



# Explosive evolution of an ancient group of Cyphophthalmi (Arachnida: Opiliones) in the Balkan Peninsula

Jérôme Murienne<sup>1\*</sup>, Ivo Karaman<sup>2</sup> and Gonzalo Giribet<sup>1</sup>

<sup>1</sup>Department of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, Cambridge, MA, USA and <sup>2</sup>Department of Biology, Trg Dositeja Obradovica, Novi Sad, Serbia

## ABSTRACT

**Aim** To investigate the phylogeny of the genus *Cyphophthalmus* in the Balkan Peninsula and to test the current recognition of 'phyletic lines' and phylogenetic groups proposed in previous studies in order to elucidate the biogeographical history of the region.

**Location** Europe, Balkan Peninsula, Adria microplate.

**Methods** Two mitochondrial (cytochrome *c* oxidase subunit I and 16S rRNA) and two nuclear (28S rRNA and 18S rRNA) markers were used to infer the phylogenetic history of the group. Molecular dating with relaxed molecular clocks was used to elucidate the relative time of diversification within the genus *Cyphophthalmus* and its constituent lineages.

**Results** Our analyses confirm the monophyly of the genus *Cyphophthalmus*, and that of the Aegean and *gorgjevici* lineages, whereas the 'Dinaric lineage' appears paraphyletic.

**Main conclusions** We show that the genus *Cyphophthalmus* is an old endemic from the Balkan biogeographical region, which gave origin to at least three main lineages. Those lineages have diversified within overlapping ranges. According to our molecular dating, they have also diversified within the same timeframe. The Dinaric Alps, although presenting a large number of species, cannot be inferred as the centre of origin of the group. Instead, the biogeographical evolution of the genus could be related to the palaeogeographic history of the Adria microplate.

## Keywords

Adria, Arthropoda, Balkan Peninsula, biogeography, Cyphophthalmi, *Cyphophthalmus*, Europe, Gondwana, Sironidae.

\*Correspondence: Jérôme Murienne, Department of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, 26 Oxford Street, Cambridge, MA 02138, USA.  
E-mail: [jmurienne@oeb.harvard.edu](mailto:jmurienne@oeb.harvard.edu)

## INTRODUCTION

The Cyphophthalmi (168 described species and subspecies worldwide: <http://giribet.oeb.harvard.edu/Cyphophthalmi/>) constitute a group of small harvestmen (Opiliones) classified into six families (Pinto-da-Rocha *et al.*, 2007), each restricted to a well-defined biogeographical region (Boyer *et al.*, 2007; Giribet & Kury, 2007). Sironidae (47 extant species) inhabit the terranes of the former Laurasia with representatives in Europe, North America and Japan, the latter restricted to the monotypic genus *Suzukiellus*, whose affinities to the family are still disputed (e.g. Giribet & Boyer, 2002; de Bivort & Giribet, 2004; Boyer *et al.*, 2007). The bulk of the European sironid species diversity is concentrated in the Balkan Peninsula, where the genus

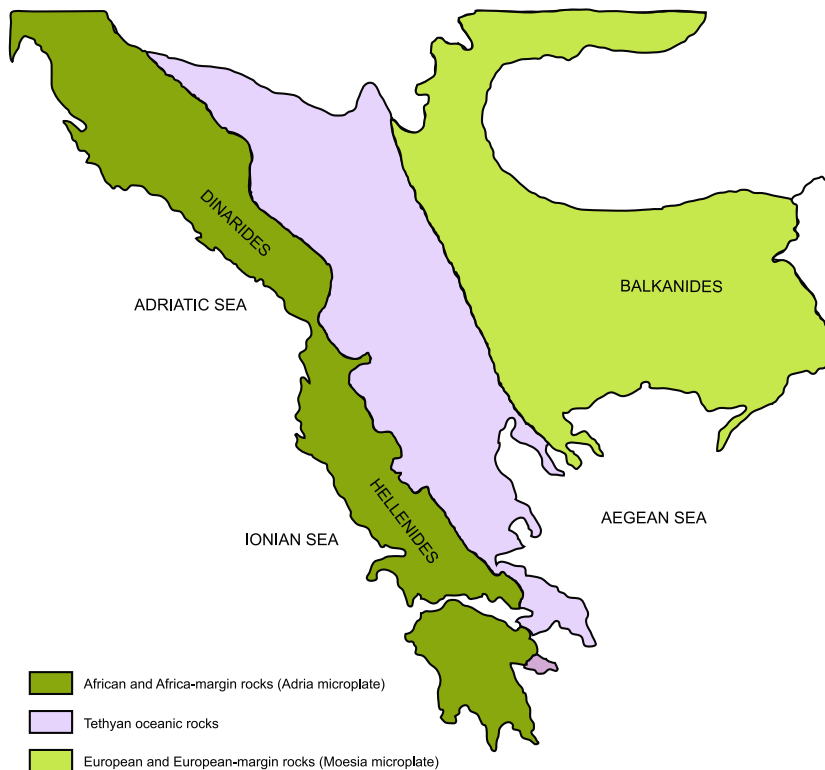
*Cyphophthalmus* diversified into 31 described species (Karaman, 2008, 2009; more species await formal description) divided into three phyletic lineages (Boyer *et al.*, 2005; Karaman, 2005b, 2009). The complex composition and distribution of the cyphophthalmid fauna (Karaman *et al.*, 1994; Boyer *et al.*, 2005; Karaman, 2005a,b), their high level of microendemism, their high diversity in the region and their often restricted distributions (many species being known from only a single cave) make Cyphophthalmi an excellent candidate taxon for the study of the origin of biodiversity in the Balkan Peninsula.

