



Gondwanian relicts and oceanic dispersal in a cosmopolitan radiation of euedaphic ground beetles [☆]



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ARTICLE INFO

Article history:

Received 26 October 2015

Revised 18 February 2016

Accepted 11 March 2016

Available online 26 March 2016

Keywords:

Anillini

Endogean beetles

Tectonic vicariance

Molecular clock calibration

Molecular phylogeny

ABSTRACT

Anillini are a tribe of minute, euedaphic ground beetles (Carabidae) characterized by the loss of eyes, loss of wings and high levels of local endemism. Despite their presumed low dispersal, they have a nearly cosmopolitan distribution, including isolated islands such as New Zealand and New Caledonia. We used a time calibrated molecular phylogeny to test, first, if the tribe as currently understood is monophyletic and, second, whether the time of divergence is compatible with an early vicariant diversification after the breakup of Gondwana. We sequenced portions of 6 mitochondrial and 3 nuclear genes for 66 specimens in 17 genera of Anillini plus 39 outgroups. The resulting phylogenetic tree was used to estimate the time of diversification using two independent calibration schemes, by applying molecular rates for the related genus *Carabus* or by dating the tree with fossil and geological information. Rates of molecular evolution and lineage ages were mostly concordant between both calibration schemes. The monophyly of Anillini was well-supported, and its age was consistent with a Gondwanian origin of the main lineages and an initial diversification at ca. 100 Ma representing the split between the eyed *Nesamblyops* (New Zealand) and the remaining Anillini. The subsequent diversification, including the split of the Nearctic *Anillinus* and the subsequent splits of Palaearctic lineages, was dated to between 80 and 100 Ma and thus was also compatible with a tectonic vicariant origin. On the contrary, the estimated age of the New Caledonian blind *Orthotyphlus* at ca. 30 ± 20 Ma was incompatible with a vicariant origin, suggesting the possibility of trans-oceanic dispersal in these endogean beetles.

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1. Introduction

Soils have been described as one of the remaining biotic frontiers (André et al., 1994), harboring an astonishing amount of life that may hold more than 1/4 of species diversity on Earth (Decaëns et al., 2006). A poorly known portion of this diversity is found in the deep layers of the soil (Jeffery et al., 2010), a complex and heterogeneous environment characterized by the absence of light, a water-saturated atmosphere and buffered temperature fluctuations (Eisenbeis and Wichard, 1987). Species inhabiting this environment (euedaphics *sensu* Eisenbeis and Wichard, 1987;

endogean *sensu* Giachino and Vailati, 2010) show varying degrees of adaptation to the conditions underground, with a general trend to size reduction, depigmentation, shortening of extremities, loss of eyes and loss of flight capacity (Eisenbeis and Wichard, 1987; Gisin, 1943).

For many euedaphic arthropods, and particularly beetles, taxonomic studies have shown a clear pattern of high diversity and micro-endemism. Illustrative cases are found within the Leptotyphlinae (Staphylinidae; Fancello et al., 2009), Bothrideridae (Dajoz, 1977), Reicheini (Carabidae; Casale, 2009) and Anillini (Carabidae; Jeannel, 1963; Giachino and Vailati, 2011; Pérez-González and Zaballos, 2013; Sokolov and Kavanaugh, 2014; Giachino, 2015). Phylogenetic community analyses based on molecular data have also shown that the adaptation to the deep soil layers promotes community phylogenetic clustering and high levels of local endemism (Andújar et al., 2015). In other groups such as

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collembolans and annelids, thought to include few species with wider distributions, recent molecular work has also revealed high cryptic diversity and strong geographical structure (Cicconardi et al., 2013).

Despite this general trend to form geographically isolated entities, some euedaphic groups show transcontinental or even worldwide distributions when considering larger clades. This is the case of the endogean ground beetles of the tribe Anillini (subfamily Trechinae), which are distributed through all continents but Antarctica, including isolated islands such as Madagascar, New Zealand, New Caledonia, Hawaii, Galapagos, the Seychelles and some Mediterranean islands. In the last global taxonomic revision of Anillini, Jeannel (1963) divided the then 139 known species in two main “divisions” (Phanerodontes and Aphaenodontes) and 11 morphological lineages, a classification subsequently modified by Jeanne (1973) and extended to 12 lineages by Vigna-Taglianti (1973). Currently, approximately 520 species of Anillini are known (JPZ, unpublished data). All known Anillini are flightless, and only four species retain eyes, of which two belong to the genus *Nesamblyops* from New Zealand; the other two are *Microdipnodes tshuapanus* Basilewsky 1960 and *Cryptorites scotti* Jeannel 1950, both from central Africa and placed by Jeannel in the large lineage of *Microtyphlus*, distributed over most of Africa, Europe and Australia.

How the Anillini have reached their current distribution is intriguing. For other taxa with subterranean species and similar continent-wide distributions, such as the Molopini ground beetles (Casale and Ribera, 2008) or the Leptodirini cave beetles (Fresneda et al., 2011), it has been shown that the traditionally recognized taxa were polyphyletic due to the inclusion of unrelated lineages exhibiting similar morphologies resulting from convergence. In the case of Anillini, Jeannel (1937, 1963) proposed the monophyly of the group and a Gondwanian origin, a proposal that has been generally accepted by other authors, although sometimes questioned (e.g. Erwin, 1982). Based on this hypothesis, the Anillini would have diversified following tectonic vicariance after the breakup of Gondwana, with subsequent dispersion only possible through temporary land bridges from sea level changes and tectonic movements or by overseas dispersion among continents and islands.

We conducted a molecular phylogenetic analysis of Anillini including 9 of the 12 main lineages proposed by Jeannel (1963) and Vigna-Taglianti (1973), and representatives of the American, Palaearctic, African, New Zealand and New Caledonian fauna, to test whether (i) the tribe Anillini as currently understood is monophyletic; (ii) the main lineages proposed by Jeannel form monophyletic entities; (iii) the estimated ages of divergence are compatible with a Gondwanian origin of Anillini and the vicariant diversification after the breakup of Gondwana; and (iv) the Mediterranean taxa and the species from New Zealand and New Caledonia show divergence times compatible with the tectonic scenario. Studying the phylogeny, biogeography and lineage ages in Anillini will provide a better understanding of the systematics and evolution of the group, and ultimately on the processes driving the high biodiversity of euedaphic mesofauna.

2. Material and methods

2.1. Sampling

We included sequences from 66 specimens of 50 species, covering 17 of the 68 described genera and nine of the 12 main lineages of Anillini (Jeannel, 1963; Vigna-Taglianti, 1973). These taxa include several representatives within each of the main groups of Anillini proposed by Jeannel (1937) (Scotodipnines and Anillines),

Jeannel (1963) (Phanerodontes and Aphaenodontes) and subtribes proposed by Jeanne (1973) (Typhlocharina, Scotodipnina and Anillina). Most genera (11) are from the west Palearctic region (from the Iberian Peninsula and Morocco to Turkey), with further exemplars from USA, Mexico, Tanzania, New Caledonia and New Zealand. We newly sequenced 54 specimens from 14 genera, whereas sequences for the remaining taxa were retrieved from Genbank, including *Nesamblyops* from New Zealand. Thirty-eight additional taxa were included as outgroups, including Bembidini, Tachyini, Trechini, Zolini, Xystosomina and Pogonini, which represent major lineages within the subfamily Trechinae that includes Anillini. *Ildobates neboti* Español, 1966 (Carabidae, Harpalinae, Zuphiini) was used to root the tree (Ribera et al., 2006; Hunt et al., 2007). Specimens were generally collected from soil samples using extraction methods (Berlese, 1905; Normand, 1909) or sifting forest litter and directly placed in absolute ethanol, either by the authors or provided by collaborators (see Tables S1 and S2 in Appendix S1 for details).

