

**Phylogeographic patterns of spider species
complex *Dysdera erythrina***

Fylogeografie pavouků druhového komplexu *Dysdera erythrina*

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MASTER THESIS

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I declare that I have composed this thesis myself and I have used the sources listed in references.



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1. INTRODUCTION

Species are the most fundamental units of biodiversity and are the main outcomes of the evolutionary process (Ereshefsky, 1992; Wiley, 1981). Very few topics in biology have raised as much debate as the species definition and the nature of species (Claridge *et al.*, 1997; Coyne & Orr, 2004; Cracraft, 1989; Ereshefsky, 1992; Hey, 2001; Howard & Berlocher, 1998; Mayr, 1982; Ridley, 2004). More than 100 books and papers have been devoted to the species problem in the last 25 years, and more than 20 different species concepts have been proposed (de Queiroz, 1998; Ereshefsky, 1992; Mallet, 2001; Mayden & Wood, 1994).

Several authors (Mayden 1997; de Queiroz 1998) have stressed the fact that evolutionary independence of lineages underlines most species concepts and their differences mainly lay in the operational aspects of the species recognition they emphasize. Following this line of reasoning, de Queiroz has recently defined the species as a separately evolving metapopulation lineage (de Queiroz, 2007). The term lineage would comprise an ancestor – descendant series or metapopulations, the inclusive population made up of connected subpopulations extended through time (de Queiroz, 2007). This proposition has found numerous adherents (e.g. Shaffer & Thompson, 2007).

The manner how to define species is essential point in taxonomy and species delimitation, process when species boundaries are determinate and new species are discovered (Wiens, 2007). On the other hand, some authors propose, that is not necessarily indispensable to define the species according to the characteristics of different species concepts, but properties of the species itself shall be used as factors delimitating its boundaries (Sites & Marshal, 2003; Sites & Marshal, 2004).

The process of species delimitations can be based on morphological characters, molecular data, combination of both, or other criteria (e.g. ecology) can be added. Different methods of delimitating species may give ambiguous or contradictory results trying to reflect the speciation process and all methods can occasionally fail to delimit the species boundaries properly (Sites & Marshal, 2003; Sites & Marshal, 2004).

Molecular based DNA species delimitation characterizes putative independent evolutionary lineages (i.e. species) on obtained data unit. Different methods have been

implemented on many species in attempt to clarify their status and phylogenetic position (e.g. Davis & Nixon, 1992; Wiens & Penkrot, 2002; Pons, *et. al.*, 2006). Molecular based DNA species delimitation helped to unravel relationship among poorly known taxa and can be also applicable in analyses and species delimitation of cryptic species complexes consisting of very closely related species that are morphologically very hard or impossible to determinate (Pons, *et. al.*, 2006). The cryptic species complexes originated in recent radiations did not have sufficient time to accumulate whole range of features as fixed morphological apomorphies, strict reproductive isolations or phenotype characters providing their easy taxonomic classification (Shaffer & Thompson, 2007).

New model of quantitative analyses based on the detecting the shift in the rate of lineage branching and assess the threshold point where stochastic lineages growth transforms into the coalescence was proposed by Pons and collaborators (2006). This approach applied on the radiated tiger beetles in Australia led to recognizing of separate evolutionary lineages and putative species (Pons *et al.*, 2006).

In this study phylogenetic methods and methods of molecular based DNA species delimitation as method proposed by Pons and collaborators (2006) and statistical parsimony (Tempelton *et. al.*, 1992), separating groups of sequences into different networks if the genotypes are connected by comparatively long branches affected by homoplasy, were implemented on woodlouse hunter spider *Dysdera erythrina* (Walckenaer, 1802).

D. erythrina is considered sibling species complex or just morphologically local variable forms of one species. This unresolved high variability of morphological characters of this species or unclear relationships of potential cryptic species inside the species complex makes *D. erythrina* highly attractive for taxonomic and genetic studies.

The distribution of the whole genera with centre of major diversity in Mediterranean suggests that *D. erythrina* group originates from this region as well. The contemporary area of occurrence from Spain to Georgia (Platnick, 2008) may be the consequence of successful area expansion after the last glaciations. The colonization carries along events leading to speciation process among other consequences and shall be reflected in genome of *D. erythrina* complex.

1.1 Characterization of the family Dysderidae with a view to *Dysdera erythrina* group

The araneomorph family Dysderidae (L. C. Koch, 1837) with primary Palaearctic distribution consists of 24 genera and 494 species, which place it among the richest spider families. Majority of this richness is found in Mediterranean. Except the primary distribution of the family in Palaearctic region and North Africa, the family occurrence is also reported from South America, USA, Hawaii etc. due to the secondary human introduction of *D. crocata*, which present-day distribution is considered cosmopolitan (Platnick, 2008).

The family Dysderidae belongs among haplogyne families and alongside with Segestriidae, Orsolobiidae and Oonopidae form the superfamily Dysderoidea (Coddington *et. al.*, 2004). The females of these taxa do not present any external sclerotized copulatory organs. The receptacula seminis of this group are connected with external openings by only one spermathecal duct. That means that the duct provides both fertilization and eggs laying. (Uhl, 2000).

Morphologically important characters of the family are presence of 6 eyes in primary state (anterior medial eyes missing), which can be secondary reduced in species inhabiting caves, and modification of the respiratory system. Beside the one pair of book lungs they present also one pair of tracheas, which communicate with the exterior by pair spiracula located near the book lungs openings.

The family Dysderidae is subdivided in three subfamilies; Harpactinae, Rhodinae and Dysderinae (Deelman-Reinhold & Deelman, 1988). The subfamily Harpactinae is considered basal group of the family.

The genus *Dysdera* (Latreille, 1804) is distinguished from the rest of taxa of the subfamily Dysderinae by series of characters; the distance of between anterior lateral eyes and anterior median eyes never extends the half of their diameter. The teeth on basal segment of chelicerae are in one row. Female internal vulva is composed of two diverticulas, the anterior has sclerotized arch connected with transversal spermatheca and the posterior with simple transversal division and membranous sac. The male bulb is cylindrical with noticeable apofisis and distal hematodocha (Deelman-Reinhold, 1988).

The Spiders species of genus *Dysdera* are ground dwellers with nocturnal activity. During the daylight they hide themselves in protective silk cocoon constructed

in the leaf-litter or under stones. They are active hunters and their principal prey are the woodlice (Cook, 1968; Řezáč & Pekár, 2007; Řezáč *et al.* 2008).

The trophic specialization on woodlice is reflected in the morphology and physiology of the genus, all species have prolonged forwardly oriented chelicerae. Varied degrees of morphological adaptation correlated with capture of prey strategies have been reported. *Dysdera* species with elongated chelicerae captured the woodlice by wedging one of the fangs on the dorsal sclerotized part of the pray body penetrating from ventral soft part with the second fang. This capture technique “pincers strategy” was reported from *D. erythrina* and *D. abdominalis*. *Dysdera* species with concave chelicerae used the “fork” tactic for pray capture. They grasped promptly the pray with their forelegs and inserted both chelicerae fangs to the soft ventral part of the pray body. This behaviour was described in *D. spinicrus*. The last known manner of attacking the pray is the “key strategy” used by *D. dubrovinii*, which disposes of the flattened chelicerae. This morphological modification makes possible insert the fangs under the dorsal sclerites of the woodlouse no matter if did evolved or not. (Řezáč & Pekár, 2007; Řezáč *et al.*, 2008).

The genus *Dysdera* consists of more than 240 species, which makes it the largest genus of the family Dysderidae (Platnick, 2008). It has Palaearctic distribution eastward as far as Kasminir and southward as far as northern Africa (Cook, 1968). The main diversity of the genus lies in Mediterranean, where outstanding radiation of *Dysdera* species took place. Many species are local endemics with very restricted area of occurrence. In spite of the grate diversity of the genus and frequent sympatry, all species maintain rather uniform morphological appearance. For the group taxonomy the characters as copulatory organs both male and female, chelicerae shape, eyes distances and size, leg spines, body size, structure and colour of carapace are employed.

Dysdera erythrina (Walkenaer, 1802) is middle-sized spider with typical morphological appearance of woodlouse hunter spiders, the carapace and leg colour varies from reddish-brown to ferruginous and abdomen is brown.

Three new species were described by Simon (1882); *D. provincialis* from France, *D. lantosquensis* from France and *D. fervida* from Corsica and Balearic Island, all three of them very similar to *D. erythrina* forming sibling species complex. None of the descriptions mentioned above has schematic picture of male of female copulatory organs as a reference (Simon, 1882). Only verbal characterization of morphological aspect based on difficultly valuable criteria as carapace structure, coloration, size and

leg spines. All of these characters can become very variable and quite hard to evaluate, which probably caused the degradation of these taxa to “local forms” of *D. erythrina* (Simon, 1914) representing the population variability of the species.

Neither the revision of whole genus *Dysdera* (Deeleman-Reinhold & Deeleman, 1988) resolved this problem, the authors confirm only the *D. erythrina* species, but do not even note any “local forms” or subspecies mentioned above. Today’s opinions on the subject are not unified. They are treated as subspecies (Platnick, 2008) or as an independent species at least in case of *D. lantoquensis* belonging to *erythrina* group (Řezáč *et al.* 2008).

The *D. erythrina* group is characterized by parallel lateral anterior margins of carapace (in dorsal view) and concave inner margin of basal cheliceral segment (Řezáč, 2007). The contemporary area of distribution of *D. erythrina* species complex includes major part of Europe and extends to Georgia (Platnick, 2008). The area of is shown in the map (see Fig. 1).



Fig.1 The contemporary area of distribution of *D. erythrina* species complex according to Ferrández (1987)

1.2 Main factors driving the distribution and genetic diversity of the genus *Dysdera*

As mentioned before, genus *Dysdera* is the richest genus of entire family Dysderidae and majority of its divergence lays in Mediterranean. The possible explanation of this divergence is the complex geological and climatic history of the region. According to the knowledge of diversity of the genera, it is presumable that climatic, geologic and geographic factors would have an impact on *D. erythrina* complex distribution, genetic variability and possible speciation process.

In spite of the moderate climate, that characterizes the Mediterranean, we can find big variability in temperature and rainfall regime. This geographical variation causes astonishing habitat diversity and the mosaic effect of the landscape provides generating and maintaining of species diversity. Isolated mountains or islands became places of interest of speciation process studies (Blondel & Aronson, 1999).

At present the Mediterranean Basin is counted among the 25 biodiversity hotspots on the Earth. Its high level of endemism; 4.3 % of endemism for vascular plants and 0.9% for vertebrate fauna, makes this region very important and worth of complex protection (Myers *et al.*, 2000).

The formation of Mediterranean lays in the upper Eocene about 40 million years before present (My BP) when movement of African plate led to its approximation to Europe and caused the closing of the gap between Europe and Africa and eventually the closure of the Thetis Ocean (Blondel & Aronson, 1999). The collision between tectonic plates started the Alpine orogenic process – the rising of the great mountains surrounding the Mediterranean Basin, which continued until Miocene (Schellard, 2002).

