**PRIMARY RESEARCH ARTICLE** 

# Diversification in tropics and subtropics following the mid-Miocene climate change: A case study of the spider genus *Nesticella*

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# Abstract

Caves may offer suitable refugia for troglophilic invertebrates during periods of unfavourable climatic conditions because of their stable microclimates. As a consequence, allopatric divergence from their epigean counterparts may occur, leading to formation of truly hypogean communities (the Climatic Relict Hypothesis). Unlike the well-studied effects of Pleistocene glaciations, we know little about how ancient climate changes drove the development of cave-dwelling organisms living at both middle and lower latitudes. We investigate the evolutionary history of the troglophilic spider genus Nesticella (Araneae, Nesticidae) in relation to Asian Neogene (23– 2.6 Ma) climatic changes. Our analyses discern clear differences in the evolution of the two main clades of Nesticella, which occur in temperate/subtropical and tropical latitudes. Eastern Asian Nesticella gradually evolved greater sedentariness and a strict subterranean lifestyle starting from the middle Miocene Epoch ( $\sim$ 15–14 Ma) in conjunction with the progressive deterioration of the climate and vegetational shifts. Caves appear to have acted as refugia because of their internally uniform temperature and humidity, which allowed these spiders to survive increasing external seasonality and habitat loss. In contrast, a uniform accumulation of lineages, long-lasting times for dispersals and the lack of a comparable habitat shifting characterized the tropical lineage. This difference in pattern likely owes to the mild effects of climate change at low latitudes and the consequent lack of strong climatic drivers in tropical environments. Thus, the mid-Miocene climatic shift appears to be the major evolutionary force shaping the ecological differences between Asian troglophilic invertebrates and the driver of the permanent hypogean communities in middle latitudes.

#### KEYWORDS

Asia, caves, habitat shift, middle latitudes, Neogene, subterranean environment, troglophiles

# 1 | INTRODUCTION

Together with local geological events, climatic oscillations are an important evolutionary driver that deeply shapes the structure of biotas (Parmesan & Yohe, 2003). Global climate changes can result in declines of natural populations and the extinction of species that

cannot withstand the new conditions. By shifting to more favourable habitats or undergoing successful evolutionary adaptations, species can avoid extinction (Hoffmann & Sgro, 2011). Long-term climate changes can force surface invertebrates to colonize subterranean environments. This process promotes isolation when their surface counterparts disappear (Climatic Relict Hypothesis) (Botosaneanu &

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Holsinger, 1991; Holsinger, 2000). The development of external unfavourable conditions (e.g. habitat loss, increasing coldness or dryness) can render caves suitable refugia for troglophilic species capable of exploiting both hypogean and epigean habitats (Bryson, Prendini, Savary, & Pearman, 2014). In these shelters, troglophiles can survive periods of intense fluctuations because of the persistence of stable microclimates. Relatively recent climate drivers, mostly Pleistocene glaciations, are generally invoked to explain the origin and current distribution of cave-dwelling invertebrates in temperate latitudes (Allegrucci, Trucchi, & Sbordoni, 2011; Bryson et al., 2014; Holsinger, 1988; Niemiller et al., 2013; Peck, 1980). In contrast, the prominent role of older environmental changes has been more rarely pointed out (Faille, Andújar, Fadrigue, Ribera, & Rocha, 2014; Faille et al., 2010; Leys, Watts, Cooper, & Humphreys, 2003), and their potential effects on the development of the modern troglofauna in both middle and lower latitudes have been far less explored.