Among the three European peninsulas that have acted as refugia during the last glaciations – Iberian, Italian and Balkan (Petit *et al.*, 2003; Tzedakis, 2004) – the Balkan Peninsula shows the highest degree of species richness and

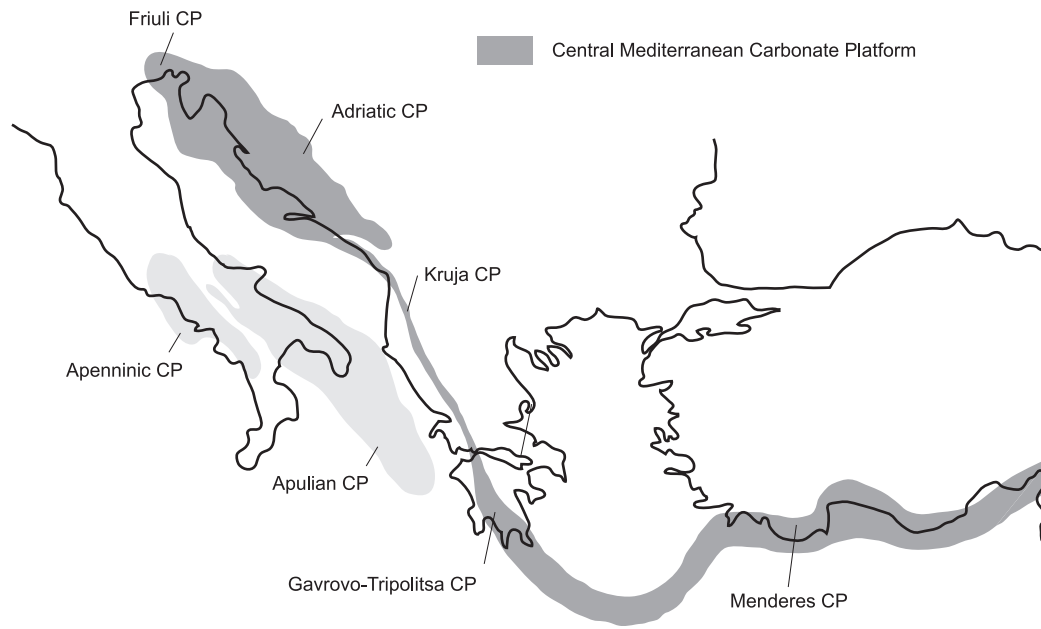
endemism and is widely recognized as a centre of biodiversity in Europe (Gaston & David, 1994). As previously noted by Džukić & Kalezić (2004), two general models can be invoked to explain this situation: climatic–ecological fluctuations and tectonic–palaeogeographic change. From a geological point of view (Fig. 1), the Balkan Peninsula includes the margin of both Eurasia (the Moesian microplate) and Gondwana (the Adria microplate), as well as remnants of the Tethys and related marginal seas (made up of oceanic crust) (Karamata, 2006). The Adria microplate is the largest lithospheric fragment in the Central Mediterranean region. It has variously been interpreted as a rigid promontory of Africa and as an independent entity (Robertson *et al.*, 1996; Pinter & Grenerczy, 2006). It was connected to Iberia in the west and to north-west Africa in the south (Wortmann *et al.*, 2001) until the Middle–Late Triassic episodes of rifting and breakup (Channell *et al.*, 1979; Robertson *et al.*, 1991; Pamić *et al.*, 1998). For most of the time the Adria microplate was in a shallow-water environment (Scheibner & Speijer, 2008) in which the Southern Tethyan Megaplatform formed before disintegrating into several carbonate platforms (Fig. 2) in the Early Jurassic (Vlahović *et al.*, 2005). Footprints of various groups of dinosaurs indicate the presence of emerged land until *c.* 125 Ma (Bosellini, 2002; Dalla Vecchia, 2002, 2008), and cycles of submergence and emergence throughout the Jurassic and Cretaceous have been recorded in some carbonate platforms (Vlahović *et al.*, 2005; Márton *et al.*, 2008). Adria and Eurasia finally collided around 65–70 Ma (Karamata, 2006). Dinarides orogenesis

occurred from the Eocene to the Miocene (Bennett *et al.*, 2008; Palinkaš *et al.*, 2008). In this context, Karaman (2005b) proposed that the diversification of the genus *Cyphophthalmus* could be explained by the dynamic events of the archipelago of the intraoceanic carbonate platform of Tethys (cycles of emergence and submergence), which has its origins in the northernmost part of Gondwana (the original position of the Adria microplate).

A molecular phylogeny of the genus *Cyphophthalmus* based on a combination of nuclear and mitochondrial markers is presented here. Several new species have been added to the first molecular study of the genus (Boyer *et al.*, 2005), allowing us to test a number of hypotheses regarding the biogeographical history of the genus in the Balkan Peninsula. If the origin of the group is related to the Adria microplate, we would expect a sister-group relationship between the genus *Cyphophthalmus* and the genus *Paramiopsalis*, which is endemic to the Iberian Peninsula, rather than with the genus *Siro*, found in Western Europe and North America. Boyer *et al.* (2005) proposed a south-eastern origin for the group. The inclusion of several specimens from Greece that were not included in the study of Boyer *et al.* (2005) will help us to test this hypothesis. Molecular dating should allow us to test whether the diversification of the group is related to recent climatic fluctuations, mountain orogenesis or the palaeogeographic history of microplates. The results also allow us to test the current recognition of lineages ('lines' *sensu* Karaman, 2005b, 2009; see also Boyer *et al.*, 2005).



**Figure 1** Simplified geological map of the Balkans (based on Channell *et al.*, 1979, and Rogers & Santosh, 2004). Major mountain chains are indicated on the map. The orogenesis of the Dinarides took place from the Eocene to the Miocene.



**Figure 2** Simplified geological map showing the present position of the carbonate platforms (CP) (based on Vlahović *et al.*, 2005).

## MATERIALS AND METHODS

### Sampling

A total of 62 individuals were included in this study. Because the monophyly of the genus *Cyphophthalmus* has already been established, by Boyer *et al.* (2005), we chose to root the tree with other members of the family Sironidae, representing the broad distribution of the family (3 species from Europe, 1 from the USA and 1 from Japan). In addition to the 15 species included in the previous study, we used 27 recently collected specimens representing 18 species (see Appendix S1 in Supporting Information). All specimens were collected alive, either by sifting litter or by direct search, and preserved in 95% EtOH. Vouchers are deposited at the Museum of Comparative Zoology (MCZ), Department of Invertebrate Zoology DNA collection (Table 1).

### DNA extraction, amplification and sequencing

The DNEasy tissue kit (Qiagen, Valencia, CA, USA) was used for tissue lysis and DNA purification following the manufacturer's protocol. Total DNA was extracted either by crushing the whole animal or one appendage in the lysis buffer, or by incubating the entire animal or appendage in the lysis buffer overnight, as described in Boyer *et al.* (2005). The intact cuticle of the animal was removed after the lysis step and kept in ethanol.