After the Alpine orogeny the large number of blocks crowded in the in the south-west Europe were detached of the Iberian plate and started to drift to their nowadays position. These blocs represented some islands of western Mediterranean as Balearic Islands, Corsica, Sardinia and areas that joined the Iberian plate or Africa as Calabria or Betic-Rif Cordillera. The islands mentioned above underwent a series of rotations as drifted slowly eastward when approximately 10 My BP in the middle Miocene reached its current position (Rosenbaum *et. al.*, 2002).

In the late Miocene (about 5.6 My BP) another dramatic change took place. The slow northward movement of Africa continued during whole Miocene and collided with south-western Europe closing the Strait of Gibraltar. The Mediterranean Sea became isolated of the Atlantic Ocean. The evaporation together with sea-level drop caused by

expanding polar ice volume provoked so-called Messinian salinity crisis. The Mediterranean Sea was practically evaporated until the Strait of Gibraltar opened again some 5.3 My BP (Krijgsman *et. al.*, 1999).

In the late Pliocene some 3.2 My BP during the global cooling trend Mediterranean - type climate known in present began to appear. This climate characterized by dry and relatively hot summers and humid cool winters was established firmly 2.8 My BP. The first ice sheets started to grow in the Northern Hemisphere 2.3 My BP and marked the start of the climatic oscillation with 100 000 – year long cycle (Blondel & Aronson, 1999).

The climatic cycles are represented by glaciations which are interrupted of relatively short warm interglacial. (Webb & Bartlein, 1992). The most information we have proceed from the last glaciation of Europe, which finished approximately 18 000 BP. The Scandinavian ice sheet in the toughest phase reached 52° N, the permafrost to 47° N, and covered the northern Europe and major part of England. There where another ice sheets in the South, that covered all high mountains of Europe as the Pyrenees, Alps, Transylvania, Caucasus and mountain range of Cantabria. Between them and the Scandinavian ice sheet extended tundra and cold steppe (Hewitt, 1996).

The consequences of climatic oscillations for fauna and flora are supposed to be dramatic. Conditions excluding life for many species most probably caused changes of species distribution on the Northern hemisphere. The conditions made retreat thermophilic species to small refugia in the south; cold-adapted species are supposed to occupy low-elevation areas. The refugia in southern Europe were in Spain, Portugal, Greece, Italy and Balkans another lied in the Caucasus, northern Turkey and around the Caspian Sea.

The interglacial warming of climate probably made another shift of species distribution. Cold-adapted species could retreat northward or remained in small and isolated mountain islands, thermophilic and arid-adapted species probably left their refugia in the south and started to colonize newly vacated areas liberated from ice and inhospitable conditions (Hewitt, 1996; 2000; 2004; Taberlet *et al*, 1998). These areas especially in central and northern Europe, cut be colonized from just one refugium or from several of them, and genomes of the colonizers did, or did not mixed. The theories concerning post glacial colonization and its genetic consequences are proposed taking into account suitable different strategies (Hewitt, 1996).

For some species – the long distance dispersants, the expansion from the southern refugia is supposed to be rather quick. The genome of the colonizers setting up the first colonies during the start of expansions would be dominant in the newly established population and genes of later migrants would not contribute importantly. Rapid type of expansion and colonization carries along great risks. Many populations may become extinct or suffer bottle-necking, which leads directly to a loss of alleles. That way the whole areas can become homozygous at certain locus, the tendency increases with following range expansion and contraction, where only a few populations survive in the locally suitable places. In contrast, slower colonizing species can keep much more allelic variability. And the process of selection of genomes across the inhabited range is supposedly due to the different environmental conditions in the inhabited area.

To the contrary, cold-adapted species most probably were obliged to find refugia at the beginning of the warm interglacial. In the south the dispersion is limited by the temperature, so the species inhabiting these regions found the refugia in mountains. Fragmentation of the range led to the isolation of populations and apparition of different genomes. The size of the inhabited area is limiting; if the region is small, populations are exposed to the bottleneck risk and loss of genetic variability as a consequence (Hewitt, 1996; 2000; 2004).

2. AIM OF STUDY

To use molecular data to characterize evolutionary lineages in spiders of the *Dysdera erythrina* species complex, and to unravel the historical and ecological factors that promoted the diversification of the group.

3. MATERIALS AND METHODS

3.1. Taxonomic sampling

Dysdera erythrina group samples were collected throughout Western, Eastern and Central Europe. The populations of Catalonian part of Iberian Peninsula were main focus point of this study. Used material proceeds from collecting between years 2001 and 2007 and was obtained from various collectors. All specimens were primarily assigned as *D. erythrina* group. The specimens with further determination in this study as *D. erythrina*, *D. lantosquensis*, *D. provincialis* and *D. fervida* species were classified by Milan Řezáč. The specimen *D. cf. erythrina* from Sardinia was considered *D. erythrina* group in this study.

The specimens were collected from under the stones or leaf-litter and were put immediately into absolute ethanol and stored at -20°C. Gathered specimens were recorded with all locality data available into Biota AppPent 161 database (Colwell, 1997). The map of collection localities is shown on the Figure 2. Detail list of localities along with specimen codes and classification is reported in the Appendix 1.



Fig. 2: Collection localities of *Dysdera erythrina* group

3.2. Selected molecular markers

3.2.1 Mitochondrial markers

Two gene fragments and one entire gene were employed as molecular markers in this study. Both protein-coding and RNA-coding markers were selected according to their variability and informative value for phylogenetic studies.

The first half of mitochondrial gene coding for subunit I of cytochrome oxidase (cox1), approximately 650-bp region, was amplified and used for the further analyses. Cox1 is considered highly conservative but variable in some regions, which makes this gene very useful for evolutionary studies (Lunt *et al.*, 1996). The function of cytochrome oxidase I lies in electron transport and proton translocation across the membrane. As the gene is protein-coding no insertions and deletion were present in selected fragment.

Sequence analyses of this gene were used previously in spiders (e.g. Arnedo *et al.*, 2000; 2001; Bidegaray – Batista *et al.*, 2007) and other arthropod groups ex. insects (Howland & Hewitt, 1995; Lunt *et al.*, 1996), crustaceans (Davolos, 2005).

Another mitochondrial genes used in this study were the terminal part coding for ribosomal large subunit (16S), transfer RNA molecule for leucine (tRNA^{Leu}) and NADH dehydrogenase subunit I (nad1). The Gaps were present in the tRNA^{Leu} looped part of this genome fragment, which require special coding in the posterior analysis signing them as absence/presence characters.

Fragment of approximate length among 890 – 900 bp (540 – 550 bp of 16S + tRNA^{Leu} and 350 of nad1) was amplified, as mentioned before, large number of deletion was found in the sequences.

3.2.2 Nuclear markers

ITS I, 28S

Two genes were selected as nuclear markers, the ITS I and the 28S. Both genes were amplified for a subset of specimens. According to the phylogenetic relationship obtained by pre-analyses of mitochondrial sequences, the least related specimens were chosen. Approximately 405 bp long fragment of ITS I and 800 bp fragment were amplified for 8 specimens. There was no base variability in the sequences of the specimens of *D. erytrina/D.provincilis* group, only specimens assigned as *D. lantosquensis* and *D. fervida* differed.

Srp54 gene intron

Introns are untranslated gene regions of genomic DNA that are spliced out in the formation of mature RNA molecules (Creer, 2007). For that reason there can be a large number of insertions, deletions and repetitions in the sequence. Estimated mutation rate of introns is approximately one quarter of the animal mtDNA (Creer *et al.*, 2003). The intron used in this study lies in the nuclear gene coding for the 54 kDa subunit of the signal recognition particle (Srp54). This particle binds newly synthesized proteins and transfers them to the endoplasmatic reticulum (Egea *et al.*, 2005).

As reported in the phylogeographic study of octocoral genus *Carijoa*, using this intron can be very good alternative if another nuclear genes, as commonly used ITS, show no significant variability. High levels of sequence variation of Srp54 were found within this group. The divergence at this locus was approximately 8-13 greater than divergence of mtDNA sequences. In *Pollicopora* species, where no variability of mitochondrial sequences was found, the Srp54 divergence reached up to 2.8%. Using

the Srp54 nuclear intron several cryptic species within the genus *Carijoa* were detected (Conception *et al.*, 2007).

Approximately 170 bp long fragment was amplified of Srp54 gene. Intron length varied approximately between 180 - 195 bp, as insertion, deletions and repetitions were presented.

3.3. Molecular methods

3.3.1 DNA extraction

For DNA extraction DNeasy® Tissue Kit (Quiagen) was used following the isolation protocol. Genomic DNA was extracted from two legs or from entire spider body pierced on the ventral part of prosoma, according to body size of the sample. Before the start of extraction itself every sample was rehydrated and dried. The time of tissue lysis varied from 3 hours in crushed legs sample to 8 hours, when using pierced body sample. The presence and intensity of extracted DNA was verified by electroforesis on 1,5% agarose gel (1,5g agarose, 100ml TBE). The gel was immersed in water – EtBr bath for a few minutes and visualized by ultraviolet light emitter. 50µl of extracted DNA were stored at -20°C and another 50µl were stored at 4°C for current using.

3.3.2 Amplification

Targeted DNA fragments were amplified using PCR method. Thermo-cycler PTC-100 (MJ Research) was used for gene amplification. Oligonucleotide primers used are listed in following table (Tab. 1).

gene	name	Sequence	reference
cox 1	C1-J-1490	5'- GGTC AACAAATCATAAAGATATTGG -3'	Folmer et al., 1994
	C1-N-2191	5'- CCCGGTAAAATTAATAAATAACTTC-3	Folmer et al., 1994
	C1-J-1546	5'- GCTATAGTGGGTACGGCTATAAG -3'	M. Arnedo, 2000
	C1-N-2194	5'- CTTCTGGATGACCAAAAAATC -3'	M. Arnedo, 2000
rrnL, L1	LR-N-13398	5'- CGCCTGTTTATCAAAAAACAT -3'	Simon et al., 1994
nad1	N1-J-12350	5'-CCTARTTGRCTARARTTRGCRSATCATCCAATTG -3'	M. Arnedo, 2000
srp54	f	5-ATGGGTGGAYATYGAAGAACTGATWGATAAAAGTCAA-3'	Jarman et al. 2002
	r	5'-TTCATGATGTTYTGGAATTGYTCATACATGTC -3'	Jarman et al. 2002
vector	Ssp6	5'- ATTTAGGTGACACTATAG -3'	--
	T7	5'- TAATACGACTCACTATAGGG -3'	--

Tab. 1: List of oligonucleotide primers used in present study

All amplifications were performed in 25µl volume of reaction mix with following component values. In case of cloned nuclear intron srp54 1.5µl of PCR H₂O extra was added to mastermix, as no DNA suspension, but cell colony was used.