2.2. DNA extraction and sequencing

DNA was extracted non-destructively from whole specimens using Qiagen extraction kits (Hilden, Germany). Voucher specimens and DNA aliquots are kept in the IBE (CSIC-UPF, Barcelona) and NHM (London). Four DNA fragments were PCR amplified and sequenced, including: (i) the 3' end of the *cox1* gene (≈ 753 bp); (ii) the 3' end of the *rnl* gene plus the complete *trnL* and the 5' end of *nad1* (≈ 779 bp); (iii) a fragment of the *SSU* nuclear ribosomal gene (≈ 630 bp); and (iv) a fragment of the *LSU* nuclear ribosomal gene (≈ 1000 bp). PCRs were made using PuReTaq Ready-To-Go PCR beads (GE Healthcare, UK) or Biotaq Polymerase (Bioline, London, UK), with 39 cycles using 48–52 °C as the annealing temperature. The primers used for each gene fragment are given in Table S3 in Appendix S1. Additional sequences for the above markers and for two additional gene fragments were retrieved from Genbank, including: (v) the 3' end of *cox1*, *trnL* and the initial portion of *cox2* (≈ 641 bp); and (vi) the nuclear internal transcriber spacer 2 (*ITS2*) (≈ 448 bp). Altogether, 203 new sequences were generated (194 for the ingroup) and 109 were obtained from public repositories (10 for the ingroup) (Andújar et al., 2011; Contreras-Díaz et al., 2007; Faille et al., 2010, 2013, 2012, 2011; Maddison and Ober, 2011; Maddison et al., 1999; Maddison, 2012; Ribera et al., 2006; Sokolov, 2007). Sequence accession numbers are provided in Table S1 in Appendix S1.

2.3. Alignment and dataset concatenation

Sequences were aligned using the online version of MAFFT 6.240 (Katoh and Toh, 2008a; Katoh et al., 2002), with the L-INS-i algorithm for the protein coding genes and Q-INS-i for ribosomal fragments (Katoh and Toh, 2008b). The correct translation to amino acids was checked in MEGA 6 (Tamura et al., 2013). Two datasets were generated: (a) the *all taxa* data set included all ingroup (Anillini) and outgroup (Trechinae + *Ildobates*) taxa for the six gene fragments (105 specimens, 4570 nt); and (b) the *ingroup* dataset included *cox1*, *rnl*, *SSU* and *LSU* sequences for only the ingroup taxa (66 specimens, 3210 nt). The *all taxa* data set included some combined conspecific sequences from different studies (Table S1 in Appendix S1). The gene fragments *cox1-trnL-cox2* and *ITS2* were only sampled for the outgroup taxa of the *all taxa* data set.

2.4. Phylogenetic analyses

All phylogenetic and calibration analyses were conducted using the CIPRES Science Gateway (Miller et al., 2010). Data matrices

were analyzed with maximum likelihood (ML) and Bayesian inference (BI) phylogenetic methods. ML trees were obtained using RAxML 7.2.7 (Stamatakis, 2006). Data sets were partitioned by gene and the fragment for the protein-coding gene *cox1* was additionally partitioned into two codon partitions, combining first and second codon positions (Andújar et al., 2012). An independent GTR + G model was applied to each data partition. For the analyses of the *ingroup* data set we rooted the trees according to the results of the *all taxa* dataset. The best scoring ML tree was selected among 100 searches on the original alignment with different randomized parsimony starting trees. Support values were obtained with 1000 bootstrap replicates (Felsenstein, 1985). BI was run in MrBayes 3.2.3 (Huelsenbeck et al., 2001). Combined data were partitioned by gene and codon position as before, and for each partition the optimal substitution model was selected using the Akaike information criterion (AIC) in jModelTest 2.1.7 (Darriba et al., 2012). BI consisted of two independent runs, each with three hot and one cold chain, for 20 million generations, whereby trees were sampled every 1000 generations. The standard deviation of split frequencies was checked to assess the convergence of results, as well as the mean and effective sampled size (ESS) of likelihood values computed with TRACER 1.6 (Rambaut et al., 2013). The 50% majority rule and strict consensus trees were calculated excluding 25% of the initial trees as a conservative burn-in, ensuring that the plateau in tree likelihood values had been reached. Trees were visualized using FigTree 1.4.2 (Rambaut, 2012), and node posterior probabilities were interpreted as support values.

2.5. Calibration analyses

We used Bayesian phylogenetic analyses in BEAST 1.8 (Drummond et al., 2012) to simultaneously estimate an ultrametric phylogenetic tree and ages of diversification. Two independent calibration schemes were used, based on rates of evolution for the genus *Carabus* (*calibration scheme 1*; applied to both the *all taxa* and the *ingroup* data sets) and fossil and geological information (*calibration scheme 2*; applied only to the *all taxa* data set).

Calibration scheme 1. We used as calibration priors the rates of molecular evolution obtained for the same DNA fragments (*cox1*, *rrnL+trnL+nad1* and *LSU* genes) and analytical settings for the genus *Carabus*, which is the closest con-familial relative in the Carabidae with evolutionary rate estimates available for the studied genes (Andújar et al., 2014, 2012). A uniform function prior on the mean substitution rate was applied for each gene, with maximum and minimum bounds matching the 95% confidence interval of the rates obtained in Andújar et al. (2012).

Calibration scheme 2. We applied priors on the ages for three nodes: (i) the age of the origin of the genus *Bembidion* was restricted to a minimum age of 56.7 Ma (Uniform function: max 300, min 55.8) based on the fossil of †*Bembidium cyaneomicans* Piton, 1940 collected in Menat (Auvergne, France) and dated from the Thanetian (58.7–55.8 Ma) (Behrensmeyer and Turner, 2013; Piton, 1940); (ii) the age of the node clustering the endemic species of *Trechus* from the volcanic island of Madeira (represented by *T. custos* Wollaston, 1854; *T. flavomarginatus* Wollaston, 1854 and *T. decolor* Jeannel, 1938) was restricted to a maximum age of 14 Ma (Uniform function: max 14, min 1) based on the age of emergence of the island (Contreras-Díaz et al., 2007); and similarly, the emergence of the island of La Gomera around 9.5 Ma ago was used as a maximum age for the colonization of the *Trechus* species from La Gomera (*T. gomerensis* Franz, 1986) and from the younger region of Anaga in Tenerife (*T. cabrerai* Jeannel, 1936); Uniform function: max 9.5, min 1) (Contreras-Díaz et al., 2007).