Target genes were selected based on previous studies of *Cyphophthalmi* and have proved to be informative at various levels in evolutionary studies. Because the first two fragments of 18S rRNA (18S hereafter) show little to no variation within Sironidae, we used only the last *c.* 650-bp fragment, amplified

by the 18Sa2.0/9R primer pair (Giribet *et al.*, 1996; Whiting *et al.*, 1997). A fragment of the 5' end (*c.* 1000 bp) of the 28S rRNA (28S hereafter) was amplified using the primer set 28SD1F/28Sb (Whiting *et al.*, 1997; Park & Ó Foighil, 2000) or alternatively with the forward primer 28Sa (Whiting *et al.*, 1997). The mitochondrial 16S rRNA (16S hereafter) was amplified using the primer pair 16Sar/16Sb (Xiong & Kocher, 1991). The mitochondrial protein-encoding gene cytochrome *c* oxidase subunit I (COI hereafter) was amplified using the primer pair LCO1490/HCO2198 (Folmer *et al.*, 1994). Because the amplification of the histone H3 gene was problematic in several previous studies, resulting in an incomplete dataset, we chose not to sequence this gene.

Polymerase chain reactions (PCRs; 50  $\mu$ L) included 2  $\mu$ L of template DNA, 1  $\mu$ M of each primer, 200  $\mu$ M of dinucleotide-triphosphates (Invitrogen, Carlsbad, CA, USA), 1 $\times$  PCR buffer containing 1.5 mM MgCl<sub>2</sub> (Applied Biosystems, Branchburg, NJ, USA) and 1.25 units of AmpliTaq DNA polymerase (Applied Biosystems). PCRs were carried out using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems), and involved an initial denaturation step (5 min at 95°C) followed by 35 cycles including denaturation at 95°C for 30 s, annealing (ranging from 44° to 49°C) for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min.

The double-stranded PCR products were verified by agarose gel electrophoresis (1% agarose) and purified with a Perfectprep PCR Cleanup 96 system (Eppendorf, Westbury, NY, USA). The purified PCR products were sequenced directly with the same primer pairs as used for amplification. Each sequence reaction contained a total volume of 10  $\mu$ L including 2  $\mu$ L of PCR product, 1  $\mu$ M of one of the PCR primer pairs, 2  $\mu$ L ABI BigDye 5 $\times$  sequencing buffer,

**Table 1** List of specimens included, with Museum of Comparative Zoology (MCZ) voucher numbers. GenBank accession numbers are indicated for each of the loci. New sequences are indicated in bold.

Voucher	18S	28S	16S	COI
DNA100459	AY639489	DQ513121	AY639550	DQ825641
DNA101383	AY918872	DQ513122	AY918877	DQ825642
DNA100461	AY639492	DQ513123	AY639552	AY639580
DNA100488	AY639490	DQ513128	AY639551	DQ825643
DNA101543	DQ513138	DQ513116	DQ518086	DQ513108
DNA100487	AY639461	DQ513120	AY639526	AY639556
DNA100499m	AY639462	AY639499	AY639527	AY639557
DNA100499f	AY639463	–	AY639528	AY639558
DNA100498m	AY639464	AY639500	AY639529	AY639559
DNA100498f	AY639465	AY639501	AY639530	AY639560
DNA100495m	AY639466	–	AY639531	–
DNA100495f	AY639467	AY639502	AY639532	–
DNA100497m	AY639468	AY639503	AY639533	–
DNA100497f	AY639469	AY639504	AY639534	AY639561
DNA100494m	AY639470	AY639505	AY639535	AY639562
DNA100494f	AY639471	AY639506	AY639536	AY639563
DNA101041	AY639472	AY639507	–	AY639564
DNA100493m	AY639473	DQ885591	AY639537	AY639565
DNA100493f	AY639474	–	–	AY639566
DNA101039	AY639475	DQ825594	–	AY639567
DNA100909	AY639476	–	AY639538	AY639568
DNA100492m	AY639477	AY639510	AY639539	AY639569
DNA100492f	AY639478	AY639511	AY639540	AY639570
DNA100500	AY639479	–	AY639541	–
DNA100501	AY639480	–	AY639542	–
DNA100910m	AY639481	AY639512	AY639543	–
DNA100910f	AY639482	AY639513	AY639544	AY639571
DNA101038	AY639483	DQ513119	–	AY639572
DNA100907m	AY639484	AY639514	AY639545	AY639573
DNA100907f	AY639485	AY639515	AY639546	AY639574
DNA100491	AY639486	AY639516	AY639547	AY639575
DNA100496	AY639487	AY639517	AY639548	AY639576
DNA100908	AY639488	–	AY639549	AY639577
DNA101342	AY918870	DQ513117	–	AY918878
DNA101343	AY918871	DQ825586	AY918876	AY918879
DNA102088	<b>FJ946373</b>	<b>FJ946398</b>	<b>FJ946347</b>	<b>FJ946425</b>
DNA102089	<b>FJ946374</b>	<b>FJ946399</b>	<b>FJ946348</b>	<b>FJ946426</b>
DNA102090	<b>FJ946375</b>	<b>FJ946400</b>	<b>FJ946349</b>	<b>FJ946427</b>
DNA102091	<b>FJ946376</b>	<b>FJ946401</b>	<b>FJ946350</b>	–
DNA102092	<b>FJ946377</b>	<b>FJ946402</b>	<b>FJ946351</b>	<b>FJ946428</b>
DNA102093	<b>FJ946378</b>	<b>FJ946403</b>	<b>FJ946352</b>	<b>FJ946429</b>
DNA102094	<b>FJ946379</b>	<b>FJ946404</b>	<b>FJ946353</b>	–
DNA102095	<b>FJ946380</b>	<b>FJ946405</b>	<b>FJ946354</b>	<b>FJ946430</b>
DNA102096	<b>FJ946381</b>	<b>FJ946406</b>	<b>FJ946355</b>	–
DNA102097	<b>FJ946382</b>	<b>FJ946407</b>	<b>FJ946356</b>	<b>FJ946431</b>
DNA102098	<b>FJ946383</b>	<b>FJ946408</b>	<b>FJ946357</b>	<b>FJ946432</b>
DNA102099	<b>FJ946384</b>	<b>FJ946409</b>	<b>FJ946358</b>	<b>FJ946433</b>
DNA102100	<b>FJ946385</b>	<b>FJ946410</b>	<b>FJ946359</b>	<b>FJ946434</b>
DNA102107	<b>FJ946386</b>	<b>FJ946411</b>	<b>FJ946360</b>	<b>FJ946435</b>
DNA102108	<b>FJ946387</b>	<b>FJ946412</b>	<b>FJ946361</b>	–
DNA102109	<b>FJ946388</b>	<b>FJ946413</b>	<b>FJ946362</b>	<b>FJ946436</b>
DNA102110	<b>FJ946389</b>	<b>FJ946414</b>	<b>FJ946363</b>	<b>FJ946437</b>
DNA102111	<b>FJ946390</b>	<b>FJ946415</b>	<b>FJ946364</b>	<b>FJ946438</b>
DNA102112	<b>FJ946391</b>	<b>FJ946416</b>	<b>FJ946365</b>	–