10x buffer (Applied Biosystems).....2.5µl
 2,5mM MgCl₂ (Applied Biosystems).....2.5µl
 10mM forward primer.....0.5µl
 10mM reverse primer.....0.5µl
 10mM DNTPs.....0.5µl
 5U/µl *Taq* polymerase (Applied Biosystems)...0.1µl
 PCR H₂O.....16.9µl
 DNA.....1.5 µl

PCR conditions are described in consecutive tables, in Table 2 for Cytochrome oxidase I (COI), in Table 3 for large ribosomal subunit fragment 16S, tRNA leucine (tRNA_{Leu}), and NADH dehydrogenase (*nad1*) and for nuclear intron Srp54 in Table 4.

step	temperature	time	cycles
1	94° C	2min	1
2	94 ° C	30s	35
3	45 ° C	35s	
4	72 ° C	1min	
5	72 ° C	5min	1
6	4 ° C	x	

Tab. 2: PCR program for COI

step	temperature	time	cycles
1	94° C	2min	1
2	94 ° C	30s	35
3	42 ° C	1min	
4	72 ° C	1min	
5	72 ° C	5min	1
6	4 ° C	x	

Tab. 3: PCR program for 16S, tRNA_{Leu} and *nad1*

step	temperature	time	cycles
1	94° C	2min	1
2	94 ° C	30s	35
3	48 ° C	1min	
4	72 ° C	35s	
5	72 ° C	6min	1
6	4 ° C	x	

Tab. 4: PCR program for srp54 nuclear gene intron

Presence of amplified fragment was verified by electrophoresis. 3µl of PCR product were loaded on 1.5 agarose gel (1.5g agarose, 100ml 1x TBE). 1.5µl of molecular weight marker EcoLadder II (Ecogen) was used as reference. Samples were removed after approximately 30 minutes at voltage of 95V and dyed in water – EtBr bath.

PCR products were diluted with PCR H₂O to final volume of 100µl and purified using MultiScreen 96-Well Filter Plates (Millipore) according to manufacturer’s guidelines. Basically the DNA cleaning consists of DNA dilution and consecutive absorption into the filter plate membrane. The DNA is retained in the filter membrane and subsequently washed out by PCR H₂O.

3.3.3 Sequencing

PCR products were sequenced in both directions using the same primers and BigDye™ Terminator version 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). The reaction mix of 10µl contained following components:

3,2mM forward primer.....1µl
 3,2mM reverse primer.....1µl
 Buffer.....3µl
 BigDye.....1µl
 Purified DNA.....3µl
 PCR H₂O.....2µl

step	temperature	time	cycles
1	96 °C	10s	35
2	50 °C	10s	
3	60 °C	4min	
4	4 °C	x	

Tab. 5: Sequence reaction program

The sequences were obtained on an automatic sequencer ABI 3700 in Serveis Científic Tècnics de la Universitat de Barcelona.

3.3.4 Cloning

To obtain sequences of the intron SRP54 of major part of the samples the cloning method was used. Cloning was performed on purified PCR products with pGem®-T (Promega) set according to manufacture's protocol. Values of the ligation mix were changed as following:

2x Rapid ligation buffer.....2.5µl
pGem®-T (50ng).....0.1µl
PCR product.....2µl
Deionized water10µl

The cells with incorporated vector were plated on LB/ampicillin/IPTG/X-Gal plates. For each sample two plates with volume of 100µl and 150µl of suspension with cells were plated. The plates were incubated at 37°C for 30 minutes, then were turned upside-down and let at the same temperature overnight.

For each sample 8 small colonies were chosen (usually the smallest ones) and used for amplification using PCR program shown in the Table 6.

step	temperature	time	cycles
1	94° C	5min	1
2	94 ° C	1min	30
3	50 ° C	30s	
4	72 ° C	3min	
5	72 ° C	5min	1
6	4 ° C	x	

Tab. 6: PCR program for cloned sequences

Successfully cloned samples amplified by PCR were purified and both side sequenced.

3.4 Sequence analysis

3.4.1 Sequence alignment

Both direction sequences were assembled and edited with Staden Package programs Pregap4 version 1.5 and Gap v4.10 (<http://staden.souseforge.net/>). Assembled sequences were pre-aligned with the other sequences manually in BioEdit Sequence

Alignment Editor (Hall, 1999). The *cox1* and *nad1* sequences did not show length polymorphism and their alignment was trivial. In mitochondrial 16S + tRNA^{Aleu} and *srp54* nuclear intron the length polymorphism was found due to the deletions in the parts of sequences. Posterior automatic alignment of 16S + tRNA^{Aleu} and *srp54* sequences was performed using online version of Mafft 6. (Kato, 2008) multiple sequence alignment (<http://align.bmr.kyushu-u.ac.jp/mafft/online/server/>) with L-INS-i manual strategy, gap-opening penalty 1.53 and offset value 0.00 (containing gap extension penalty) parameters. The gaps were considered informative characters in this study. They were codified as two states – present or absent using GapCoder program (Young & Healy, 2002) based of gap codification method (Simmons & Ochoterena, 2000). Automatic alignments were connected using Winclada program version 1.00.08 (Nixon, 2002).

3.4.2 Mitochondrial alignments dataset

A total number of 94 *Dysdera erythrina* group individuals were analyzed in the present study. Only 8 haplotypes appeared more than once in studied *Dysdera erythrina* group individuals, so 86 unique haplotypes were used, including also haplotypes with partly missing information. Three samples were short of *cox1* partition, four of 16S rRNA + tRNA^{Aleu} and two of *nad1*.

Two different matrices containing only unique haplotypes were created for subsequent phylogenetic analyses. Both of them included *D. erythrina* group but differed in number of outgroups. The first one “m77” contained only *D. erythrina* group haplotypes with complete sequence information (*cox1*, 16S rRNA + tRNA^{Aleu}, *nad1*) *Dysdera cf. erythrina* from Sardinia was used for tree rooting according to its phylogenetic position shown in the analyses where also other outgroups were used. This matrix was used for haplotype network analyses delimiting evolutionary lineages within *D. erythrina* group.

The second matrix “m98” consists of all *D. erythrina* group haplotypes, even those their part of information is missing, and 12 outgroups representing another 6 *Dysdera* species of 12 localities of Mediterranean region especially The Canary Islands and Iberian Peninsula. The outgroups are represented by *D. adriatica*, *D. inermis*, *D. silvatica*, *D. gomerensis*, *D. calderensis*, *D. valentina* and *D. crocota*. The Canary Islands species (*D. adriatica*, *D. inermis*, *D. silvatica*, *D. gomerensis*, and *D. calderensis*) were chosen deliberately because their further use as calibration points in

lineage age estimation. Two specimens of *D. inermis*; one from Iberian Peninsula and one from Morocco were put into the analyses because their genetic differences reflect the opening of the Strait of Gibraltar. This event is geologically dated and can be used as a fixed calibration point. Two more *Dysdera* species (*D. valentina* and *D. crocota*) distantly related to *D. erythrina* group were used. *D. adriatica* was utilized to root the parsimony tree as in the previous analyses of other species of the genus *Dysdera*.

3.4.3 Nuclear intron dataset

The matrix “msrp54” contained the nuclear intron sequences amplified only for a subset of the studied specimens. The selection was made according to pre-analyses of mtDNA sequences. Every mitochondrial evolutive lineage of *D. erythrina* group was represented by the intron sequence of selected specimen. The final matrix contained 37 distinct sequences; 23 individuals were represented by single alleles and 11 by two or more alleles (see Table 7).

specimen	number of alleles	specimen	number of alleles
K328	1	V48	1
K477	2	V49	1
K498	1	V51	2
K499	1	V58	1
LB237	1	V59	1
LB241	1	V62	1
LB247	1	V64	1
V1	1	V69	1
V10	1	V7	2
V19	1	V71	1
V21	2	V73	2
V25	2	V75	1
V26	1	V76	1
V3	2	V76	2
V31	2	V79	1
V32	3	V82	3
V34	1	V9	1
V40	1		

Tab. 7: The specimens and corresponding number of alleles of srp54 intron

3.5 Phylogenetic methods

The alignments were analysed using Maximum parsimony (MP) and model based analyses; Maximum Likelihood (ML) and Bayesian Method (BM). The program Mega 4 (Tamura *et al.* 2007) was used to obtain general information about the genetic variability of the sequences in the dataset.

3.5.1 Maximum parsimony (MP)

Maximum parsimony is a method based on search of the phylogenetic tree with the smallest number of evolutionary changes and its outcome is topologically the shortest tree – the tree with minimum state changes. Parsimony analyses were performed on “m98” dataset using the TNT program version 1.1 (Goloboff *et al.* 2003) and Nona (Goloboff, 1993), the Nona program is integrated in WinClada v 1.00.08 (Nixon, 2002). The heuristic search was used with a number of 1000 Wagner trees with 100 interactions followed by tree bisection and reconnection branch swapping (TBR). Five trees were held for each interaction up to maximum number of 10 000.

The branch support was obtained by Jackknifing (Farris *et al.*, 1996). Resampling was based on 1000 replicates with individual heuristic search with 15 iterations of Wagner trees. Five trees were held per iteration up to a total maximum of 10 000. Clades that appeared in less than 50% of resample cases were collapsed.

3.5.2 Model based methods

The evolutionary models used for better estimation of phylogenetic relationship between groups vary in the complexity from the simplest model - Jukes and Cantor model (JC), where the frequency of bases is considered equiprobable and transitions and transversions nucleotide changes occurs with the same probability. To the contrary the variation of these factors is considered in the most complex model General Time-Reversal Model (GRT).

To find the most suitable evolutionary model for the model test based analyses the Modeltest program (Posada & Crandall, 1998) was used. For each gene of the dataset was found appropriate model with the least number of parameters according to Akkaike information criterion (AIC).

3.5.2.1 Bayesian inference

Bayesian method is based on posterior probabilities of already existing trees implementing Markov Chain Monte Carlo (MCMC) algorithm. This approach compares the probabilities of the trees randomly created at the beginning of the process and their posterior growth. At determined moment the alternative trees are compared and topology of the tree with higher probability is accepted for further analyses.

Bayesian inference analyses were performed using program Mr. Bayes version 3 (Ronquist & Huelsenbeck, 2003). For each gene appropriate model of nucleotide substitution was selected by Modeltest as following; *cox1* TVM + I + G, *16S* TrN + I + G, *nad1* HKY + G. The program Mr. Bayes do not implement all the evolutionary models analyzed in Modeltest and hence the TVM + I + G, and the TrN + I +G were assimilated to GTR + I + G. Unlinked evolutionary models were defined along with a Mk model for the gaps scored as absence/presence characters. Two independent searches were ran to evaluate convergence of the results. Each one comprised 8 Markov Chain Monte Carlo chains (one heated, 7 cold) for 10 million generations, sampling every 1000 generations.

The Tracer program, version 1.3 (Rambaut & Drummond, 2003) was used to asses that MCMC chains had accomplished stationary and the convergence of parameters. Ten firs 10% of the trees were discarded as burn-in for the analysis.

3.5.2.1 Maximum likelihood (ML)

Maximum Likelihood (Felsenstein, 1981) approach is based on the search of the tree with most likely topology given the data and given the evolutionary model.

Maximum Likelihood analyses were performed with the RAxML 7.0.0 (Stamatakis, 2006) program version. Heuristic search consisted of ten random additions of taxa. For each gene the General Time Reversal model with invariants and among-site rate variation (GTR + I + G) was assigned to each gene fragment (this is the only model implemented in RAxML). The gaps were removed from the matrix as they are not considered in this method. Resampling by 100 replicates using Bootstrapping (Felsenstein, 1985) was performed on the best tree obtained by ML analyses.