For each dataset and calibration scheme, analyses were conducted applying the best fitting model of substitution to each gene and codon position partition (*cox1* fragment; combined first and

second codon positions; Andújar et al., 2012). A Yule speciation prior was applied and analyses were run for 30 million generations sampling one tree every 5000 generations. In the molecular clock settings only gene partitions were considered, and three independent analyses were conducted under three alternative molecular clock hypotheses, applying (1) an uncorrelated lognormal (ULN) or (2) a strict clock (SC) to all genes, or alternatively (3) using an ULN for nuclear and a SC for mitochondrial genes. For each of these, two independent runs in BEAST were conducted. Alternative molecular clock settings were compared with Bayes factors as estimated with the stepping-stone and the path-sampling algorithms in BEAST (Baele et al., 2013) and with the SHM estimator in TRACER 1.6. Consensus trees were estimated with TreeAnnotator (Drummond et al., 2012) discarding the 50% initial trees as a burn-in fraction, after checking ESS of likelihood, evolutionary rates and root age values, and ensuring that the tree likelihood values had reached a plateau. Posterior probabilities were considered as a measure of node support.

For the *all taxa* data set under the *calibration scheme 1*, *Ildobates neboti* was used to root the tree, whereas for the analyses on the *calibration scheme 2*, due to convergence problems a starting tree was used as a fixed topology only allowing for the estimation of branch lengths (by removing the operators *arrowExchange*, *wideExchange*, *wilsonBalding*, *subtreeSlide*). The consensus tree obtained from the analysis under the *calibration scheme 1* showing the best Bayes Factor value was used as the fixed topology. All calibration analyses on the *all taxa* data set (for both calibration schemes) were duplicated with and without an additional minimum age prior of 136.4 Ma on the root, based on the oldest known fossil for the subfamily Harpalinae (included as outgroup) (Behrensmeyer and Turner, 2013; Fujiyama, 1978). For the *ingroup* data set trees were rooted according to the results of the *all taxa* dataset. No other topological constraints were applied.

3. Results

The best fitting models of evolution as estimated in jModeltest for each gene fragment and data set alignment (*all taxa* data set: 105 taxa, 4570 bp; *ingroup* data set: 66 taxa, 3210 bp) are shown in Table 1. Trees obtained for the different datasets and phylogenetic methods were highly congruent, and the incomplete sampling for the *cox1-trnL-cox2* and *ITS2* gene fragments did not produce any obvious artefacts in the tree searches or the topologies obtained. For the *all taxa* dataset, all ML and Bayesian analyses with both MrBayes and BEAST consistently resulted in a polyphyletic Bembidiini due to the distant position of the genera *Sinechostichus* and *Erwiniana*, and well supported monophyletic Trechini, Zolini, Tachyini and Anillini, but with very weak support for the relationships among them and with the single representatives of Xystosomini and Pogonini (Figs. 1 and 2).

All analyses conducted on the *all taxa* dataset resulted in a monophyletic Anillini, with *Nesamblyops* (clade P1) as sister to

Table 1

Best fitting model of nucleotide change as estimated in JModeltest using the AIC criterion for the *all taxa* and the *ingroup* datasets.

	<i>All taxa</i> dataset	<i>Ingroup</i> dataset
<i>cox1</i>	GTR + I + G	GTR + I + G
<i>cox1</i> (1st + 2nd positions)	GTR + I + G	GTR + I + G
<i>cox1</i> (3rd positions)	GTR + G	GTR + G
<i>rrnL + trnL + nad1</i>	GTR + I + G	GTR + G
<i>LSU</i>	GTR + I + G	GTR + I + G
<i>SSU</i>	SYM + I + G	GTR + I + G
<i>cox1 + trnL + cox2</i>	GTR + I + G	n.a.
<i>ITS2</i>	HKY + G	n.a.