The Neogene (23-2.6 Ma) saw large-scale climate change involving significant environmental episodes and a general cooling of both ocean and land (Potter & Szatmari, 2009). Environmental changes at the regional scale in Asia are thought to be at least partially driven by the progressive collision between the Indian and Asian plates and the subsequent tectonic deformations associated with orogenesis of the Qinghai-Tibetan Plateau. Conceivably increases in elevation of the plateau since the late Eocene and other contemporary events such as the retreat of the Paratethys and global cooling, likely led to dramatic effects on the Asian monsoonal circulation by changing atmospheric flow (Chatterjee, Goswami, & Scotese, 2013; Clift et al., 2008; Zhang, Jiang, Zhang, & Yu, 2015; Zhang, Wang, Guo, & Jiang, 2006). The altered moisture patterns during the second half of the Miocene Epoch led to greater seasonality in East Asia (Tang et al., 2015), including the aridification of inland Asia (Miao, Herrmann, Wu, Yan, & Yang, 2012; Sun, Gong, Tian, Jia, & Windley, 2015; Zhuang, Hourigan, Koch, Ritts, & Kent-Corson, 2011) and the gradual creation of a shallow latitudinal temperature gradient and extensive changes in vegetation cover (Jacques, Shi, & Wang, 2013; Pound, Haywood, Salzmann, & Riding, 2012). Molecular evolutionary research supports the roles played by Neogene climatic changes and contemporaneous major geological events in shaping the evolutionary history of numerous organism communities in Asia (Che et al., 2010; Favre et al., 2015; Jiang, Deng, Li, & Xu, 2015; Wang, Schneider, Zhang, & Xiang, 2012). Most such studies have focused on epigean organisms and not speciation of the Asian hypogean fauna.

We explore the evolution of a group of ancient troglophilic organisms that require very specific conditions of temperature and humidity: the "scaffold-web spiders" *Nesticella* Lehtinen & Saaristo, 1980 (family Nesticidae). These species have a wide geographical distribution that covers both middle and low latitudes, and they occur worldwide in regions characterized by a tropical and humidsubtropical climate. In Asia, some species extend their distributions into temperate latitudes. In contrast to most other nesticids, which are mainly restricted to subterranean habitats, species of *Nesticella* typically have both epigean or endogean lifestyles in being only partially cave dwelling and more frequently occurring in rainforest litter (Lehtinen & Saaristo, 1980). Nevertheless, Nesticella living in subtropical environments of southern China seem to have developed a much stronger bond with the hypogean ecosystem than their tropical counterparts, and also exhibit a surprisingly wide local diversity that has no parallel in other regions of Asia (Lin, Ballarin, & Li, 2016; Zhang & Li, 2013). Despite the general absence of specialized morphological adaptations, these species generally occur in small geographical areas and are permanently restricted to caves or other comparable habitats; basically, they are absent from the surface. Zhang and Li (2013), who mainly focused on the lineages inhabiting the Yunnan-Guizhou Plateau area in southern China, hypothesized that the Chinese clade had an old origin and its components underwent a rather recent and major radiation. Underground colonization of the plateau occurred through multiple, independent episodes, possibly influenced by the last glaciations. However, the precise tempo and mode of the evolution of these unusually specialized hypogean lineages could not be precisely established because of the lack of comparison with epigean populations.

The remarkable differences in diversity, distribution and habitat preference of modern *Nesticella* in the Asian tropics and subtropics raise interesting questions about the processes that led to their ecological disparities, despite their close phylogenetic relationships. Consistent with the assumed ancient history of the genus and its pronounced troglophilic habits, here we hypothesize that the increasing seasonality and habitat changes that gradually affected eastern Asia since the mid-Miocene played a central role in inducing the formation of strict hypogean lineages, but only in middle latitudes.

Our study aims to (i) reconstruct a precise phylogeny and spatiotemporal evolution of *Nesticella*, including both hypogean and epigean species, based on a comprehensive set of nuclear and mitochondrial genes; (ii) investigate the timing and mode of adaptation to caves; and (iii) test for the possibility that mid-Miocene climate change played a leading role by comparing the evolutionary history of distinct lineages of *Nesticella* in different latitudes and climate zones.