**Table 1** Continued

Voucher	18S	28S	16S	COI
DNA102113	–	<b>FJ946417</b>	<b>FJ946366</b>	<b>FJ946439</b>
DNA102114	<b>FJ946392</b>	<b>FJ946418</b>	<b>FJ946367</b>	<b>FJ946440</b>
DNA102476	<b>FJ946393</b>	<b>FJ946419</b>	<b>FJ946368</b>	–
DNA102477	<b>FJ946394</b>	<b>FJ946420</b>	<b>FJ946369</b>	–
DNA102479	<b>FJ946395</b>	<b>FJ946421</b>	<b>FJ946370</b>	–
DNA102480	–	<b>FJ946422</b>	<b>FJ946371</b>	–
DNA102532	<b>FJ946396</b>	<b>FJ946423</b>	–	<b>FJ946441</b>
DNA102533	<b>FJ946397</b>	<b>FJ946424</b>	<b>FJ946372</b>	–

and 2  $\mu$ L ABI BigDye Terminator v3.0 (Applied Biosystems). The sequencing reactions involved an initial denaturation step for 3 min at 95°C, and 25 cycles (95°C for 10 s, 50°C for 5 s, and 60°C for 4 min). The BigDye-labelled PCR products were cleaned using Performa DTR Plates (Edge Biosystems, Gaithersburg, MD). The sequence reaction products were then analysed using an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems).

### Sequence editing

Chromatograms were edited and overlapping sequence fragments were assembled using SEQUENCHER 4.7 (Gene Codes Corporation 1991–2007, Ann Arbor, MI). BLAST searches (Altschul *et al.*, 1997), as implemented in the NCBI website (<http://www.ncbi.nlm.nih.gov/>), were conducted to check for putative contamination. The software package MACGDE: Genetic Data Environment for MACOSX (Linton, 2005) was used to determine fragments based on internal primers and secondary structure features (Giribet & Wheeler, 2001; Giribet & Boyer, 2002). All new sequences have been deposited in GenBank under the accession numbers specified in Table 1.

### Phylogenetic analyses

Phylogenetic analyses were conducted under direct optimization (Wheeler, 1996) with the program POY 4.1 (Varón *et al.*, 2008). 18S rRNA showed a high degree of conservation with no length variation and was considered as pre-aligned. 28S and 16S rRNA were respectively divided into 18 and 4 fragments according to internal primers and secondary-structure features. The COI sequences were first aligned according to conservation of the amino acid sequence. As previously noted by Boyer *et al.* (2005), COI sequences of Sironidae show an unusual length variation. A first region of 33 nucleotides shows a clear deletion of 3 nucleotides in *Paramiopsalis* and *Cyphophthalmus*, when compared with other sironid and non-sironid Cyphophthalmi. A second region of 42 nucleotides shows a deletion of 3 nucleotides in the genus *Cyphophthalmus*. A third region of 48 nucleotides shows a deletion of 6 nucleotides in *Siro valleurum* and of 3 nucleotides in *Siro rubens*, and a 3-nucleotide deletion in *C. serbicus*, *C. eratoae*

and *C. corfuanus*. These three ambiguous regions were treated under direct optimization, whereas the remaining part of COI, showing no length variation and a high degree of conservation at the protein level, was treated as pre-aligned.

Instead of manually defining a specific strategy (see Murienne *et al.*, 2008, for commands), we used the `max_time` command, which implements a default search strategy that effectively combines tree building with tree bisection–reconnection (TBR) branch swapping, parsimony ratchet (Nixon, 1999) and tree fusing (Goloboff, 1999). The strategy was implemented on the Harvard odyssey cluster using the Load Sharing Facility queuing system (`bsub -o poy.out -n 8 -R"span[ptile=8]" -q normal -a openmpi/search.poy`), where `-n` is the total number of cores requested and `ptile=8` allows the jobs to be grouped so the processes take all eight cores on a node. In parallel environments, `poy` will exchange trees between processes only at the end of each search command. We thus used four replicates of the search (`max_time:1:0:0`) routine. This series of commands attempts as many builds, swaps, ratchets and fusings as possible within the specified total time of 4 days, trees being exchanged between processors at the end of each search (Varón *et al.*, 2008). Because the relative importance of the various partitions has already been explored in Boyer *et al.* (2005), we present only the results from the combined analysis. To be consistent with the previous methodology and to be able to compare the results, we used the 121 weighting scheme as in Boyer *et al.* (2005), where indels receive a cost of 2, transversions receive a cost of 2 and transitions receive a cost of 1. The resulting implied alignment (Wheeler, 2003; Giribet, 2005) was then used in TNT (Goloboff *et al.*, 2008) to estimate nodal support with 500 bootstrap replicates (Felsenstein, 1985).

A classical two-step analysis was also performed. Sequences were aligned using MUSCLE 3.6 (Edgar, 2004) with default parameters. Fully duplicate sequences (DNA100494m and DNA100498m) were removed. Ambiguous regions previously reported for COI (fragments not pre-aligned under direct optimization) were discarded. For the other genes, ambiguous regions were removed using GBLOCK 0.91B (Castresana, 2000) with options `-t=d -b5=h`. Concatenation of the separate data was performed with PHYUTILITY (Smith & Dunn, 2008). The resulting matrix was submitted to a maximum likelihood analysis using RAxML 7.0.4 (Stamatakis, 2006) with a GTR +  $\Gamma$  model (Yang, 1993) applied to each partition and a rapid bootstrap procedure (Stamatakis *et al.*, 2008). The analyses were performed on the cluster of the CIPRES project at the San Diego super-computer centre: [http://www.phylo.org/sub\\_sections/portal/](http://www.phylo.org/sub_sections/portal/) (accessed 10 April 2009). We chose the GTR model because it is the most common and general model for real-world DNA. Although many authors have used the GTR + I +  $\Gamma$  model to incorporate rate heterogeneity (Gu *et al.*, 1995), it is well known (Yang, 2006) that adding a proportion of invariable sites creates a strong correlation between  $p_0$  and  $\alpha$ , making it impossible to estimate both parameters reliably (Sullivan *et al.*, 1999; Mayrose *et al.*, 2005).