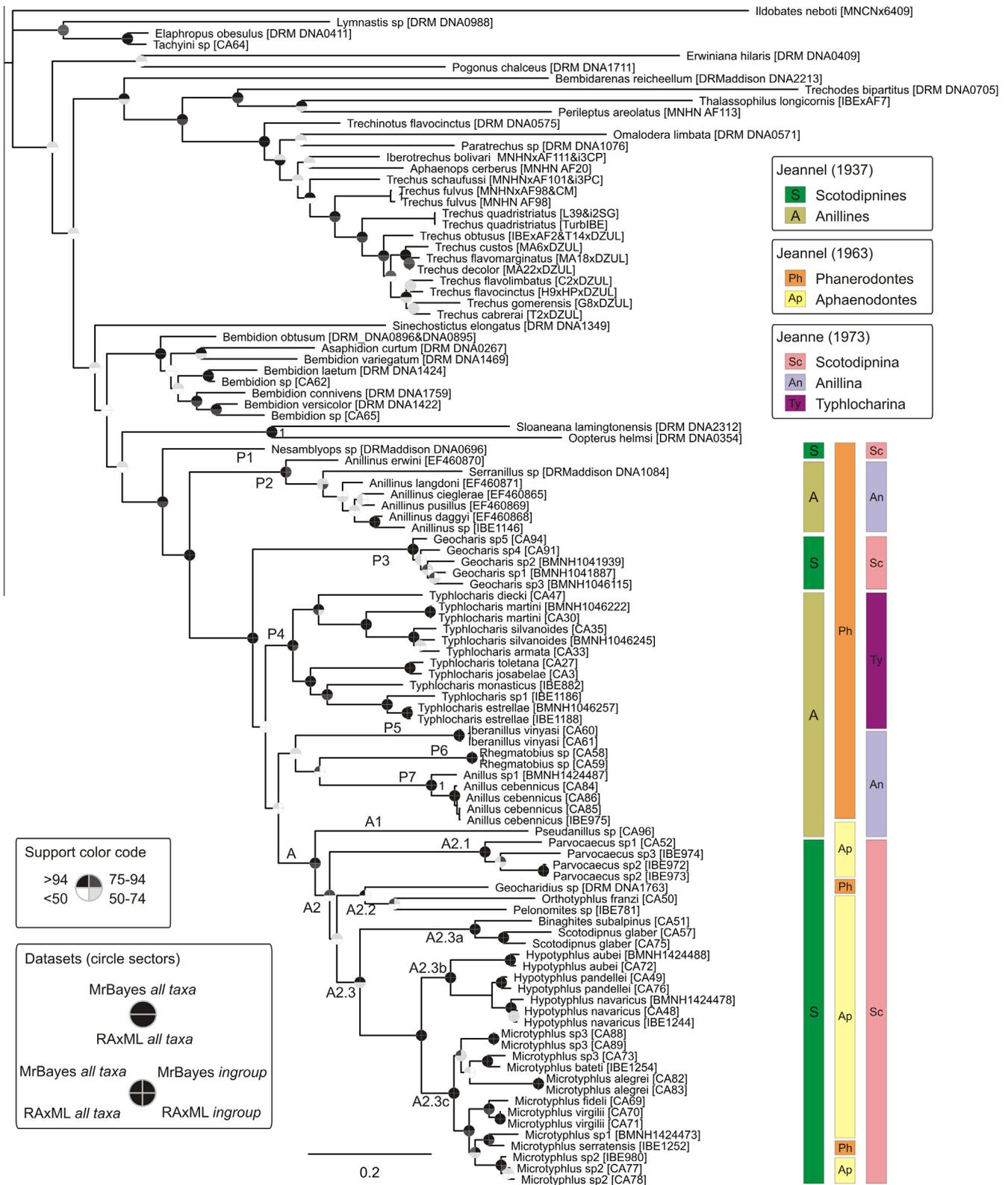


Fig. 1. Bayesian 50% majority rule consensus tree from the *all taxa* dataset including Anillini and other Trechinae lineages as obtained in MrBayes. Solid circles in nodes represent posterior probabilities and bootstrap values for Bayesian and maximum-likelihood analyses for both *all taxa* and the *ingroup* datasets (see legend in figure). Text labels on nodes indicate main lineages as referred in the text. Right bars represent classification into Anillines/Scotodipnines (Jeannel, 1937), Phanerodontes/Aphaenodontes (Jeannel, 1963) and Anillina/Scotodipnina/Typhlocharina (Jeanne, 1973) as defined by the labial tooth and the umbilicate series. Voucher numbers are indicated in brackets.

the remaining taxa. *Nesamblyops* was consequently used to root the trees of Anillini in all subsequent analyses without outgroups (*ingroup* data set). We also obtained highly consistent results for

the internal relationships of Anillini between both datasets (Figs. 1 and 2; Figs. S1–S6 in Appendix S1). The North American *Anillinus* + *Serranillus* were found as a well supported clade (P2) as sister

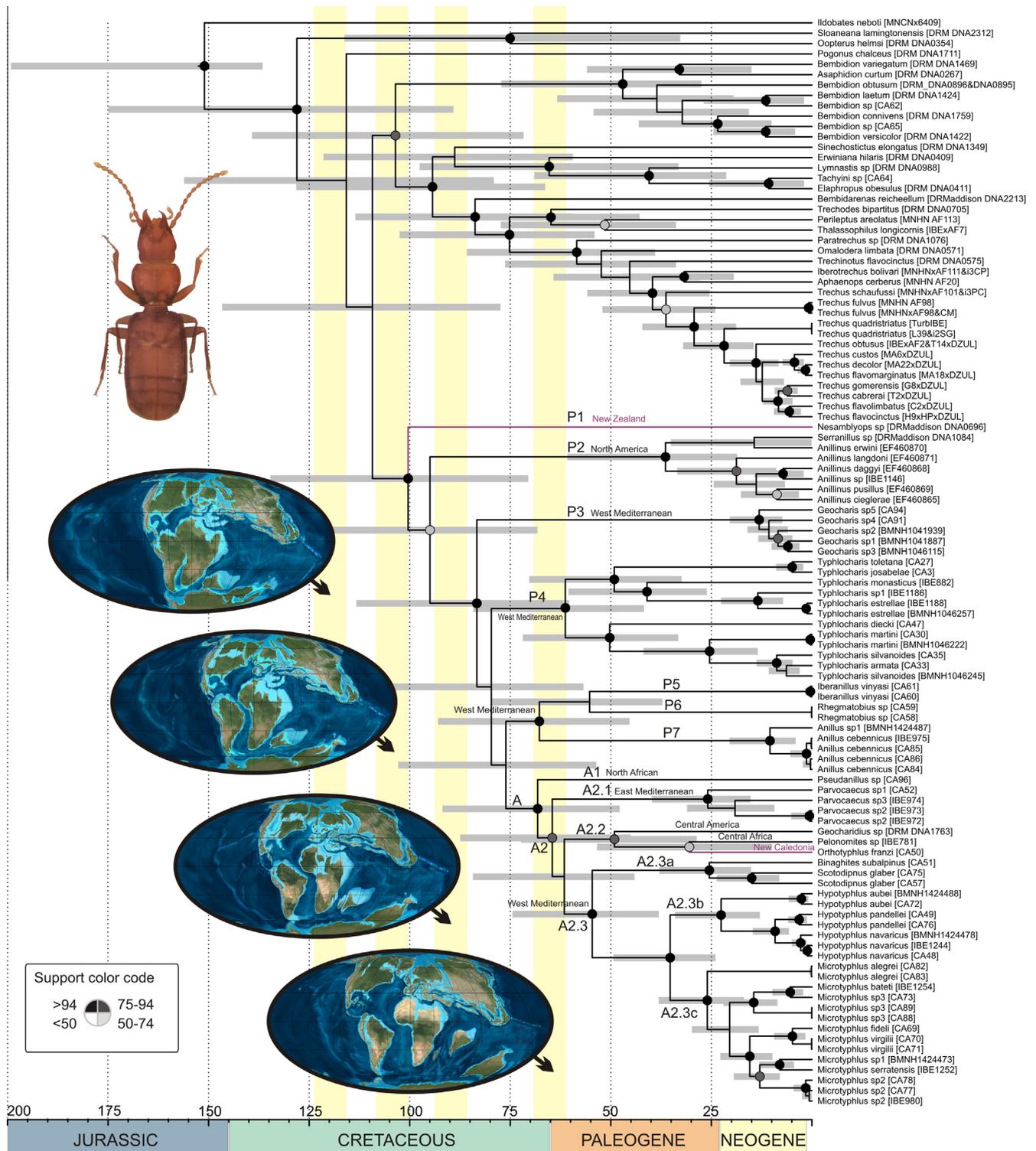


Fig. 2. Ultrametric time-calibrated tree from the *all taxa* dataset including Anillini and other Trechinae lineages as obtained in BEAST for the Calibration scheme 2 (node age calibration priors on *Bembidion* and *Trechus* lineages) together with the additional prior on the root age (split between Trechinae and Harpalinae; see text for details). Solid circles in nodes represent posterior probabilities. Text labels on nodes indicate main lineages as defined in the text. The beetle image corresponds to *Geocharis* sp. voucher number BMNH1041887. Palaeogeographic maps for 120 Ma, 105 Ma, 90 Ma and 65 Ma have been obtained from ©Ron Blakey, Colorado Plateau Geosystems. Voucher numbers are indicated in brackets.

to the remaining Anillini. Within the latter, we found six well supported clades (or single-species lineages) with poorly resolved relationships among them, corresponding to (i) *Geocharis* (P3); (ii) *Typhlocharis* (P4); (iii) *Iberanillus* (P5); (iv) *Rhegmotobius* (P6); (v) *Anillius* (P7); and (vi) a large clade (Clade A) including all 'aphaenodontes' (Jeannel, 1963) and *Geocharidius*. Lineages P5, P6

and P7 were recovered as a clade independently of the phylogenetic method and dataset, but always with weak support. Within clade A we found *Pseudanillus* (A1) as sister to the remaining taxa (Clade A2). Within clade A2, we found (i) a clade with *Parvocaeus* (A2.1); (ii) a clade clustering *Geocharidius*, *Orthotyphlus* and *Pelonomites* (A2.2), only supported in the *all taxa* dataset and the Bayesian

inferences; and (iii) a supported clade (A2.3) including a monophyletic *Scotodipnus* + *Binaghites* (clade A2.3a), *Hypotyphlus* (A2.3b), and *Microtyphlus* (A2.3c) (Fig. 1).