# 2 | MATERIALS AND METHODS

### 2.1 | Taxon sampling

A total of 162 individuals of *Nesticella* belonging to 48 species were sampled (list of specimens and GenBank accession numbers, Appendix S1: Table S1; Appendix S2 gives further information). They represent ~66% of the current 72 described species (World Spider Catalog, 2017) and ~82% of all Asian species. Taxon sampling maximized representation in the Asian tropics and subtropics for comparing their evolutionary histories. Species from Madagascar, Sri Lanka and Fiji were used as representatives of Africa, South Asia and Oceania, respectively. In addition, samples of *Wraios longiembolus* Ballarin & Li, 2015 were added after preliminary results showed it shared a possible recent common ancestor with *Nesticella*. Representative samples of nine more nesticids belonging to the genera *Carpathonesticus* Lehtinen & Saaristo, 1980; *Cyclocarcina* Komatsu, 1942, *Hamus* Ballarin & Li, 2015, *Kryptonesticus* Pavlek & Ribera, 2017, *Nesticus* Thorell, 1869, *Speleoticus* Ballarin & Li, 2016 and *Typhlonesticus* Kulczyński, 1914 from eastern Asia and Europe were included in the phylogenetic analysis as outgroup taxa to better evaluate the monophyly of *Nesticella* and resolve its historical relationships within the family. Further specimens of the families Linyphiidae (six species) and Pimoidae (five species) were selected to root the phylogeny following recent molecular evidence showing their close relationship to Nesticidae (Garrison et al., 2016; Wheeler et al., 2016).

#### 2.2 DNA sequencing and phylogenetic analysis

Six gene regions were amplified selectively: two mitochondrial gene fragments encoding cytochrome c oxidase I (COI) and 16S rRNA (16S), and four nuclear gene fragments encoding histone 3 (H3), 28S rRNA (28S), 18S rRNA (18S) and Actin 5C (Act5c). Standard primers used in molecular analysis of spiders as well as oligonucleotides tested effectively on Nesticella (Zhang & Li, 2013) were adopted. In addition, new primers were specifically designed for H3 (H3aR1, 5'-ATATCCTTGGGCATAATGGTGAC-3') and Act5c (Act2F, 5'-GATCTGGCATCACACCTTCTAC-3': Act252F-2. 5'-ACNAACTGG-GAYGAYATGGAGAA-3'). More detailed information was provided in Appendix S2. The complete list of primers and procedures were listed in Appendix S1: Table S2. Raw sequences were edited and assembled using BIOEDIT v.7.2.5 (Hall, 1999). Alignment was performed on the online version of MAFFT v.7.0 (Katoh & Standley, 2013) using the algorithms G-INS-i for highly conserved sequences (COI. H3. Act5c) and Q-INS-i for sequences with more variable regions (16S, 18S, 28S); all other settings were left as default. The Q-INS-i algorithm was specifically designed for high-divergence sequences and was selected to increase alignment accuracy. All aligned fragments were visually inspected and protein-coding nucleotide sequences were translated into amino acids to reduce possible mismatches. Genes that showed a high number of problematic alignments (28S, 16S) were trimmed using the program TRIMAL (Capella-Gutierrez, Silla-Martinez, & Gabaldon, 2009) set with the automated heuristic method, as suggested by the authors. The resulting sequences were combined using MESQUITE v.3.04 (Maddison & Maddison, 2015).

Preliminary investigation using DAMBE 6 (Xia, 2017) suggested the presence of saturation in the 3rd codon position of *COI*. Because saturation may effect phylogenetic inference and estimated times of divergence in deep branches (Breinholt & Kawahara, 2013; Nilsson, Härlid, Kullberg, & Janke, 2010), its impact on the tree was tested by keeping and removing the codon in the analysis.

Maximum Likelihood (ML) and Bayesian Inference (BI) methods were chosen to infer the phylogeny of *Nesticella*. The most suitable partition schemes and substitution models for the phylogenetic reconstructions were found by using PARTITIONFINDER v.1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012) and the Akaike information criterion.