## Age estimation and rates of diversification

Our calibration scheme is based on several lines of evidence. (1) Sironidae are distributed in Laurasia (Boyer *et al.*, 2007), indicating an old age for the group under the assumption of vicariance. Laurasia separated from Gondwana following the opening of the Atlantic Ocean (173 Ma). (2) *Paramiopsalis* is restricted to old geological terranes in the Iberian Peninsula (Murienne & Giribet, 2009). (3) The breakup of the Adria microplate began in the Late Triassic. (4) The *Paramiopsalis*/*Cyphophthalmus* split is found at 257 Ma when calibrating the origin of Cyphophthalmi at 400 Ma (the oldest fossil of its sister-group) on Boyer *et al.*'s (2007) phylogeny. We thus chose to calibrate the *Paramiopsalis*/*Cyphophthalmus* split at 200 Ma based on palaeogeographic evidence: the breakup of the Adria microplate.

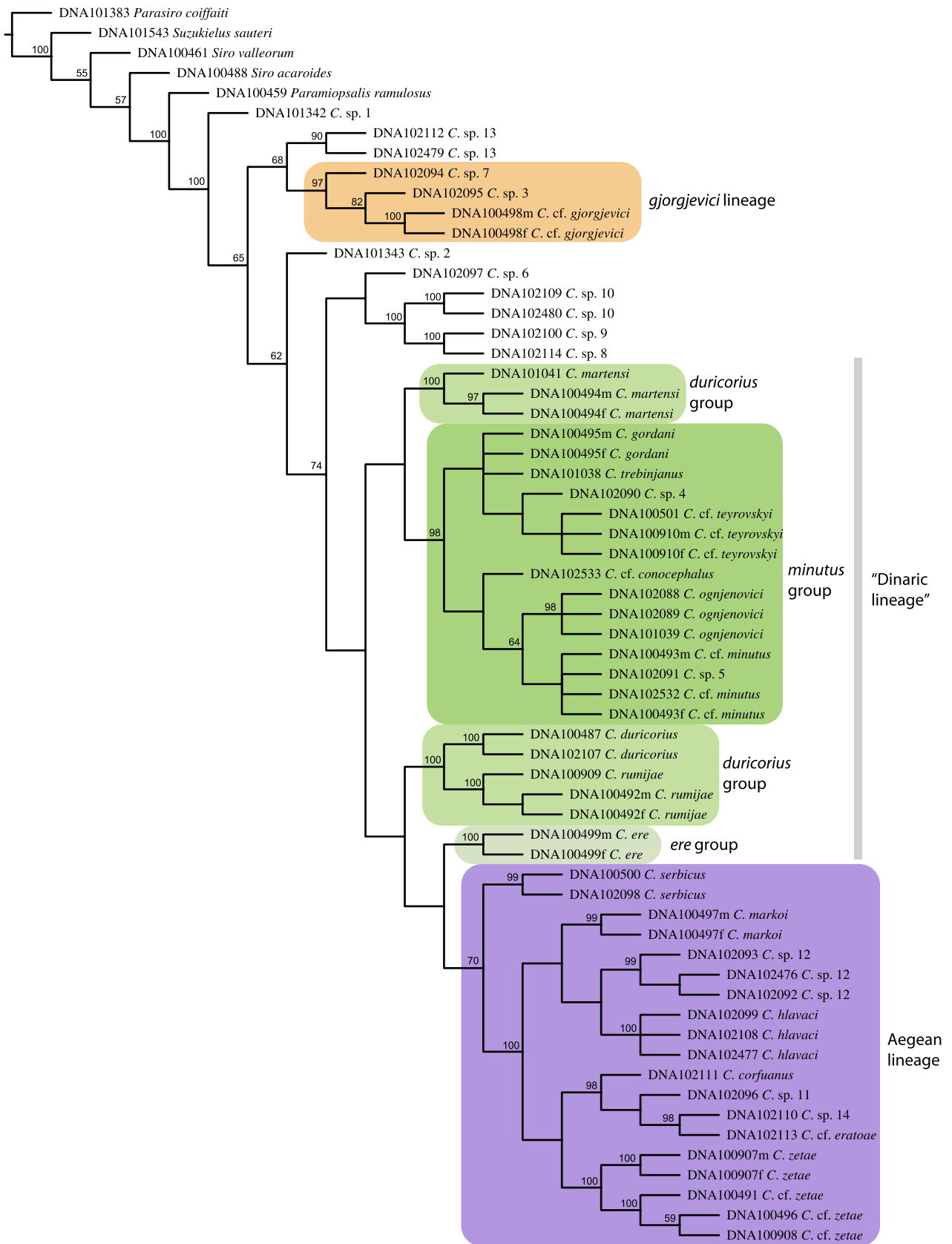
The ages of clades were estimated on the maximum likelihood tree (keeping only one specimen per species) using standard likelihood methods as implemented in the program `r8s 1.71` (Sanderson, 2003, 2006). We used a cross-validation procedure (Sanderson, 2002) to select the best method among those offered by the program. We tested one clock-like method, the Langley–Fitch method (Langley & Fitch, 1974), and two relaxed-clock methods, nonparametric rate smoothing (Sanderson, 1997) and penalized likelihood (Sanderson, 2002). For the penalized likelihood method, the degree of autocorrelation within lineages was estimated using cross-validation, and the smoothing parameter  $\lambda$  defined accordingly. We also tested the performance of two penalty functions, the additive penalty function, which penalizes squared differences in rates across neighbouring branches in the tree, and the log penalty function, which penalizes the squared difference in the log of the rates on neighbouring branches.

The search was then performed using the commands `num_time_guesses=3` (3 initial starting conditions) and `check-Gradient` in order to validate the results. The program RAxML 7.0.4 was used to generate 100 bootstrap datasets based on the optimal topology. Those 100 topologies thus only vary in branch lengths. Divergence estimates were then calculated for each of the 100 bootstrap replicates using `r8s 1.71` to obtain standard deviations on each node using the `profile` command.