Results from the 15 alternative calibration analyses (12 for the *all taxa* and 3 for the *ingroup* dataset) are summarized in Figs. 3 and 4 and Tables 2, S4 and S5 in Appendix S1. For the different calibration schemes and datasets, the ULN clock was always favoured, but representing only a minor improvement in the BF value over the combined application of SC and ULN to the mitochondrial and nuclear genes respectively (SC-ULN clock) (Table 2). The use of the SC for all genes always resulted in a major decrease of the BF value, accompanied by major differences in the estimates of both ages of diversification and rates of molecular evolution (Table 2). BF results were consistent among the estimation methods used (Stepping Stone, Path Sampling and Marginal Likelihood; Table S4 in Appendix S1).

For the *all taxa* dataset under the favoured ULN clock, both calibration schemes resulted in highly congruent results when the age of the root was constrained. Based on the calibration *scheme 2*, the time for the common ancestor of Anillini was estimated at 100.4 Ma (95% HPD: 70.4–134.5 Ma), representing the split between the fully eyed *Nesamblyops* from New Zealand and the remaining Anillini. The subsequent diversification into the main lineages P2, P3, P4, (P5 + P6 + P7) and A was dated to between 77.7 and 96.9 Ma (95% HPD: 53.5–129.8 Ma), and the split of *Pseudanillus* (A1) from the remaining 'aphaenodontes' + *Geocharidius* (A2) was dated at 69.6 Ma (95% HPD: 47.7–91.7 Ma). The split of the New Caledonian *Orthotyphlus* was dated at 30.5 Ma (95% HPD: 9.9–53.3 Ma). Rates of molecular evolution estimated with the calibration *scheme 2* for the genes *rrnL*, *ITS2* and *LSU* were very

similar to those previously reported for the genus *Carabus* (Andújar et al., 2012) and used here as priors in the calibration *scheme 1* (Table 3). On the other hand, the rate estimated for the gene *cox1* was higher than that reported in some recent studies (Andújar et al., 2012; Papadopoulou et al., 2010; see Section 4).

Ages of diversification and rates of molecular evolution obtained for the ULN and the SC-ULN clocks were similar (when other parameters were the same), with a large overlap on their distributions but higher variances for the ULN clock (Figs. 3 and 4). The alternative calibration schemes had an important effect on the results when the age of the root was not constrained, resulting in younger ages and higher evolutionary rates for *scheme 2* (internal calibration points based on fossil and biogeographic information) than for *scheme 1* (rates extrapolated from the ground beetle genus *Carabus*). Hence, the additional constraint of the root age based on the fossil information had the main effect of increasing the ages of diversification, lowering the rates of molecular evolution. This resulted in very similar ages and rates for both calibration schemes.

Calibration analyses conducted on the *ingroup* dataset estimated older ages for the diversification of the Anillini (Fig. 3). Nevertheless, the notable increment of the variance resulted in a large overlap with the ages obtained for the *all taxa* dataset.

4. Discussion

4.1. Are Anillini monophyletic?

From our results we can clearly conclude that Anillini is a monophyletic lineage with a trans-continental distribution of

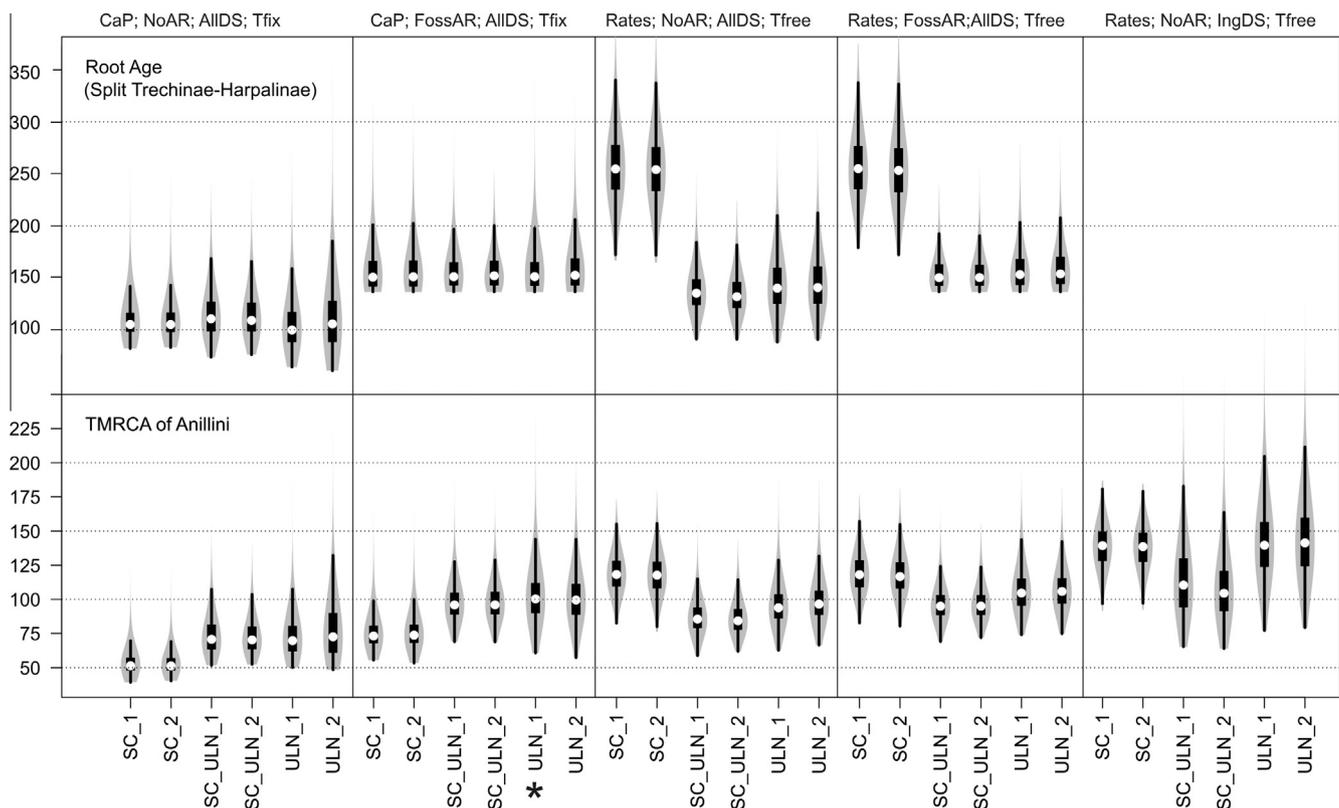


Fig. 3. Violin plots (Hintze and Nelson, 1998) obtained in R with the library *vioplot* representing the mean (white dot), standard deviation (sd; black bar), 2ndsd (black line) and the density distribution (gray shape) for the root age (split between Trechinae and Harpalinae) and for the time of the most recent ancestor (TMRCA) of Anillini as obtained in the calibration analyses conducted with BEAST after discarding the burn-in fraction. The vertical axes represent million years before present. The calibration analysis represented in Fig. 2 is marked with an asterisk. SC: Strict molecular clock applied to all genes; ULN: Uncorrelated log-normal clock applied to all genes; SC_ULN: Strict clock applied to mitochondrial genes uncorrelated log-normal to nuclear genes; CaP: Age calibration priors as in calibration *scheme 2*; RaC: Rate calibration priors as in calibration *scheme 1*; NoAR: Root age prior not included; FossAR: Fossil root age priors included; AllDS: *All taxa* data set; IngDS: *ingroup* data set; Tfix: Tree topology constrained; Tfree: Tree topology non-constrained.

