from zero for the clades occurring solely on Cyprus, supporting the existence of gene flow among them. A detailed taxonomic study including morphological analysis and character depiction of representatives from all main clades is needed in order to revisit current taxonomic status of the focal taxon which in view of the evidence provided herein, evidently splitting what is today considered as Armadillo officinalis into three distinct species.

Cladochronological analysis indicates that the first separation of the widespread Mediterranean clade from the rest originates back to the Burdigalian (20.43–15.97 Mya). During this period, a sea corridor was formed, separating the northern from the southwestern Near East regions (Cornacchia et al., 2021.). Thus, we could assume that the

Table 3

Calculated D statistic	setting the same	"species id"	to individuals belonging to the	identified phylogenetic clades.

P1	P2	Р3	Dstatistic	Z-score	p-value	f4-ratio	BBAA	ABBA	BABA
CY1	CY2	CY3	0.018158	0.706584	0.479825	0.031868	1201.64	1796.04	1731.98
CY4	CY1	CY2	0.090538	3.87443	0.000107	0.086234	2190.2	1679.2	1400.38
CY2	CY1	CY5	0.018058	0.584373	0.558969	0.011231	5678.38	1376.5	1327.67
CY3	CY1	CY4	0.055452	2.42242	0.015417	0.116986	1866.92	1644.13	1471.37
CY3	CY1	OFF	0.04262	1.6802	0.092919	0.02169	6575.37	1211.43	1112.39
CY4	CY1	OFF	0.059344	2.21674	0.026641	0.030565	6910.89	1372.45	1218.68
CY4	CY2	CY3	0.100703	4.80491	1.55E-06	0.149008	1334.13	2382.19	1946.3
CY3	CY2	OFF	0.100972	3.91015	9.22E-05	0.054019	7394.02	1486.75	1214.05
CY4	CY2	OFF	0.075786	2.73326	0.006271	0.047012	6557.87	1769.22	1519.95
CY4	CY3	OFF	0.016845	0.682333	0.495029	0.008975	7495.57	1488.47	1439.15

paleogeography of the area promoting isolation and diversification of an ancestral *Armadillo officinalis* clade in the southeastern part of its distribution, leading to a distinct ISR + Cypriot clade, whereas the widespread OFF clade remained to the north of the sea corridor and has given the nominal species which has been described from type material from Italy. Later on, the separation of the ancestral clade that gave rise to the endemic Cypriot clades from that of Israel occurred in the Langhian - Serravallian (15.97–11.63 Mya). At the same time, the Cypriot populations that belong to the widespread OFF clade, show variable affinities to populations from other regions, suggesting multiple introductions to the island. Therefore, time-calibrated phylogenetic analyses coupled with population genetic patterns indicate multiple colonisations of Cyprus by 'A. officinalis', one during the MSC, probably facilitated by the paleogeography of the area at the time, and the others being recent, and probably human-mediated.

Concluding, cryptic within-island divergence of populations was revealed, indicating the occurrence of high diversity, not evident in morphology. The complex geological history of Cyprus, coupled with the long and continuous presence of humans for >10,000 years, can provide an explanatory framework for this diversification. The unexpected cryptic divergence of an apparently well-defined taxon raises questions also on the status of other Cypriot taxa with similarly restricted dispersal abilities, calling for genetic/genomics studies. We note that vivid genetic divergence was also reported from the, similarly sized, island of Crete in the case of the land snail species complex Albinaria cretensis (Bamberger et al., 2022). Our work has shed light into evolutionary processes taking place on this isolated, oceanic island that, despite its biogeographic characterization, lies in a closed sea, surrounded by three continents. Judging from our findings herein, we can expect that many cryptic Oniscidea species with subtle or no morphological differences could be discovered around the globe in future studies.

CRediT authorship contribution statement

Andreas C. Dimitriou: Conceptualization, Data curation, Formal analysis, Investigation, Methodology. Aglaia Antoniou: Methodology, Formal analysis, Writing – review & editing. Ioannis Alexiou: Methodology. Nikos Poulakakis: Methodology, Resources, Writing – review & editing. Aristeidis Parmakelis: Resources, Data curation, Methodology, Writing – review & editing. Spyros Sfenthourakis: Conceptualization, Resources, Data curation, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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