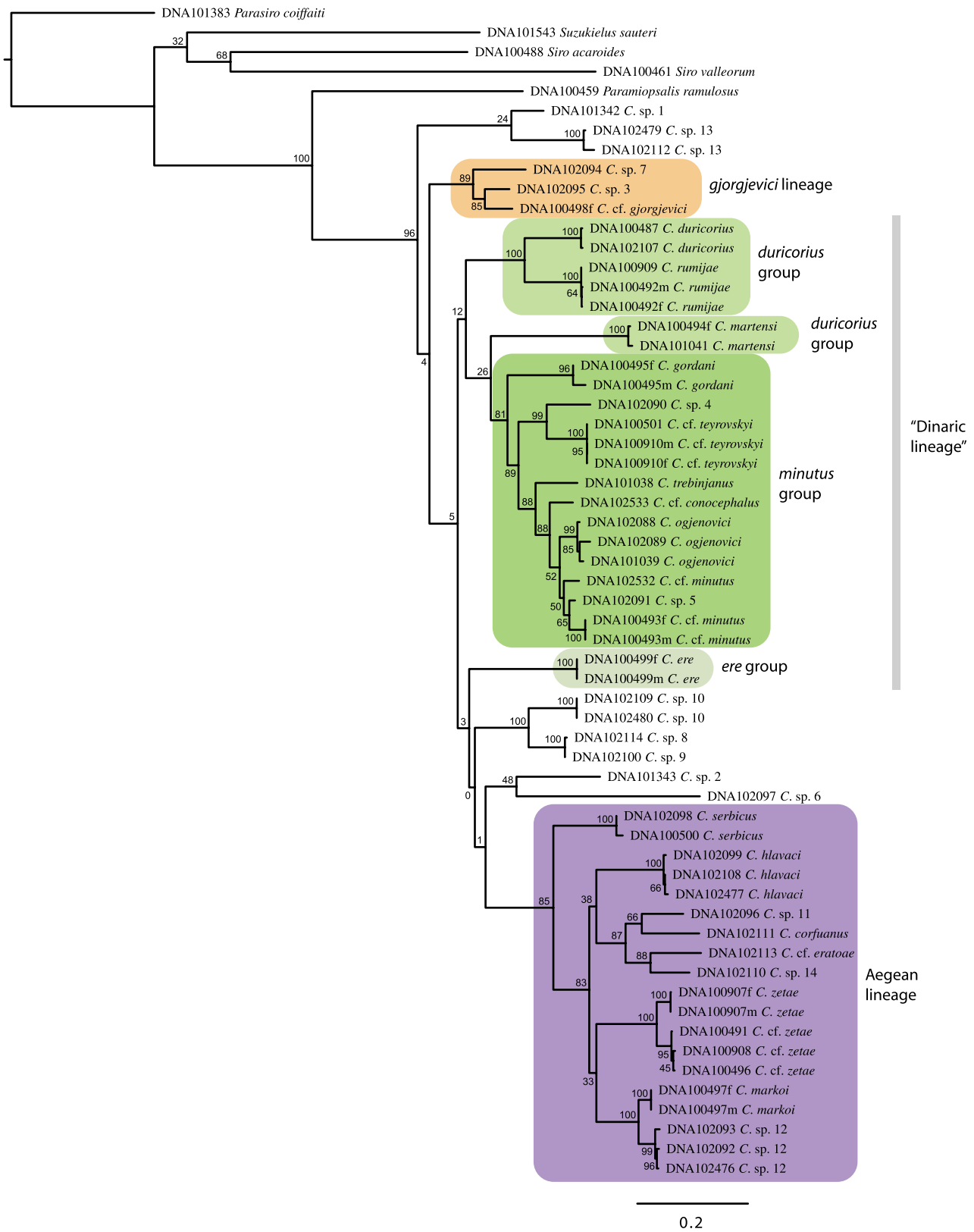
Temporal shifts in diversification rates were analysed using the R package LASER (Rabosky, 2006a). The package was used to compare the fit of alternative diversification models (Rabosky, 2006b). Diversification parameters were computed using the best-fitting model among two rate-constant and five rate-variable diversification models. The package was also used to draw a lineage-through-time plot (Harvey *et al.*, 1994).

## RESULTS

During the 4 days of tree searching under direct optimization, `poy` conducted 548 builds + TBR, 9026 fusing rounds and 288 ratchet rounds. Shortest trees were found 2339 times for a tree length of 4972, resulting in 12 equally most parsimonious trees. The strict consensus tree (Fig. 3) shows the monophyly



**Figure 3** Strict consensus for the 12 equally parsimonious trees obtained under direct optimization. Bootstrap frequencies are indicated on nodes. Shading indicates the various lineages of *Cyphophthalmus* used in the text.



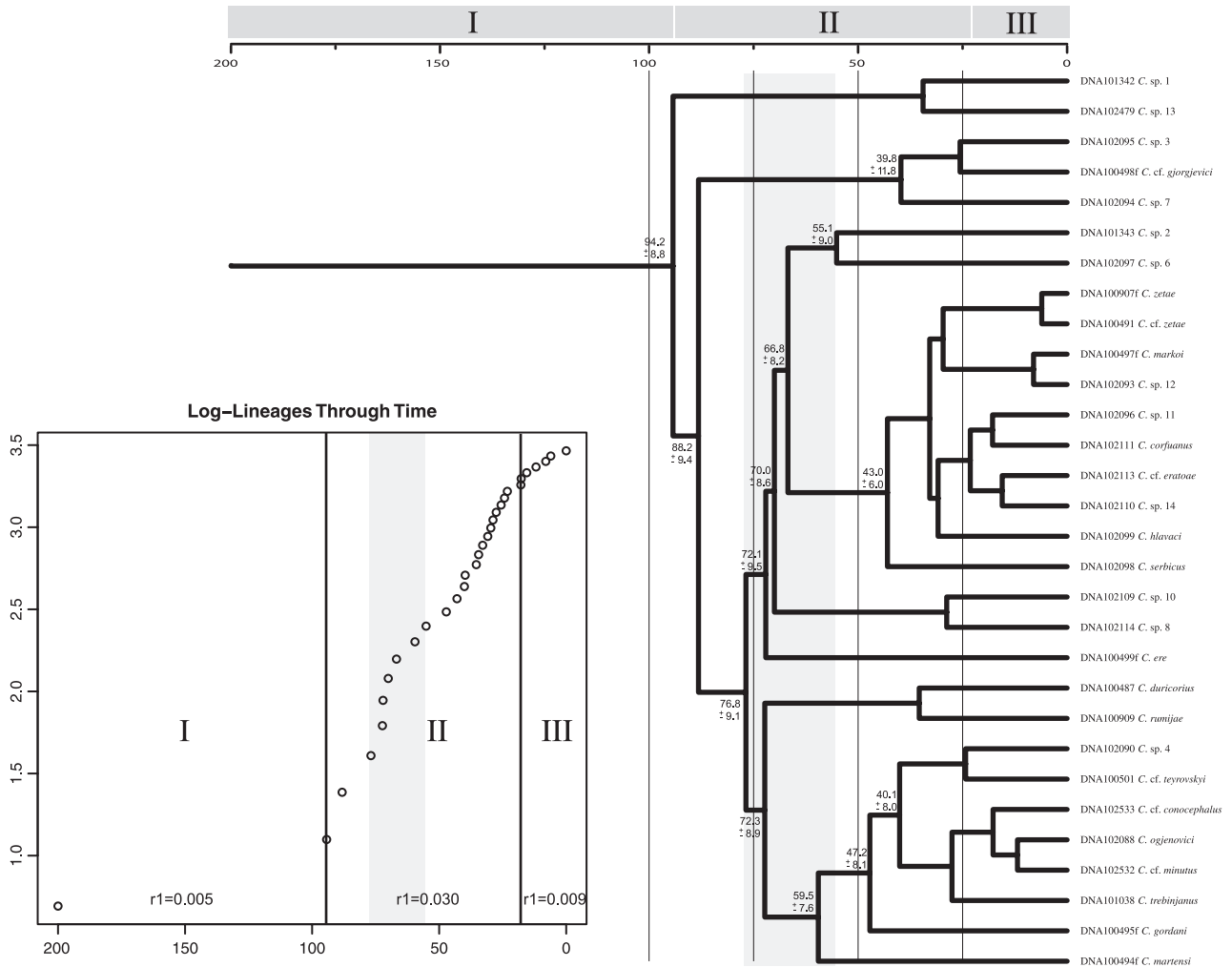
**Figure 4** Optimal tree using maximum likelihood with a GTR +  $\Gamma$  model. Bootstrap frequencies are indicated on nodes. Shading indicates the various lineages of *Cyphophthalmus* used in the text, as in Fig. 3. The scale bar indicates corrected genetic distance.