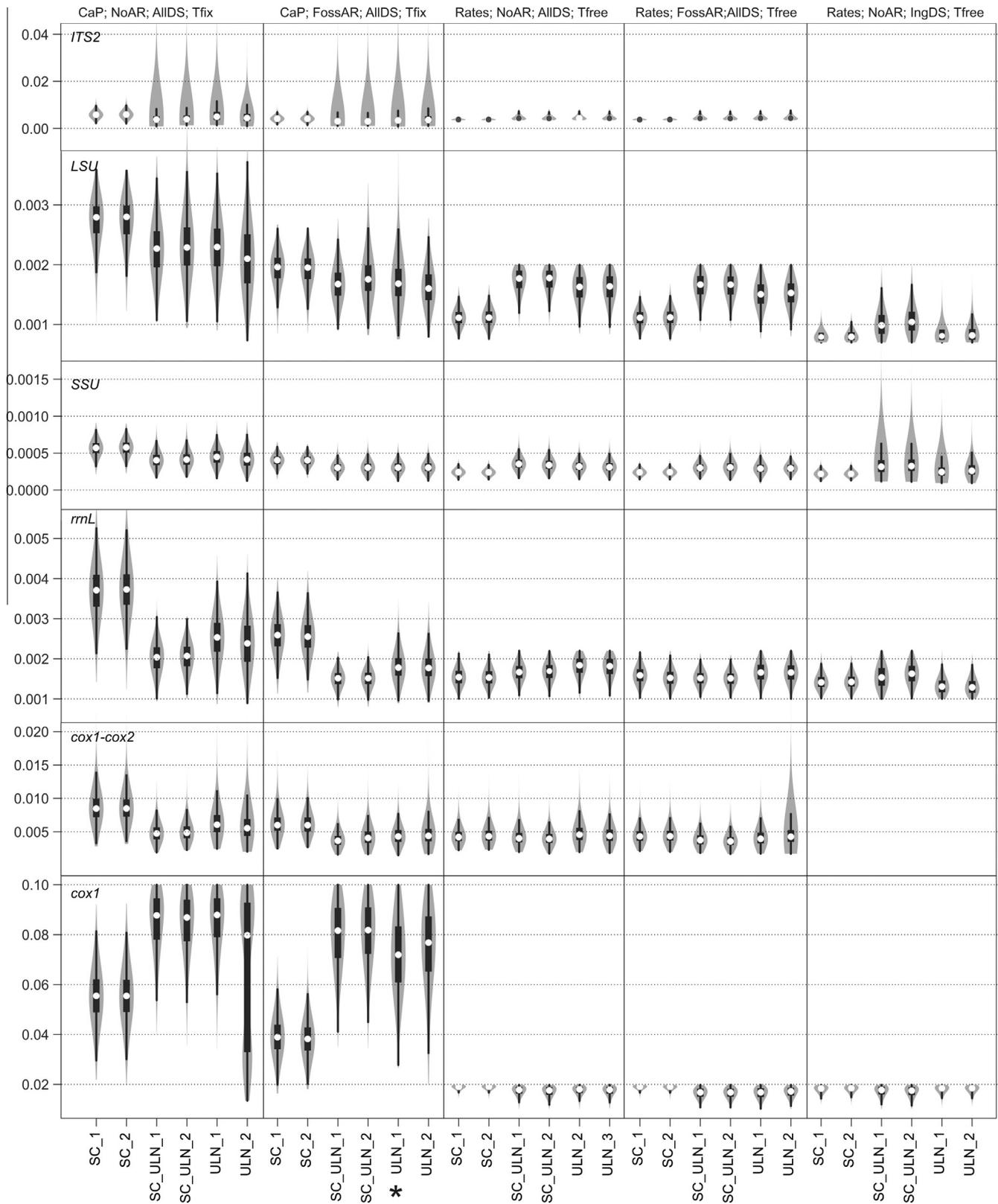


Fig. 4. Violin plots for the rates of molecular evolution of *cox1*, *cox1-cox2*, *rml*, *SSU*, *LSU* and *ITS2* gene fragments as obtained in the calibration analyses with BEAST. The vertical axes represent substitutions per site per million years per lineage (subs/s/Ma/l). The calibration analysis represented in Fig. 2 is marked with an asterisk. Details on graph and acronyms as in Fig. 3.

mostly endogean species. This is in agreement with the current treatment of the group and in contrast with other formerly recognized widespread taxa including subterranean lineages shown to be polyphyletic by recent molecular data, e.g. beetles in the tribe

Molopini (Casale and Ribera, 2008) and Leptodirini (Fresneda et al., 2011).

The monophyly of the tribe Anillini was first proposed in the early studies of Jeannel (1937, 1963), and although since then it

Table 2

Bayes factor values obtained in BEAST using the Step-Stone sampling method for the 30 calibration analyses conducted.

	SC	SC	SC_ULN	SC_ULN	ULN	ULN
CaP;NoAR; AllDS;Tfix	−40377.81	−40378.04	−39986.90	−39970.94	−39922.88	−39911.67
CaP;FossAR; AllDS;Tfix	−40381.53	−39985.23	−39974.80	−39985.29	−39917.30	−39928.02
RaC;NoAR; AllDS;Tfree	−40750.54	−40760.92	−40434.48	−40425.77	−40392.50	−40381.53
RaC;FossAR; AllDS;Tfree	−40725.29	−40729.63	−40422.55	−40425.72	−40356.27	−40363.04
RaC;NoAR;IngDS;Tfree	−23651.03	−23660.36	−23533.38	−23526.92	−23488.33	−23496.57

CaP: age calibration priors (Calibration scheme 2); RaC: rates of *Carabus* priors (Calibration scheme 1); NoAR: no root age prior; FossAR: Fossil root age priors; AllDS: All taxa data set; IngDS: *Ingroup* data set; Tfix: tree topology fixed; Tfree: tree topology free.

Table 3Rates of molecular evolution for Trechinae and other coleopteran lineages for the genes *cox1*, *rrnL*, *ITS2* and *LSU*. Values correspond to substitutions per site per million years per lineage (subs/s/Ma/l) for the mean and 95% HPD interval as obtained in BEAST.

	Trechinae ^a	<i>Carabus</i> (Andújar et al., 2012)	Tenebrionidae (Papadopoulou et al., 2010)	Leptodirini (Ribera et al., 2010)
<i>rrnL</i>	0.00178 (0.00117–0.00248)	0.0016 (0.001–0.0022)	0.0054 (0.0036–0.0072) ^b	
<i>cox1</i>	0.0719 (0.0472–0.0999) 0.0126 (0.0074–0.0177) ^c	0.0145 (0.01–0.0198)	0.0177 (0.0139–0.0215) ^b	0.0204 (0.0151–0.0260)
<i>ITS2</i>	0.00338 (0.00122–0.00866)	0.0057 (0.0035–0.0081)		
<i>LSU</i>	0.00168 (0.00111–0.00242)	0.0013 (0.0007–0.002)		

^a Here obtained with the Calibration Scheme 2, root constraint and ULN clock model.