3.6 Lineage age estimation

The program r8s, version 1.6 (Sanderson, 2003) was used to test the most suitable approach of the tree calibration. The firs approach – Langley and Fitch (LF) is the strictest and considers the rate constancy for all taxa, second Penalized likelihood (PL) approach depends on smoothing constant setting, which makes it more or less relaxed and the third (nPrs) implements the rate correlation only on sister taxa.

The cross-validation test was performed on the best tree obtained by Maximum Likelihood analyses with branch length information computed by Treefinder program (Jobb, 2008). The Langley and Fitch (LF) model was selected for given data.

The formation of the Canary Islands was used as calibration points for estimating absolute divergence time. The calibration points in this study were based on colonization of newly originated volcanic islands as these dispersal events are now reflected in genetic differences. *D. silvatica*, *D. calderensis* and *D. gomerensis* inhabiting La Gomera, La Palma and El Hierro islands were chosen for the calibration. The colonization occurred from the La Gomera not earlier than 2 Myr in case of La Palma and 1.2 Myr in case of El Hierro, when their origin is dated.

The opening of the Strait of Gibraltar approximately 5.3 Myr ago was used as fixed calibration point, as the dispersal events are not that tightly associated with geology as vicariant events. The *D. inermis* specimens from Morocco and Iberian Peninsula populations were used.

3.7 Species delimitation

3.7.1 Haplotype network

Haplotype network estimation was performed with the TCS program, version 1.21 (Clement *et al.* 2000). The network is estimated from DNA sequences using statistical parsimony. Changes along the branches were calculated for matrix “m77” and matrix “msrp54” using the default setting connection limit of 95% parsimony and gaps codified as absence/presence characters.

3.7.2 Species delimitation using mtDNA branching times

The calibrated tree obtained from r8s was used to implement the method proposed by Pons and collaborators (2006), based on the upturn in branching rates at the transition between-species to within-species rate of lineage branching. The code implementing the model in R using functions from the APE library was kindly provided by T. Barraclough (unpublished).

3.8 Morphological approach

3.8.1 Morphological measurements

All specimens in adult stage used in this study were submitted to morphological measurements using stereomicroscope Leica MC 16a. Carapace length, carapace width, cheliceral length, length of cheliceral fang, femur of the one of the forelegs and metatarsus of the one leg of the last pair, were measured. Type of chelicerae was also recorded. Two different stages were recognized; concave state and convex state.

The data obtained were analysed in the program Primer version 5.2.2 performing non-metric Multi Dimensional Scaling (MDS) on the quantitative variables.

4. RESULTS

4.1 Mitochondrial DNA

The matrix 77 dataset comprises of total number of 1491 bases; 631 bases representing cox1, 527 of 16 S + tRNA_{Aleu} and 331 of nad1. The complete number of variable positions in this dataset was 542 (cox1: 230, 16 S + tRNA_{Aleu}: 139, nad1: 173). The number of parsimony informative characters was less and was represented by total number of 427 (cox1: 197, 16 S + tRNA_{Aleu}: 96, nad1: 135).

The matrix 98 dataset comprises of total number of 1522. Complete number of variable positions was 712, which 567 were parsimony informative characters. This dataset contained also gaps codified as absence/present characters, which represented 25 characters.

Genetic divergence of *Dysdera erythrina* group

The overall p-distance for all genes included in the dataset was 9.3% (S. E. 0.005). The most variable gene was the nad1 (p-distance = 14.3%, S. E. 0.011), followed by the cox1 (10.4 %, S.E. 0.006). The 16S + tRNA_{Aleu} segment was the least variable part of the dataset (p-distance value = 5.5%, S. E. 0.006).

Genetic variability between the main *D. erythrina* group lineages are shown in table (Tab. 8) alongside with the number of specimens representing each lineage.

Intralineage variability of *D. lantosquensis* was 2.3%, while in the *D. erythrina/provincialis* reached 8.9%.

	<i>D. cf. erythrina</i> , Sardinia	<i>D.</i> <i>fervida</i>	<i>D.</i> <i>lantosquensis</i>	<i>D.</i> <i>erythrina/provincialis</i>
<i>D. cf. erythrina</i> , Sardinia (1)		0.01	0.009	0.008
<i>D. fervida</i> (1)	0.168		0.009	0.008
<i>D. lantosquensis</i> (6)	0.167	0.137		0.008
<i>D. erythrina/provincialis</i> (68)	0.165	0.154	0.145	

Tab. 8: The p-distances between groups shown in the lower triangle, upper triangle shows the standard errors. Number of haplotypes included in the comparison brackets.

4.1.1 Phylogenetic analyses

4.1.1.2 Parsimony

The maximum parsimony analysis was conducted on the matrix “m98” yielded 84 most parsimonious trees 3128 steps long (CI-0.47, RI-0.88). The strict consensus of the trees along with jackknife supports above 50% is shown in Fig. 3. The strict consensus derived from the most parsimonious trees reveals several well supported mitochondrial haplotype lineages. The single haplotype sampled the Sardinia tentatively identified as belonging to the *D. erythrina* complex is shown as the sister group to the remaining haplotypes. Both *D. fervida* and *D. lantosquensis* haplotypes form monophyletic groups, which are in turn supported as sister clades.

The remaining haplotypes, which includes of the specimens identified as *D. erythrina* s.s. and *D. provincialis*, form a well-supported clade that can be further divided into 7 well-supported monophyletic groups (hereafter referred as A, B, which includes a single haplotype, C, D, E, F and G). Relationships among lineages are poorly supported, except for the sister group relationship of F and G clades.

The position of the clades as their specimen composition does not show any clear geographic pattern. For example, the clade A consists of specimens from Navarra, Prague, Montsec and Corredor NP. Also location of the specimens from the same zone or even locality varies a lot. Specimens from Montseny NP are spread across the tree;

they are present in A, B, C, F and G clades. The same occurs with specimens from Corredor NP (clades A and C) and specimens from Navarra (clades A and G).

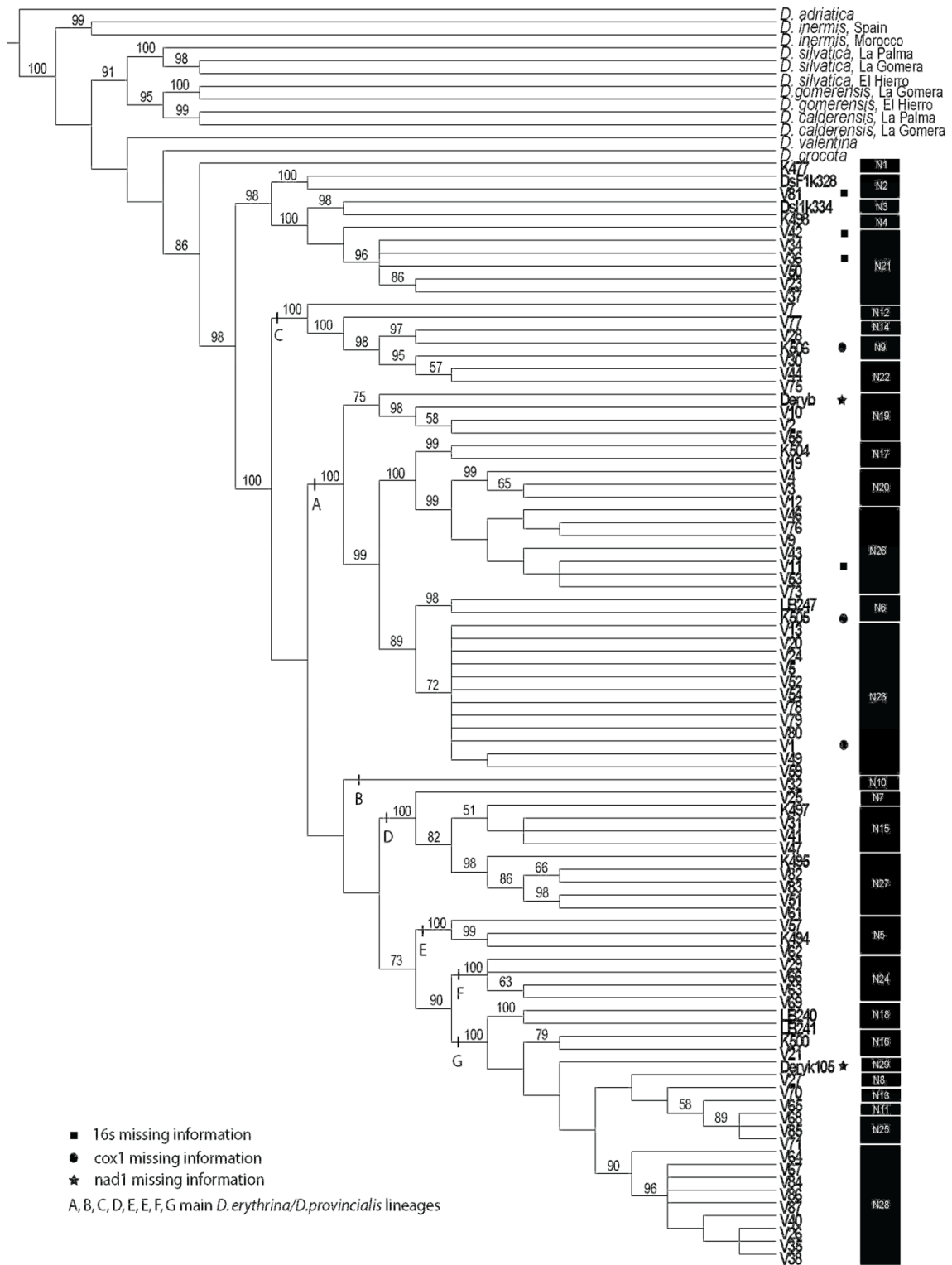


Fig. 3: Strict consensus tree from the 84 trees obtained in parsimony analyses. Numbers above nodes correspond to jackknife support (only values above 50% shown). Groupings on the right side of terminal names refer to independent networks obtained with statistical parsimony haplotype network analyses (N1 to N29) A, B, C, D, E, F, G referred to main mitochondrial haplotype lineages of the *D. erythrina/provincialis* clade discussed in relation to the nuclear gene intron *srp54* data.

4.1.1.2 Model based analyses

The best models selected by for each gene fragment with the corrected Akkaike information criterion (AICc) as implemented in the program Modeltest are shown below:

cox1:	16S + tRNA _{Leu} :	nad1:
<u>Model selected:</u> TVM+I+G	<u>Model selected:</u> TrN+I+G	<u>Model selected:</u> HKY+G
-lnL = 4640.3560	-lnL = 2376.5532	-lnL = 2786.9614
K = 172	K = 168	K = 170
AICc = 9754.9355	AICc = 5247.7212	AICc = 6275.0410
<u>Base frequencies:</u>	<u>Base frequencies:</u>	<u>Base frequencies:</u>
freqA = 0.2282	freqA = 0.3938	freqA = 0.3312
freqC = 0.1053	freqC = 0.1554	freqC = 0.2307
freqG = 0.2300	freqG = 0.1147	freqG = 0.0732
freqT = 0.4365	freqT = 0.3361	freqT = 0.3649
<u>Substitution model:</u>	<u>Substitution model:</u>	<u>Substitution model:</u>
Rate matrix	Rate matrix	Ti/tv ratio = 3.3704
R(a) [A-C] = 3.0835	R(a) [A-C] = 1.0000	
R(b) [A-G] = 12.9290	R(b) [A-G] = 2.1347	
R(c) [A-T] = 2.0032	R(c) [A-T] = 1.0000	
R(d) [C-G] = 1.0739	R(d) [C-G] = 1.0000	
R(e) [C-T] = 12.9290	R(e) [C-T] = 6.5553	
R(f) [G-T] = 1.0000	R(f) [G-T] = 1.0000	
(I) = 0.5479	(I) = 0.4872	(I) = 0
(G) = 0.8972	(G) = 0.5081	(G) = 0.3521

The best tree found in 10 independent rounds (-log L = 15802, 814829) along with bootstrap support are shown in Fig 4. Results of the Bayesian analyses (not shown) closely resembled those of the maximum likelihood analyses. Posterior probabilities of the compatible clades are shown in Fig. 4. (the same as ML tree).