of the genus *Cyphophthalmus* with 100% bootstrap frequency (BF hereafter) and with its sister-group represented by the Iberian genus *Paramiopsalis* (Juberthie, 1962; Boyer *et al.*, 2005, 2007; Murienne & Giribet, 2009).

The general topology is broadly similar to the one obtained by Boyer *et al.* (2005). *Cyphophthalmus* sp. 1 from Bulgaria (the second most eastern species) is sister to all of the remaining species. The *gjorgjevici* lineage is monophyletic (97% BF). The Aegean lineage (*serbicus* group *sensu* Boyer *et al.*, 2005) is monophyletic (70% BF). It is found to be sister to *C. ere* with a low support value, as in Boyer *et al.* (2005). The Dinaric lineage is paraphyletic, with *C. duricorius*, *C. rumijae* and *C. ere* separated from the other species. Within the 'Dinaric lineage', the *minutus* group is monophyletic (98% BF). The *duricorius* group is polyphyletic, with *C. martensi* forming a lineage distinct from *C. duricorius* and *C. rumijae*. Most of the main lineages identified are well supported by bootstrap values. By contrast, deeper nodes appear with low support values or with bootstrap frequencies below 50%.

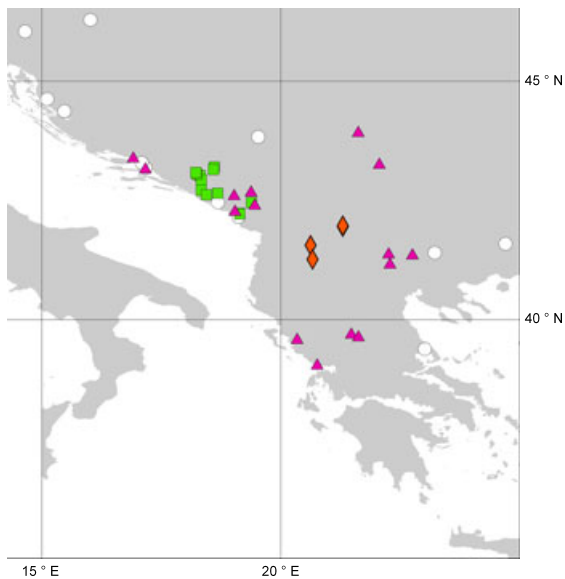
The maximum likelihood analysis gave a tree (Fig. 4) of  $Ln L = -13985.79$ . The topology is broadly similar to the one obtained under Direct Optimization. *Cyphophthalmus* sp. 1 is not retrieved as sister to the remaining species but groups with *Cyphophthalmus* sp. 13, the two species constituting the sister-group to all other species of *Cyphophthalmus*. The *duricorius* group is paraphyletic, whereas it was polyphyletic under direct optimization. Regarding support values, the same phenomenon is observed under maximum likelihood as with direct optimization. The main lineages present high support values whereas the deeper nodes present very low bootstrap frequencies.

The cross-validation process found the best-fitting method to be penalized likelihood in combination with the Powell algorithm, a smoothing value of 32 and an additive penalty function. The resulting chronogram is presented in Fig. 5. The diversification of the genus is dated at 94.3 Ma, thus 105.7 Myr after the origin of the group. Most of the major lineages originated within a short timeframe corresponding to the collision of the Adria microplate with the Moesia



**Figure 5** Chronogram based on the maximum likelihood tree and lineage-through-time plot with net speciation rates estimated under the yule3rate model for species of *Cyphophthalmus*. The grey area represents all the nodes for which dates and confidence values are within the timeframe of the collision between the Adria and the Moesia microplates.





**Figure 6** Distribution map indicating the localities of the samples of *Cyphophthalmus* species used in this study. The Aegean line is represented with triangles, the *minutus* group is represented with squares and the *gjorgjevici* lineage is represented with diamonds.

microplate (65–70 Ma). The grey area of Fig. 5 represents all the nodes for which dates and confidence values are within this timeframe.

LASER identified yule3rate as the best-fitting model. Temporal shifts are represented on the lineage-through-time plot as vertical bars, delimiting three zones. Speciation rates are indicated for each of the zones. As for the chronogram, the grey area represents all the nodes for which dates and confidence values are within the collision timeframe. According to the scenario suggested by the yule3rate model, the genus *Cyphophthalmus* began diversifying (zone I) with a net diversification rate of 0.005 speciation events per million years ( $\text{Myr}^{-1}$ ). A shift in net diversification took place 94 Ma (zone II), with the rate shifting dramatically to 0.030 speciation events  $\text{Myr}^{-1}$ . The net diversification rate shifted again around 23 Ma (zone III), decreasing to 0.009 speciation events  $\text{Myr}^{-1}$ .