^b Interval represents 2*standard deviation.

^c Rate obtained with no codon position partitioning for the *cox1* gene. See Section 4.

has been rarely questioned, Erwin (1982) suggested that Anillini were most likely a grade within a related tribe (Tachyiini), related to *Lymnastis*, *Paratachys* and other genera representing putatively early splits in the evolution of the lineage. Anillini are minute, depigmented, apterous and almost always eyeless ground beetles, usually separated from other closely related groups by characters which are not exclusive, such as the narrow and minute terminal segment of the palpi and the lack of recurrent striola at the apex of the elytra (Jeannel, 1963). These characters are considered to be prone to convergent evolution correlated with adaptation to the deep soil layers or a reduction in body size. The anophthalmia, depigmentation and lack of wings are equally found in organisms living in subterranean environments, or even in forest litter, and many other lineages show these traits among the Trechinae (Faille et al., 2013).

The only diagnostic morphological character of Anillini, the widely opened and straight shape of the basal bulb of the aedeagus, has been recently found also in the tribe Lovriciini (Giachino et al., 2011). This shared character has led to the recent proposal of a sister relationship of Anillini and Lovriciini, a hypothesis we could not test here. Our results reject other taxa traditionally considered as potential sister groups to Anillini, including Lymnastini (proposed by Jeannel, 1963) or *Paratachys* (Erwin, 1982), corroborating previous conclusions based on larval morphology and molecular data (Grebennikov and Maddison, 2005; Maddison and Ober, 2011). Nevertheless, our phylogenetic analysis does not establish the sister taxon to Anillini unambiguously, as relationships between the main lineages of Trechinae were not strongly supported and not all relevant taxa were sampled (e.g. Lovriciini), which leaves some uncertainty about the origin and age of the lineage.

The internal phylogeny of Anillini according to our results is characterized by several ancient lineages and a general structure mostly concordant with an arrangement in “Phyletic Series” proposed by Jeannel (1963), although the main characters he proposed (presence/absence of a labial tooth or the pattern on the elytral umbilicate series) cannot be reliably used for a systematic subdivision of the group into monophyletic entities. The first five splits in the phylogeny (clades P1, P2, P3, P4, and (P5 + P6 + P7)) include only Jeannel’s ‘Phanerodontes’ (species with a labial tooth), whereas the derived clade A includes all of the ‘Aphaenodontes’ together with two additional taxa with labial tooth. The presence

of the labial tooth could thus be considered as a plesiomorphic character state, found also in most other Trechinae and retained in the early lineages within Anillini. The loss of the labial tooth is a derived character showing two reversals to the ancestral character state among the sampled taxa: one in the genus *Geocharidius* and another in some species of the genus *Microtyphlus*, in both cases explaining the misplacement of these taxa in the classification of Jeannel (1963). In the case of *Geocharidius*, Vigna-Taglianti (1973) proposed this genus as an independent phyletic series, distinct from that of *Geocharis* where it was placed by Jeannel (1963). Similarly, the division within the genus *Microtyphlus* proposed by Jeannel (1963) according to the presence/absence of labial tooth was not accepted by many authors (Jeanne, 1973; Español, 1974). Other cases of morphologically related taxa including species with and without labial tooth have been proposed in recent publications (Moore, 1980; Giachino, 2008; Giachino and Vailati, 2011; Pérez-González and Zaballos, 2013), demonstrating the lability of the character.

The morphology of the labial tooth of Anillini is more complex than initially considered, and we have identified four character states: (i) fully developed tooth; (ii) total absence of the tooth, (iii) tooth with different degree of development within a genus, or even within a single species; (iv) presence of a hyaline tooth, not fully visible with light microscopy. The latter situation could be problematic, since species reported as “toothless” may indeed have a hyaline labial tooth (Zaballos and Casale, 1997).

Some of the main lineages recovered are in good agreement with Jeannel’s Series, such as clades P1 (Series ‘*Zeanillus*’), P2 (Series ‘*Anillinus*’) or P4 (Series ‘*Typhlocharis*’), but with some exceptions in the monophyly of Series ‘*Geocharis*’ (clade P3) and ‘*Microtyphlus*’ (nested within clade A). The sequencing of additional genera within the ‘Aphaenodontes’ will be key to further test the validity of these clades. Of particular interest are the oculated species from central Africa, which Jeannel (1963) placed without hesitation in the Series ‘*Microtyphlus*’.

4.2. Origin and diversification of Anillini: Gondwanian relicts or oceanic dispersal?

Our estimated age for the common ancestor of Anillini (around 103 Ma, in the middle Cretaceous) is early enough for a

Gondwanian origin, as originally proposed by Jeannel (1963). The assumption of the monophyly of Anillini, together with the conviction that the current species could not disperse over sea barriers, led this author to propose the ancient origin of the group. He also hypothesized that an humiculous, fully eyed and winged ancestor would have allowed the initial dispersion of the lineage, leading to subsequent local diversifications and multiple independent colonisations of the endogean environment. Erwin (1982) agreed in the need of a winged ancestor, but disagreed about the monophyly and age of the Anillini. The requirement of ancient ages for the origin of a monophyletic Anillini was one of the arguments for his proposal of their para- or polyphyly. Our results are suggestive of an ancient origin and a complex evolutionary history, involving both vicariance facilitated by plate tectonics and long distance colonization events.

The basal split separated the endemic New Zealand genus *Nesamblyops* from the rest of the Anillini. Jeannel (1963) placed *Nesamblyops* into Series *Zeanillus*, which included only New Zealand representatives. *Nesamblyops* is one of the few Anillini with eyes, consistent with an early split of the lineage retaining the plesiomorphic state. The estimated age of 103 Ma (range 135–70 Ma) is consistent with a Gondwanian origin and the separation of New Zealand from the continent during the late Cretaceous, starting at around 83 Ma with the formation of the Tasman Sea (Neall and Trewick, 2008). Other non related genus within Anillini, *Pelodiaetodes* Moore, 1980 has also been proposed as a Gondwanian relict based on its morphological affinities to other genera from Australia and Madagascar (Sokolov, 2015). There has been much dispute about the origin of the New Zealand biota and the relative roles of vicariance (Craw et al., 1999) and long distance dispersal (Landis et al., 2008; Pole, 1994). The alleged total or partial submersion of the island during the Eocene (Waters and Craw, 2006) would imply a recent origin of the biota through overseas dispersal, a hypothesis corroborated with molecular data in some groups (e.g. Hurr et al., 1999; Winkworth et al., 2002; De Queiroz, 2005) including Coleoptera (Pons et al., 2011). However, recent molecular studies have also provided well documented cases of ancient vicariance (Carr et al., 2015; Giribet and Boyer, 2010; Murielle et al., 2014). Overall, data support a dual scenario, as initially proposed by Fleming (1962), with both vicariance and long distance dispersal operating for different groups (Carr et al., 2015). The Anillini provide an additional example of an ancient lineage inhabiting New Zealand with a putative Gondwanian origin.