Both maximum likelihood analyses and Bayesian inference (10% of the initial trees discarded as burn-in) recovered the monophyly of the *D. erythrina* complex with high support. The remaining relationships matched those found in parsimony analyses, including the basal position of *D. cf. erythrina* from Sardinia haplotype, the sister-group relationship of *D. fervida* and *D. lantosquensis*, and the main haplotype lineages of the *D. erythrina/D. provincialis* clade. Relationships among some of these lineages differ from those in the parsimony analyses, albeit none of them with high support. The remaining difference involved haplotype relationships within lineages.

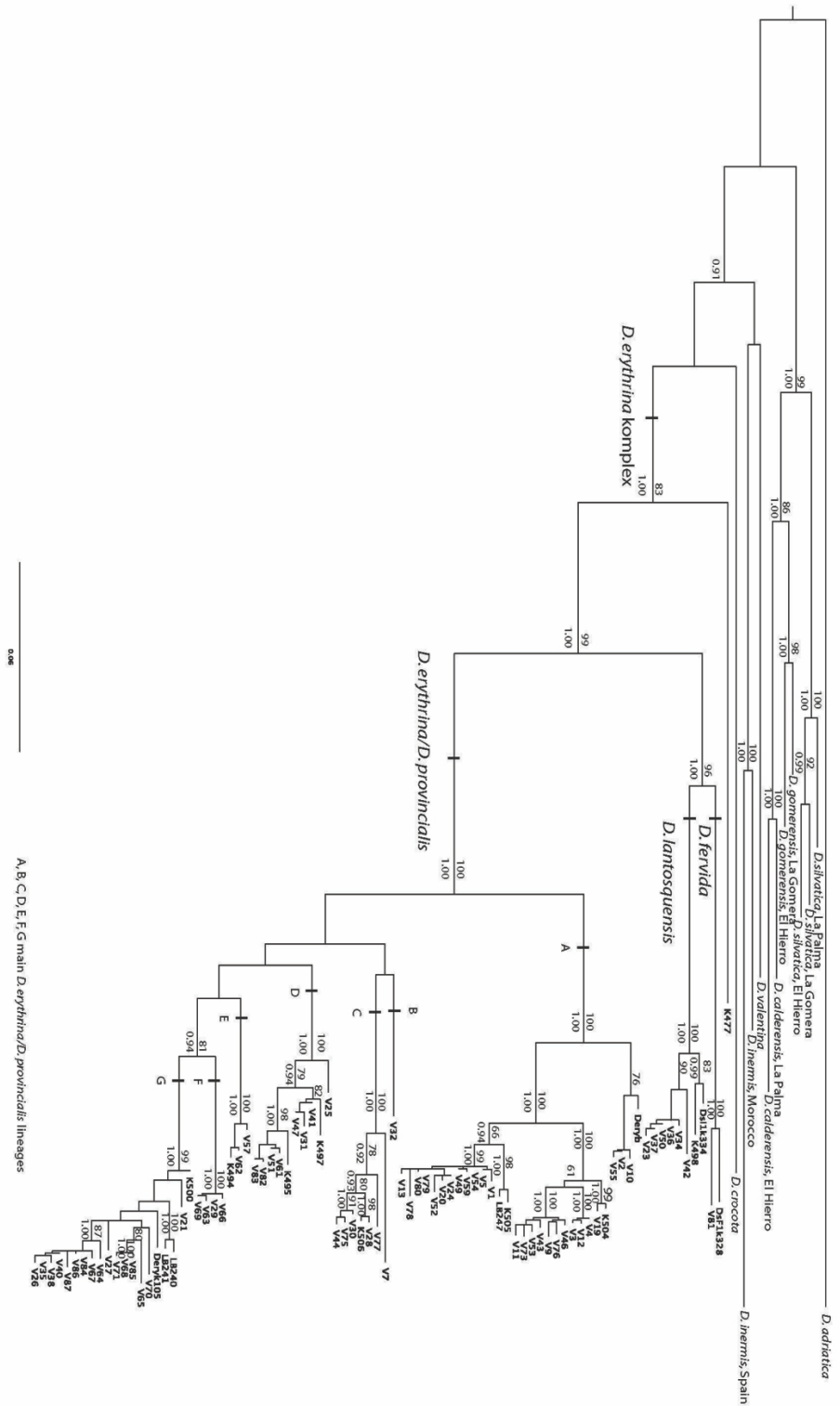


Fig. 4: The best maximum likelihood tree (- log L = 15802.814829). Values above branches correspond to bootstrap support (only values above 50% reported), and below branches refer to Bayesian posterior probability (only values above 90% reported). A, B, C, D, E, F, G referred to main mitochondrial haplotype lineages of the *D. erythrina/provincialis* clade discussed in relation to the nuclear gene intron *srp54* data.

4.1.2 Haplotype lineage age estimation

Cross validation analyses in r8s on the ML tree selected the Langley-Fitch as the best method for tree calibration, suggesting that the data accommodated to a global molecular clock. The resulting chronogram (ultrametric tree) based on fixed and constrained calibration points (see Methods) is shown in Fig. 5. Bootstrap confidence intervals (direct age estimation, standard deviation, maximum and minimum) for selected clades are summarized in the Table 10.

Formation of the *D. erythrina* complex occurred approximately 7.43 million years ago (My), (12.7-7 My) Basal splitting among continental lineages occurred 5.91 My ago (12.7-5.9, My) and the origin of *D. fervida* and *D. lantosquensis* date back to 4.44 My ago (8.3-4.2, My). The *D.erythrina/D. provincialis* clade split about 3.31 My (12.7-2.9, My), while main haplotype lineages in these clade originated about or during the Plio-pleistocene glacial cycles, mean values of the nodes fall in the range 2.4 and 0.26 My (detail list in Fig. 5 – Table 10)

4.1.3 Lineage delimitation

Putative independent evolutionary lineages (i.e. species) were characterized by means of statistical parsimony networks of mitochondria haplotypes and by the analyses of their branch lengths. Statistical parsimony implemented in the TCS program on the complete haplotypes identified 29 independent networks, based on connection limit of 16 steps; i.e. branches of 17 steps and beyond are considered to fall outside of the 95% confidence interval for these connections to be nonhomoplastic. Incomplete haplotypes were subsequently assigned to the networks based on their phylogenetic position in standard parsimony analyses. Corresponding networks are shown on the parsimony tree (see Fig. 3)

The calibrated tree obtained from r8s was used to implement the method proposed by Pons and collaborators (2006), based on the upturn in branching rates at the transition between-species to within-species rate of lineage branching. This method identified 39 putative independent lineages, of which 22 were represented by single individuals (see Fig.5). Confidence limits for the estimated number of lineages ranged 34 to 41. The GMYC model was preferred over the null model of uniform branching rates (logL 0 290.4803, compared to null model logL = 286.2553; $2\Delta L = 8.45$, χ^2 test, d.f. = 3, $P < 0.05$).

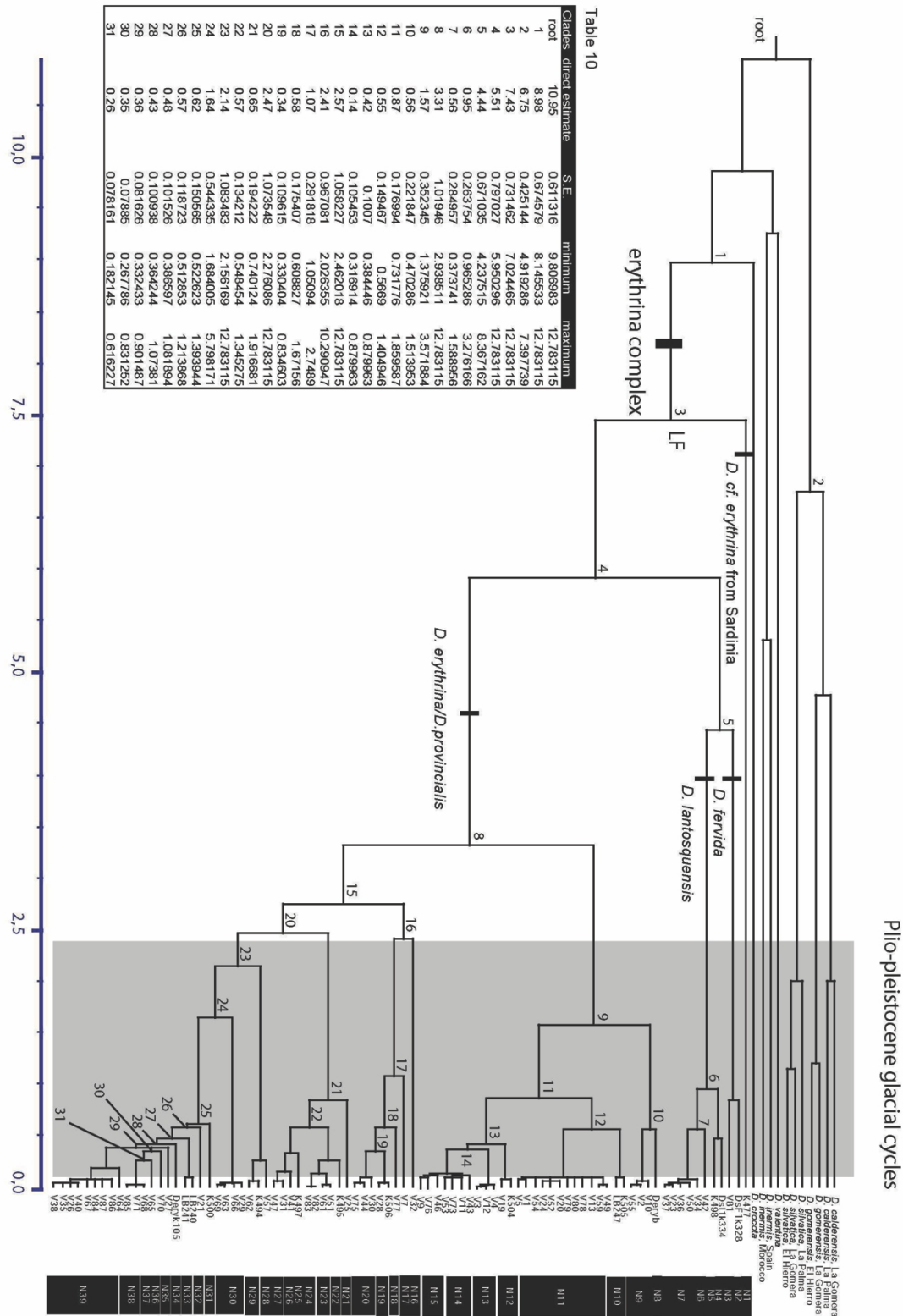


Fig. 5: Chronogram based on ML tree obtained in R8S using LF method and constrains discussed in the text. Ages in the bar below refer to million years. Time span corresponding to Plio-prlistocene glacial cycles highlighted in grey. Ages estimate along with standard errors of the labeled nodes are shown in the Table 10. Groupings on the right side of terminal names (N1 to N39) refer to independent lineages obtained using the species delimitation method proposed by Pons and collaborators (2006).