## DISCUSSION

Although most of the hypotheses explaining the high biodiversity in the region refer to the Balkans as a glacial refugium (Taberlet *et al.*, 1998; Petit *et al.*, 2003; Hewitt, 2004; Schmitt, 2007) or a zone of exchange (Magyari *et al.*, 2008), we provide evidence that the Balkan Peninsula is also home to an old endemic biota. This situation is attested by the high rate of specific endemism in several groups, such as spiders with 27% endemism (Deltchev, 2004), amphibians with 28% endemism, and reptiles with 21% endemism (Džukić & Kalezić, 2004). Evidence from phylogenetic studies and the presence of deep genetic divergence in populations (Oosterbroek & Arntzen, 1992; Cooper *et al.*, 1995; Oliverio *et al.*, 2000; Seddon *et al.*,

2001; Ursenbacher *et al.*, 2008) also suggest long biogeographical isolation. In this context, the long and complex palaeogeographic history of the region (Rage & Roček, 2003; Rokas *et al.*, 2003; Parmakelis *et al.*, 2006; Kuhlemann, 2007), as well as its high habitat heterogeneity, topographic diversity and great climatic variation are of fundamental importance in explaining the Balkan biodiversity.

The genus *Cyphophthalmus* is sister to the genus *Paramiopsalis* of the Iberian Peninsula. The inferred early diversification of the genus *Cyphophthalmus* (94 Ma) is also consistent with the rifting of the Adria microplate and the presence of a dynamic archipelago on the carbonate platforms (Fig. 2) (Karaman, 2005b). It appears that at least three distinct groups have diversified with overlapping ranges (Fig. 6). Furthermore, these groups appear to have diversified within the same timeframe (Fig. 5) and with high speciation rates compared to the rates in zones I and III. Those nodes are also the ones showing the lowest support in our phylogenies (Figs 3 & 4), once again suggesting a rapid diversification. This provides evidence that, as for the *Cyphophthalmi* of New Zealand and Southeast Asia, the genus *Cyphophthalmus* underwent explosive evolution (*sensu* Romer, 1960; Hennig, 1966) in the Balkans. This term, as opposed to explosive radiation, refers to an explosive diversification without change in morphology. In the case of *Cyphophthalmus*, we can correlate the explosive evolution of the group with the collision of the Adria microplate and Eurasia to form the present-day Balkan Peninsula (Fig. 1). This situation echoes the evolution of the crested newts (*Triturus cristatus* superspecies), for which the four European species originated near-simultaneously in the Balkan region (Arntzen *et al.*, 2007), although the origin of that group is much more recent, in the Miocene.

The Southern Dinaric Alps is the region with the highest number of species of *Cyphophthalmus*. However, the *Cyphophthalmus* species of the Dinaric Alps have an apical position in the phylogeny of the group and belong to several distinct lineages. It is thus clear that the Dinaric Alps is not the centre of origin of the group. Boyer *et al.* (2005) proposed a south-eastern origin for the group. Our results show that some of the most eastern species (*Cyphophthalmus* sp. 1 and *Cyphophthalmus* sp. 2 from Bulgaria, and the members of the *gjorgjevici* lineage) indeed represent some early offshoots in the phylogeny. However, *Cyphophthalmus* sp. 13 from Dalmatia groups with these species. In addition, a number of species belonging to the Aegean lineage are also present in the east. Even if we expect a centre of origin in the west in the case of an Adria microplate origin, the observed explosive evolution coupled with some potential extinction could blur the biogeographical pattern within the Balkan Peninsula. Specimens of *Cyphophthalmus* from Turkey were not available for this study, and their future inclusion may help us to understand the centre of origin of the group. Whether the specimens known from Turkey are derived or basal remains a mystery. They were both assigned to the species *C. duricorius* by Gruber (1969) and more recently elevated to species rank and assigned to the Aegean phyletic lineage by Karaman

(2009), although their exact position is still to be tested phylogenetically.

The situation for the Balkan Peninsula, with an explosive evolution of only one genus of Cyphophthalmi, is very different from the one for the Iberian Peninsula. This territory contains four of the eight genera currently recognized in the family Sironidae, a generic diversity and morphological disparity of Cyphophthalmi not found in any other region of the world so far (Murienne & Giribet, 2009). Although these two European peninsulas have usually been depicted as glacial refugia, we provide evidence that old endemic lineages in these two territories have undergone very different diversifications: one – the Balkan Peninsula – by hosting an old genus with subsequent explosive evolution; and the other – the Iberian Peninsula – by hosting many ancient genera, each with few species. These differences could be related to the very different palaeogeographic histories of the two peninsulas.

## ACKNOWLEDGEMENTS

We are indebted to Sarah Boyer for preliminary work with the genus *Cyphophthalmus*, which directed many of the research questions addressed in this article, and to Plamen Mitov, Jochen Martens and Axel Schoenhofer, who generously provided specimens of *Cyphophthalmus*. Dan Rabosky provided help with the LASER package. TNT was made freely available through the generosity of the Willi Hennig Society. Miquel A. Arnedo and an anonymous referee made suggestions that helped to improve this article. This study was partly supported by the Ministry of Science, Technologies and Development of the Republic of Serbia, grant no.143037 to I.K. This research was supported by a Marie Curie International Outgoing Fellowship to J.M. (221099) within the 7th European Community Framework Program. This material is based upon work supported by the National Science Foundation (grant no. 0236871) to G.G.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Locality and collecting information for the *Cyphophthalmus* specimens used in this study.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

## BIOSKETCHES

**Jérôme Murienne** is a Marie Curie fellow at Harvard University (USA). He is interested in the phylogeny and biogeography of terrestrial invertebrates. A particular focus of his research is the evolution of Gondwanan fauna.

**Gonzalo Giribet** is Professor of Organismic and Evolutionary Biology and Curator of Invertebrates at the Museum of Comparative Zoology, Harvard University (USA). He is interested in the origins and maintenance of invertebrate diversity, in both marine and terrestrial environments, and in theoretical aspects of systematics and biogeography.

**Ivo Karaman**, at the University of Novi Sad (Serbia), is interested in Opiliones and other soil arthropod fauna from the Balkan region and in their biogeography.

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Editor: Bradford Hawkins