Other early splits in the phylogeny of Anillini are also compatible with a vicariant origin due to plate fragmentation. Thus, the North American *Anillinus* group was estimated to date from ca. 95 Ma (confidence interval: 68–130 Ma), compatible with the early Tertiary break-up of the north Atlantic (Ziegler, 1988). Subsequent splits occurred mostly within Palaeartic lineages, with some Mediterranean genera among the oldest lineages dating from 78 to 97 Ma. This includes the Sardinian *Rhegmatobius*, sister to the genus *Iberanillus* in the Mediterranean coast of the Iberian peninsula. Both the age of the separation (early Eocene) and the geographic distribution of the two genera are compatible with a vicariant split due to the tectonic separation of the tyrrhenian plates during the early Oligocene (Schettino and Turco, 2006), as has been reported for other groups of subterranean Coleoptera (e.g. Ribera et al., 2010).

On the contrary, the estimated phylogenetic position and age of the New Caledonian *Orthotyphlus* (ca. 35 Ma; confidence interval: 10–55 Ma) are incompatible with a vicariant origin resulting from the fragmentation of New Caledonia and the Australian plate ca. 83 Ma (Neall and Trewick, 2008). This result is in good agreement with molecular data suggesting a recent origin for the New Caledonian fauna and flora through long distance colonization (Cruaud

et al., 2012; Grandcolas et al., 2008; Papadopoulou et al., 2013), and also with geological evidence indicating that all of New Caledonia must have been submerged during part of the Eocene, with its final emersion at 37 Ma (Pelletier, 2007; Schellart et al., 2006). Indeed, *Orthotyphlus* was included in the derived clade A, together with *Pelonomites* and *Pseudanillus* from Central and North Africa respectively, *Geocharidius* from Central America and the Mediterranean representatives of the *Microtyphlus* Series. Even if relationships within this clade may be uncertain, the estimated age for the initial diversification of this group (clade A: ca. 69 Ma; confidence interval: 50–90 Ma) requires long range overseas dispersal of several taxa to explain its presence in South America, Africa, Eurasia and New Caledonia (and probably in Australia, of which no representative could be obtained).

Different mechanisms have been proposed for the long distance colonization of minute soil organisms, most prominently within soil among roots of rafting vegetation that drift with oceanic currents (Fraser et al., 2011; Holzapfel and Harrell, 1968; Nikula et al., 2013). Passive or active aerial dispersion seems a less likely option, although the possibility of winged species within the Anillini (or among the ancestral species) cannot be dismissed entirely. Two genera from central Africa (*Cryptorites* and *Microdipnodes*) that were unambiguously assigned to the *Microtyphlus* phyletic series by Jeannel (1963) maintain functional and even fully developed eyes, despite being wingless (no data is available about this character for *Microdipnodes*). The confirmation of the phylogenetic position of the eyed African genera within the *Microtyphlus* lineage would suggest a scenario where the evolutionary loss of eyes occurred multiple times independently as a result of the adaptation to life below ground. Molecular data on these taxa, together with further sampling of Southern hemisphere lineages including representatives of the Malagasian, Indian, South American and Australian faunas, will be key for further understanding the colonization routes of the Anillini.

4.3. The molecular clock in Anillini

Our conclusions are contingent on the estimations of the age of divergence of the main lineages of Anillini. There is an increased use of Bayesian dating methods that provide the opportunity to introduce priors on the ages of diversification of particular taxa and the rates of molecular evolution, and allow for the simultaneous optimization of the topology, the substitution rates and the time frame for the evolution of lineages (Drummond et al., 2006). This approach facilitates complex calibration analyses, but the results from the combination of different priors, and their effect on the topology and the estimated ages are not always well understood when using actual, rather than simulated data (Andújar et al., 2012; Duchêne et al., 2014; Ho and Duchêne, 2014). We have addressed these uncertainties by exploring the effect of different combinations of calibration priors (constraints in diversification ages vs. constraints in substitution rates) and molecular clock priors (strict vs. uncorrelated lognormal clocks). Our results were in general consistent with previous studies, showing a marked effect of the calibration schemes and priors in the final results. In spite of this variation, and with the exception of the analyses conducted with the unfavoured SC in the *calibration scheme 1*, all calibration analyses were compatible with a Gondwanian origin of Anillini. Similarly, calibration analyses resulted in estimates for the split of *Orthotyphlus* to be more recent than the initial tectonic separation of New Caledonia, with only the *ingroup* dataset resulting in confidence intervals old enough for a tectonic vicariant origin of *Orthotyphlus*. In general, we found a trend toward older age estimates when the rates of molecular evolution were constrained (using rates estimated in related groups of

Coleoptera), and younger age estimations when only recent calibration points were used. The need to include age priors for the root or some deep nodes to increase the accuracy of the calibration has already been suggested (Duchêne et al., 2014), probably to reduce the errors arising from the saturation of the model of nucleotide substitution. Our results here point in the same direction. The use of an additional age constraint to the root resulted in highly congruent results between analyses conducted with alternative calibration constraints (diversification ages vs. substitution rates), also increasing the consistency of the estimated rates of molecular evolution with that of other studies. Specifically, the calibration analyses based on age constraints (including that of the root) resulted in rates of molecular evolution consistent with those reported for the ground beetle genus *Carabus* for the same gene fragments (Andújar et al., 2012), and this calibration also was consistent with other previous studies in Coleoptera (Cieslak et al., 2014; Papadopoulou et al., 2010; Ribera et al., 2010). An exception was the rate estimate for the *cox1* gene when only prior constraints on the ages for the selected nodes (Calibration Scheme 2) were used, which was unrealistically high. As it has been shown in previous studies, Bayesian partitioned analyses may have difficulties in the branch length estimation of some variable genes (Brown et al., 2010; Marshall, 2010), and thus we interpret this *cox1* rate as potentially an analytical artefact resulting from the codon position partitioned model. Additional analyses where the *cox1* gene was not subjected to codon position partitioning resulted in a rate of 0.0126 substitutions per site per million years per lineage (subs/s/Ma/l), in good agreement with previous studies, including the rate we applied in the Calibration Scheme 1 (Table 3). Similar higher than expected rate values have been observed for the *nad5* gene in Andújar et al. (2012) and for the *cox1* gene in Pons et al. (2010). Despite these discrepancies, the accumulation of calibration analyses with congruent estimations using independent age priors for different lineages points to some generalities in the evolutionary rates of Coleoptera, even if a standard strict molecular clock can probably be discarded.

5. Author contributions

CA. designed the study. CA, AF, IR, JPZ, SPG contributed to the sampling. CA and AF conducted the laboratory work. CA, IR and APV designed the analyses. CA conducted the analyses. CA and IR drafted the manuscript, which was reviewed and approved by all authors.

Acknowledgments

We thank all collectors in Table S2 for providing material for the study, Rocío Alonso (IBE Barcelona) and Alex Aitken (NHM London) for their support in the molecular laboratory, and two anonymous Referees for valuable comments. This study was partially supported by the NERC grant NE/M021955.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2016.03.013>.

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