4.2 Nuclear intron *srp54* analysis

Nucleotide sequences of the *srp54* intron were obtained for a subsample of 34 individuals, representing the main mitochondrial haplotype clades in the *D. erythrina* complex. In some specimens, the direct chromatograms revealed multiple peaks suggesting the presence of two or more alleles. PCR amplification of these individuals were cloned and several (1 to 5) colonies sequenced. Total length of the *srp54* fragment analyzed was 184 bp, including the alignment gaps, 72bp correspond to flanking exons and 112 positions to the intron. The fragment contained 48 variable positions of which 30 were parsimony informative.

Overall p-distance among sequences was 4.8% (S.E. 0.008), genetic divergence between is summarized in Table 11.

	<i>D. fervida</i>	<i>D. cf. erythrina</i> , Sardinia	<i>D. lantosquensis</i>	<i>D. erythrina/provincialis</i>
<i>D. fervida</i>		0.011	0.014	0.007
<i>D. cf. erythrina</i> , Sardinia	0.025		0.014	0.009
<i>D. lantosquensis</i>	0.043	0.043		0.013
<i>D. erythrina/provincialis</i>	0.034	0.045	0.056	

Table 11: The p-distances between groups shown in the lower triangle, upper triangle shows the standard errors.

Genetic divergences between alleles of the same individual are summarized in Table 12. Some intra-individual allelic divergences were greater than those observed between different species. For example, p-distance between alleles of specimen V51 (7%) exceeds the maximum difference reported among *D. fervida* and *D. erythrina/provincialis* which was 5.6%. On the other hand, cloning revealed three different alleles in specimens V32, 76 and V82.

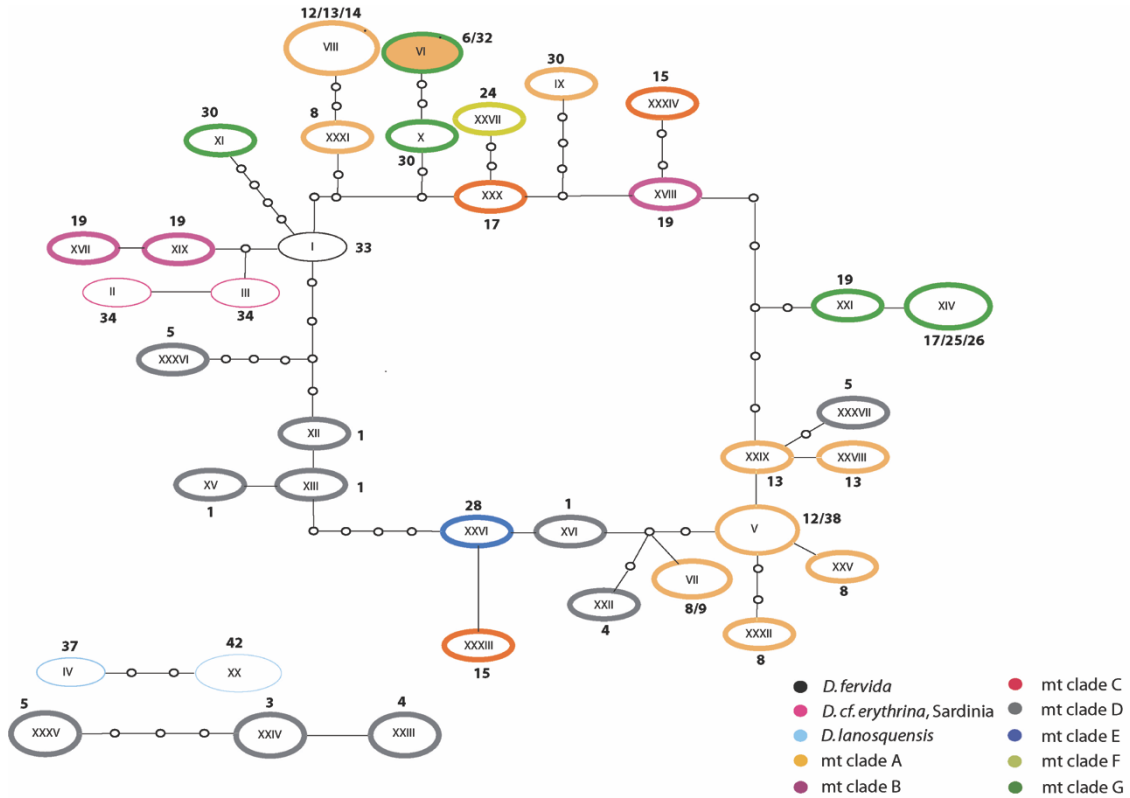
specimen	d	S.E.	clones
V21	0.055	0.017	a, b
V25	0.006	0.005	a, b
V31	0.043	0.015	a, b
V32	0.017	0.008	a, b, c
V51	0.070	0.021	a, b
V7	0.030	0.014	a, b
V73	0.006	0.006	a, b
V76	0.048	0.016	a, b, c
V82	0.078	0.016	a, b, c

Table 12. The p-distances between alleles of the same specimens

Analyses of the 37 alleles with statistical parsimony resolved three independent networks. Results are shown in Fig. 6 and summarized in Table 13.

Result revealed contrasting patterns of correlation between allelic relationships and geographical or mitochondrial haplotype closeness in the *D. erythrina/D. provincialis* lineage. For example some alleles of the mitochondrial lineage A were closely related, while others show closer affinities with other mitochondrial clades. A similar pattern was discovered in the mitochondrial clade G. Specimens LB241 and LB247, which pertain to different mitochondrial clades, shown the same allele (VI). The phylogenetic relationship among the species in the *D. erythrina* group obtained I the mitochondrial analyses are blurred in the in the analyses of the nuclear intron. *D. lantosquensis* alleles form an independent network, but *D. cf. erythrina* from Sardinia and *D. fervida* are included in the same network as most of the alleles of the *D. erythrina/D. provincialis* lineage, and seems to be closely related to particular alleles of the clade G and B. On the other hand, three alleles of the mitochondrial clade D were included in the independent network.

Geographic patterns are equally idiosyncratic. Some closely related alleles show geographical affinities, while localities separated by more than 1.500 km (e.g. Etxalar and Prague) may also share the same alleles.



Aleles	Specs	Locality	mt Clade	Aleles	Specs	Locality	mt Clade
I	K328	33	<i>D. fervida</i>	XV	V31a	1	D
II	K477a	34	<i>D. cf. erythrina</i> from Sardinia	XVI	V31b	1	D
III	K477b	34	<i>D. cf. erythrina</i> from Sardinia	XVII	V32a	19	B
IV	K498	37	<i>D. lantosquensis</i>	XVIII	V32b	19	B
V	K499	38	A	XIX	V32c	19	B
V	LB237	38	A	XX	V34	42	<i>D. lantosquensis</i>
V	V3e	12	A	XX	V48	42	<i>D. lantosquensis</i>
V	V79	38	A	XXI	V40	19	G
VI	LB241	6	G	XXII	V51a	4	D
VI	LB247	32	A	XXIII	V51b	4	D
VII	V1	8	A	XXIV	V58	3	B
VII	V49	9	A	XXV	V59	8	A
VIII	V10	14	A	XXVI	V62a	28	E
VIII	V3a	12	A	XXVII	V69	24	F
VIII	V76j	13	A	XXVIII	V73k	13	A
VIII	V9	12	A	XXIX	V73L	13	A
IX	V19	30	A	XXX	V75	17	C
X	V21a	30	G	XXXI	V76g	8	A
XI	V21c	30	G	XXXII	V76h	8	A
XII	V25a	1	D	XXXIII	V7a	15	C
XIII	V25b	1	D	XXXIV	V7b	15	C
XIV	V26a	26	G	XXXV	V82a	5	D
XIV	V64	25	G	XXXVI	V82b	5	D
XIV	V71	17	G	XXXVII	V82d	5	D

Figure 6: Nuclear intron *srp54* allele network. Table below (Tab.13), information of the alleles distribution of the analyzed specimens, mitochondrial haplotype clade of the specimens and locality.

4.3 Morphological approach

The results of Multi Dimensional Scaling (MDS) of the investigated morphological characters are shown in Fig. 7. The plot reveals differences in morphological characters in *D. fervida* and *D. lantosquensis*, which are clearly separated from the most of the specimens of *D. erythrina/D. provincialis* group.

Some specimens identified as *D. provincialis* (V86, V70) and some other included in the *D. erythrina/D. provincialis* (e.g. V32, V2, V30, V83) also seem to separate from the bulk of the group. However, most specimens identified as *D. erythrina* or *D. provincialis* are pooled in the large continuous cloud. The specimens separated from the *D. erythrina/D. provincialis* group pertain each to the different mitochondrial clade, where they are unique to show differences. The specimen V32 pertains to mitochondrial clade B, which presents independent lineage with unclear position within the *D. erythrina/D. provincialis* group.

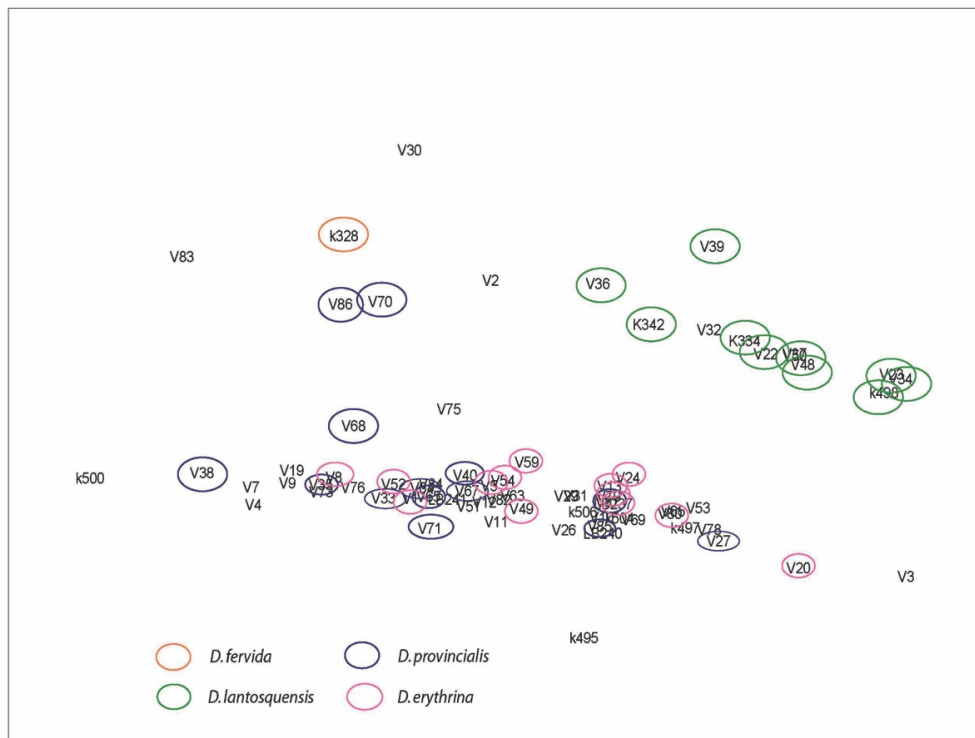


Fig. 7: MDS plot of the morphological measurements of the *D. erythrina* group. Specimens labeled according to their assignment to *D. fervida*, *D. lantosquensis*, *D. provincialis* or *D. erythrina* (specimens identified by M. Řezáč). Remaining individuals belong to the *D. erythrina/D. provincialis* mitochondrial clade with no further species assignment.

5. DISCUSSION

Analyses of mitochondrial sequence data of a trough geographic sample of spiders of the *D. erythrina* group revealed unexpected levels of genetic diversity. Phylogenetic analyses of these data identified a previously unknown lineage endemic to Sardinia that is the sister taxa to remaining species of the group. Mitochondrial data also corroborates species status of *D. fervida* and *D. lantosquensis*, and provides further support for a clade including representatives identified as *D. erythrina* and *D. provincialis*. Two quantitative methods for species delimitation exclusively based on sequence data, independent networks defined in statistical parsimony and the analyses of mtDNA branching times, suggested as many as 29 and 39 (confidence interval 34 to 41), respectively, independent evolutionary lineages in the *D. erythrina* complex.

It should be pointed out; however, that genetic divergence on itself does not constitute a definitive proof for specie status. Comparative information of the phenology, ecology and morphological features of the different genetic populations provides key evidence to demonstrate the independence of the evolutionary trajectories of the different lineages. Because of the maternal inheritance and its haploid nature, mtDNA markers are sensitive and likely to record historical processes than nuclear loci. Less effective population size of maternally inherited markers provides the monophyly establishing when species is divided more quickly than nuclear markers and the evidence of the gene flow barrier can be reported due to the absence of recombination (Moore, 1995; Wiens & Penkrot, 2002). However, under the certain circumstances mitochondrial markers may not accurately reflect population history (Irwin, 2002; Funk & Omland, 2003; Ballard & Whitlock, 2004; Lin & Danforth, 2004). This may be particularly true in low dispersal organism, or in species with sedentary females and vagile males, as in the case of mygalomorph spiders (Hendrixson & Bond, 2005). Genealogical concordance across multiple loci is the ultimate test to distinguish between stochastic and deterministic causation in accounting for phylogeographic patterns (Kuo & Avise, 2005). Unfortunately, some of the nuclear markers investigated in present study, such as 28S and ITS I, did not provide enough information to corroborate the results of the mitochondrial analyses.

At first sight, the levels of genetic divergences found in the *srp54* intron were more promising. However, the allele relationship suggested by the network analyses

revealed a lack of congruence with both geography and mitochondrial clades. In addition, the alleles found in some individuals show similar levels of divergence as those found in different species. These data may indicate the possible existence of hybridization events between some of the lineages. The individual V51 can serve as an example of such a case; one of its alleles form an independent network along with other alleles of the same mitochondrial clade, while the remaining allele shows affinities to alleles of completely different mitochondrial clade. However, the lack of geographical continuity of some of these putative events casts some doubts on the possibility of ongoing hybridization and seems more compatible with an unsorted ancestral polymorphism. On the other hand, the presence of more than two alleles in some of the individuals analyzed, along with the recovering of some independent networks point towards the existence of pseudogene. More data is required to test this probability. Alternatively, extra alleles may be the result of cloning artifacts.

The analyses of morphological features have shown that some mitochondrial lineages can be distinguished morphologically. *D. fervida* and *D. lantosquensis* follow the pattern obtained in the analyses of mitochondrial data and it is observed that all individuals of these lineages group apart from the *D. erythrina/D. lantosquensis* clade. It seems that at least some individuals of *D. erythrina/D. provincialis* clade also provide diagnostic morphological characters, but these individuals come from different mitochondrial lineages and are unique to show differences within their corresponding lineage. This observation may draw a general conclusion that lineages of *D. erythrina/D. provincialis* mitochondrial clade do not seem to display diagnostic features in the characters examined, except may be the single specimen in the lineage B.

The major part of lineage divergence observed in *D. erythrina/D. provincialis* clade seems to rise during the Plio-pleistocene glacial cycles, suggesting that possible isolation of populations could lead to origin of this rich diversity. Unfavorable life condition for xerothermic fauna most probably led to local extinction and retreat to more suitable refugia, which were also affected by decrease of temperature and humidity (e. g. Hewitt 1995; 2004). The fragmentation of the population and subsequent isolation in the refugia could generate the mitochondrial diversity and its fixation in *D. erythrina/ D. provincialis* clade. On the other hand, the separation of *D. cf. erythrina*; the basal lineage of whole *D. erythrina* complex and sister group of the complex; *D. fervida* and *D. lantosquensis* dates back to the former past.

The current distribution of *D. erythrina* species complex shows no clear geographic pattern. The results indicate large overlapping of the geographical ranges of lineages. The absence of geographic pattern of distribution suggests that some mechanisms maintaining the lineages boundaries and avoiding the hybridization could be in play. Possible reproductive isolation barrier could be provided by existence of the chromosomal races preventing merging of genetic pool after secondary contact. The chromosome number and autosome morphology is different for 7 studied species of the genus *Dysdera* (Řezáč *et al.*, 2007). The studies revealed analogical situation in the genus *Scytodes* pertaining to the family Scytodidae (Araujo *et. al.*, 2007); which belongs among haplogyne groups same as family Dysderidae. It is possible that variability of chromosome number and autosome morphology exists also on intraspecific level as reported from *D. crocata* recently introduced to South America. However, in case of *D. crocata* recent chromosomal divergence is concerned with no impact on the reproduction (Rodríguez Gil *et. al.*, 2002).

Other factors maintaining the separation of the lineages could be existence of reproductive barriers not contemplated in this study as reproductive behavior, phenology or some ecological preferences.

The current distribution of *D. erythrina* species complex also may be due to the secondary contact of previously separated populations. The population range expansion could be caused by expansion of suitable xerothermic habitats preferred by this group and its subsequent following. Another possibility how to explain secondary contact of the previously separated populations is human mediated introduction. *Dysdera* species are reported to be prone to passive accidental transport with human material because of their tendency to attach the silk cocoons, where they dwell during the daylight, to the large objects laying on the ground (Řezáč *et al.*, 2008). If the species is adapted to the arid environment, shows no difficulties to survive the transport and subsequently inhabit synanthropic habitats as in case of *D. crocata*, currently reported as cosmopolite. That seems the most suitable explanation for the presence of *D. fervida* in Grand Canyon (U.S.A.), but may also explain the presence of identical haplotypes in localities separated by 1.500 km (Exalar - northern Spain and Prague).

6. CONCLUSION

Phylogenetic analyses of four mitochondrial genes, along with morphology, provide strong evidence that *Dysdera lantosquensis* and *D. fervida* constitute independent evolutionary lineages.

Performed analyses revealed an existence of new additional lineages from Sardinia (in this study denoted as *D. cf. erythrina* from Sardinia).

Formerly described species of *D. erythrina* and subspecies *D. provincialis* cannot be distinguished solely on the basis of somatic characters (morphological measurements) although mitochondrial data suggest multiple evolutionary lineages with no clear geographic pattern segregation.

The major part of the genetic diversity of *D. erythrina/D. provincialis* lineages originated in relatively recent past compared with *Dysdera lantosquensis* and *D. fervida*.

Further studies covering the areas not focused in this project are needed to illuminate the great diversity of *D. erythrina/D. provincialis* lineages.

7. FURTHER PROSPECTS

It seems essential to link mitochondrial lineages with other criteria as phenology, ecology, behavior or other phenotypic characters not considered in this study which could lead to lineages segregation or on the contrary, to the extensive interbreeding after the periods of population divergence, where glacial cycles could play an important role.

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List of appendixes

Appendix 1: Table of collection localities, specimen codes, amplified genes and classification of the used specimens of *D. erythrina* group.

Appendix 1: List of specimen codes, collection localities and amplified gene fragments

DNA Code	Species Name	Cox1	nad1	16 S	srp54	mt haplotypes	n alleles	Locality Name	State/Prov	Country	Loc. Code
V31	<i>D. erythrina</i> group	+	+	+	clones		a,b	Serra de Prades, Montblanc	Catalonia	France	1
k497	<i>D. erythrina</i> group	+	+	+	-			Serra de Prades, Barranc de la Font d'en Garro	Catalonia	Spain	2
V25	<i>D. erythrina</i> group	+	+	+	clones		a,b	Serra de prades, trail from la Barta to Rojals	Catalonia	Spain	2
V41	<i>D. erythrina</i> group	+	+	+	-			Serra de prades, trail from la Barta to Rojals	Catalonia	Spain	2
V47	<i>D. erythrina</i> group	+	+	+	-			Serra de prades, trail from la Barta to Rojals	Catalonia	Spain	2
V58	<i>D. erythrina</i> group	-	-	-	+			Montsec, Ager, dirt road to Coll d'Ares	Catalonia	Spain	3
V72	<i>D. erythrina</i> group	+	-	-	-	V51 or V61		Montsec, Ager, dirt road to Coll d'Ares	Catalonia	Spain	3
V51	<i>D. erythrina</i> group	+	+	+	clones		a,b	Montsec de Rúbies; Hostal Roig trail	Catalonia	Spain	4
V61	<i>D. erythrina</i> group	+	+	+	-			Montsec de Rúbies; Hostal Roig trail	Catalonia	Spain	4
V82	<i>D. erythrina</i> group	+	+	+	clones		a,b,c	Estani de Montcortes	Catalonia	Spain	5
V83	<i>D. erythrina</i> group	+	+	+	-			Estani de Montcortes	Catalonia	Spain	5
LB241	<i>D. erythrina</i> group	+	+	+	+			Road Grande to Anso	Navarra	Spain	6
LB240	<i>D. erythrina</i> group	+	+	+	-			La Jarrieta, recreative zone	Navarra	Spain	7
V1	<i>D. erythrina</i> group	-	+	+	+			Etxalar, Bidasoa basin	Navarra	Spain	8
V13	<i>D. erythrina</i> group	+	+	+	-			Etxalar, Bidasoa basin	Navarra	Spain	8
V49	<i>D. erythrina</i> group	+	+	+	+			Etxalar, Bidasoa basin	Navarra	Spain	8
V5	<i>D. erythrina</i> group	+	+	+	-			Etxalar, Bidasoa basin	Navarra	Spain	8
V52	<i>D. erythrina</i> group	+	+	+	-			Etxalar, Bidasoa basin	Navarra	Spain	8
V54	<i>D. erythrina</i> group	+	+	+	-			Etxalar, Bidasoa basin	Navarra	Spain	8
V59	<i>D. erythrina</i> group	+	+	+	+			Etxalar, Bidasoa basin	Navarra	Spain	8
V8	<i>D. erythrina</i> group	+	-	-	-	V49 or V54		Etxalar, Bidasoa basin	Navarra	Spain	8
V77	<i>D. erythrina</i> group	+	+	+	-			Begues	Catalonia	Spain	9
V2	<i>D. erythrina</i> group	+	+	+	-			Serra de Collserola, nr. Cerdanyola del Vallès	Catalonia	Spain	10
K105	<i>D. erythrina</i> group	+	+	+	-			Sant Llorenç del Munt	Catalonia	Spain	11
V55	<i>D. erythrina</i> group	+	+	+	-			St. Llorenç del Munt	Catalonia	Spain	11
V12	<i>D. erythrina</i> group	+	+	+	-			P.N. Corredor, Canyamàs, Dosrius	Catalonia	Spain	12
V3	<i>D. erythrina</i> group	+	+	+	clones		a	P.N. Corredor, Canyamàs, Dosrius	Catalonia	Spain	12
V4	<i>D. erythrina</i> group	+	+	+	-			P.N. Corredor, Canyamàs, Dosrius	Catalonia	Spain	12
V43	<i>D. erythrina</i> group	+	+	+	-			P.N. Corredor, Canyamàs, Dosrius	Catalonia	Spain	12

V9	<i>D. erythrina</i> group	+	+	+	+			P.N. Corredor, Canyamàs, Dosrius	Catalonia	Spain	12
V53	<i>D. erythrina</i> group	+	+	+	-			Montseny, Cardedeu	Catalonia	Spain	13
V73	<i>D. provincialis</i> *	+	+	+	clones		a,b	Montseny, Cardedeu	Catalonia	Spain	13
V76	<i>D. provincialis</i> *	+	+	+	clones		a,b	Montseny, Cardedeu	Catalonia	Spain	13
V10	<i>D. erythrina</i> group	+	+	+	+			Montseny, loc GPS edu	Catalonia	Spain	14
V7	<i>D. erythrina</i> group	+	+	+	clones		a,b	Montnegre	Catalonia	Spain	15
V46	<i>D. erythrina</i> group	+	+	+	-			Motseny, La Costa del Montseny	Catalonia	Spain	16
V65	<i>D. erythrina</i> group	+	+	+	-			Montseny, càmping les Illes near Tordera river	Catalonia	Spain	17
V68	<i>D. erythrina</i> group	+	+	+	-			Montseny, càmping les Illes near Tordera river	Catalonia	Spain	17
V71	<i>D. erythrina</i> group	+	+	+	+			Montseny, càmping les Illes near Tordera river	Catalonia	Spain	17
V75	<i>D. erythrina</i> group	+	+	+	+			Montseny, càmping les Illes near Tordera river	Catalonia	Spain	17
V29	<i>D. erythrina</i> group	+	+	+	-			Montseny, Turó del'Home	Catalonia	Spain	18
V28	<i>D. erythrina</i> group	+	+	+	-			Montseny, Can Cevera near Montseny town	Catalonia	Spain	19
V30	<i>D. erythrina</i> group	+	+	+	-			Montseny, Can Cevera near Montseny town	Catalonia	Spain	19
V32	<i>D. erythrina</i> group	+	+	+	clones		a,b,c	Montseny, Can Cevera near Montseny town	Catalonia	Spain	19
V33	<i>D. provincialis</i> *	+	+	+	-	V35		Montseny, Can Cevera near Montseny town	Catalonia	Spain	19
V35	<i>D. provincialis</i> *	+	+	+	-			Montseny, Can Cevera near Montseny town	Catalonia	Spain	19
V38	<i>D. provincialis</i> *	+	+	+	-			Montseny, Can Cevera near Montseny town	Catalonia	Spain	19
V40	<i>D. provincialis</i> *	+	+	+	+			Montseny, Can Cevera near Montseny town	Catalonia	Spain	19
V11	<i>D. erythrina</i> group	+	-	-	-			Montseny, La Castanya	Catalonia	Spain	20
k506	<i>D. erythrina</i> group	-	+	+	-			Montseny, Sot de Bernal	Catalonia	Spain	21
V87	<i>D. erythrina</i> group	+	+	+	-			Montseny, Turé Gros	Catalonia	Spain	22
V84	<i>D. erythrina</i> group	+	+	+	-			Montseny, Matagalls	Catalonia	Spain	23
V85	<i>D. erythrina</i> group	+	+	+	-			Montseny, Matagalls	Catalonia	Spain	23
V86	<i>D. erythrina</i> group	+	+	+	-			Montseny, Matagalls	Catalonia	Spain	23
V44	<i>D. erythrina</i> group	+	+	+	-			Montseny, Santa Fe del Monteny	Catalonia	Spain	24
V63	<i>D. erythrina</i> group	+	+	+	-			Montseny, Santa Fe del Monteny	Catalonia	Spain	24
V66	<i>D. erythrina</i> group	+	+	+	-			Montseny, Santa Fe del Monteny	Catalonia	Spain	24
V69	<i>D. erythrina</i> group	+	+	+	+			Monsteny, Santa Fe del Monteny	Catalonia	Spain	24
V64	<i>D. erythrina</i> group	+	+	+	-			Montseny, Viladrau, Mas Martí	Catalonia	Spain	25
V67	<i>D. erythrina</i> group	+	+	+	-			Montseny, Viladrau, Mas Martí	Catalonia	Spain	25
V70	<i>D. erythrina</i> group	+	+	+	-			Montseny, Viladrau, Mas Martí	Catalonia	Spain	25
V26	<i>D. provincialis</i> *	+	+	+	clones		a,b	Cantonigròs	Catalonia	Spain	26

V27	<i>D. provincialis</i> *	+	+	+	-			Cantonigròs	Catalonia	Spain	26
k494	<i>D. erythrina</i> group	+	+	+	-			Castellar de n'Hug	Catalonia	Spain	27
k495	<i>D. erythrina</i> group	+	+	+	-			Riu	Catalonia	Spain	28
V57	<i>D. erythrina</i> group	+	+	+	-			Riu	Catalonia	Spain	28
V62	<i>D. erythrina</i> group	+	+	+	clones		a	Riu	Catalonia	Spain	28
k504	<i>D. erythrina</i> group	+	+	+	-			La Jonquera	Catalonia	Spain	29
V19	<i>D. erythrina</i> group	+	+	+	+			Coll de Banyuls	Languedoc	France	30
V21	<i>D. erythrina</i> group	+	+	+	clones		a,b	Coll de Banyuls	Languedoc	France	30
k500	<i>D. provincialis</i> *	+	+	+	-			Mt. Canigó, rd. Vallmanyà-Bastida	Languedoc	France	31
k505	<i>D. erythrina</i> group	-	+	+	-			P.N. des Cévennes, Mont Aigual	Provence	France	32
LB247	<i>D. erythrina</i> group	+	+	+	+			P.N. des Cévennes, Mont Aigual	Provence	France	32
k328	<i>D. feryvida</i> *	+	+	+	+			Iles d'Hyères, Ile de Port Cros	Provence	France	33
K477	<i>D. erythrina</i> n.sp.	+	+	+	+			Montgorgioni, Mt. Arci	Sardinia	Italy	34
V20	<i>D. erythrina</i> *	+	+	+	+			Lórrach, Well am Rhein		Germany	35
K334	<i>D. lantosquensis</i> *	+	+	+	-			Monte Argentario	Tuscany	Italy	36
k498	<i>D. lantosquensis</i> *	+	+	+	+			Parco Regionale della Maremma	Tuscany	Italy	37
V42	<i>D. erythrina</i> group	+	-	-	-			Parco Regionale della Maremma	Tuscany	Italia	37
LB237	<i>D. erythrina</i> *	+	+	+	+	V78		Prague, Motol	Bohemia	Czech Republic	38
V24	<i>D. erythrina</i> *	+	+	+	-			Prague, Divoká Šárka	Bohemia	Czech Republic	38
V78	<i>D. erythrina</i> *	+	+	+	-			Prague, Divoká Šárka	Bohemia	Czech Republic	38
V79	<i>D. erythrina</i> *	+	+	+	+			Prague, Divoká Šárka	Bohemia	Czech Republic	38
V80	<i>D. erythrina</i> *	+	+	+	-			Prague, Divoká Šárka	Bohemia	Czech Republic	38
V23	<i>D. lantosquensis</i> *	+	+	+	-			Pardubice, Kunětická hora	Bohemia	Czech Republic	39
V37	<i>D. lantosquensis</i> *	+	+	+	-			Missina hill above Pécs		Hungary	40
K342	<i>D. lantosquensis</i> *	+	-	+	-	V37		Slovak kras, Hrušov		Slovakia	41
V22	<i>D. lantosquensis</i> *	+	-	-	-	V23		Slovak kras, Hrušov		Slovakia	41
V34	<i>D. lantosquensis</i> *	+	+	+	+			Balaton		Hungary	42
V36	<i>D. lantosquensis</i> *	+	-	-	-			Balaton		Hungary	42
V39	<i>D. lantosquensis</i> *	+	+	+	-	V50		Balaton		Hungary	42
V48	<i>D. lantosquensis</i> *	+	+	+	+	V50		Balaton		Hungary	42
V50	<i>D. lantosquensis</i> *	+	+	+	-			Balaton		Hungary	42
V81	<i>D. feryvida</i> *	+	-	-	-			Grand Canyon	Arizona	USA	43

dadrk450	<i>D. adriatica</i>	+	+	+	-			Materija	Kozina	Slovenia	x
dcaGl130	<i>D. calderensis</i>	+	+	+	-			Bco. de Juel	La Gomera	Canary Islands	x
dcaPk103	<i>D. calderensis</i>	+	+	+	-			Juan Adalid	La Palma	Canary Islands	x
dcrok418	<i>D. crocata</i>	+	+	+	-			Hoz de Peregrina, rio Dulce	Castilla-La Mancha	Spain	x
dgoGl132	<i>D. gomerensis</i>	+	+	+	-			Cañada de Jorge	La Gomera	Canary Islands	x
dgoHl133	<i>D. gomerensis</i>	+	+	+	-			Casa foresta de Frontera	El Hierro	Canary Islands	x
diI3k228	<i>D. inermis</i>	+	+	+	-			Tarifa, dirt rd. From Mirador del Estrecho to Facinas	Andalusia	Spain	x
diM1k226	<i>D. inermis</i>	+	+	+	-			4 km south from Tanger, road from Xauen	Tanger	Morroco	x
dsiGk94	<i>D. silvatica</i>	+	+	+	-			Bco. de Juel	La Gomera	Canary Islands	x
dsiHx117	<i>D. silvatica</i>	+	+	+	-			Hoya del Pino	El Hierro	Canary Islands	x
dsiPk16	<i>D. silvatica</i>	+	+	+	-			Pista de Machin	La Palma	Canary Islands	x
dvalk471	<i>D. valentina</i>	+	+	-	-			Cocentaina, Serra Mariola, rd. Borona	Valencia	Spain	x