Six putative partitions were suggested as follows: COI+Act5c+H3 first and second codon positions: COI third codon position (when included); and Act5c+H3 third codon positions and one for each remaining gene. The GTR+I+G model was recommended for all partitions with two exceptions: COI third and Act5c+H3 third codon positions used the GTR+G model. ML was performed on RAXML v.8.2.0 (Stamatakis, 2014) under a GTRGAMMAI model and the six partitions scheme, using 1,000 rapid bootstrap replicates and a random starting tree. BI was implemented in MRBAYES v.3.2.5 (Ronquist & Huelsenbeck, 2003) following the parameters obtained from PartitionFinder and performing two simultaneous runs of four Monte Carlo Markov chains (MCMCs)-one cold and three heated chainsfor 50 million generations. Trees were sampled every 5,000th generation and the first 25% of sampled trees was discharged as burn-in. The results were checked by using TRACER v.1.6 (Rambaut, Suchard, Xie, & Drummond, 2014) to ensure stationarity.

# 2.3 | Divergence-time estimation

To estimate absolute divergence times for branches on the phylogeny, a reduced data set of 70 taxa was implemented that consisted of 50 ingroup samples, including species of *Nesticella* and *Wraios* as well as 20 other nesticids, linyphilds and pimoids in the outgroup. Analyses used one specimen for each species to preclude identical sequences and, thus, zero-length branches. *Nesticella mogera* was the exception because samples from both north and south of the Yangtze River served to calibrate the clock based on the geological origin of the river.

Two palaeogeographic events that presumably shaped the evolutionary history of Nesticidae were selected to time-calibrate the phylogeny: the separation of the Japanese archipelago from mainland Asia and the formation of the Yangtze River in China. In addition, nesticid and pimoid fossils from Baltic amber and the estimated origin of Nesticidae obtained from the literature were selected for divergence-time estimation (details in Appendix S2). To further confirm the accuracy of the inferred chronogram and the correct use of specific calibration points (e.g. nesticid fossils and the opening of the Sea of Japan), a separate chronogram was estimated by a BEAST analysis using the same data set, settings and procedure adopted in the final analysis but selecting only three calibration points derived from previous studies on spiders (estimated origin of Nesticidae, pimoid fossils, Yangtze River).

Substitution saturation was reported to negatively influence the estimation of divergence time in deep branches and possibly effect estimated times of shallow nodes (Beaulieu, Oliver, O'meara, & Beaulieu, 2015; Nilsson et al., 2010; van Tuinen & Torres, 2015). The inclusion of *COI* third codon position led to different estimated ages of the basal nodes in comparison with divergence times predicted previously. Therefore, the *COI* third codon position (399 bp) was subsequently excluded in the final analysis.

A Bayesian MCMC approach was conducted twice in BEAST v.1.8.2 (Drummond, Suchard, Xie, & Rambaut, 2012) under a

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lognormal relaxed clock and a Yule tree prior with the same nucleotide substitution models and partitions suggested by PARTION-FINDER v.1.1.1 (Lanfear et al., 2012) for the phylogenetic analysis. For each analysis, four parallel runs were performed for a total of 50 million generations with a random starting tree, with sampling every 5,000th generation and while discharging the first 5,000 trees as burn-in. Chain stationarity was ensured using TRACER v.1.6 (Rambaut et al., 2014) by checking that the effective sample size was >200 for all the parameters. The resulting 10,000 trees and the Log files derived from the two repeated analysis were combined using the program LOGCOMBINER v.1.8.2 present in the BEAST package. The consensus tree was composed with TREEANNOTATOR v.1.8.2, which was also included in the BEAST package, while burning the first 1,000 trees. The final chronogram was edited using FIGTREE v.1.4.2 (available at: http://tree.bio.ed.ac.uk/software/f igtree/). The result of the final analysis was used subsequently as a starting tree to estimate the ancestral range and reconstruct the age of cave adaptation.

### 2.4 Diversification rate through time

A multiple lineage-through-time plot (LTT) served to display graphically the overall pattern of diversification in *Nesticella* and to compare visually lineage accumulation together with temperature shifts through the epochs. Three different data sets were implemented, one including the whole genus and two others treating tropical and subtropical/temperate groups of species separately. Each plot was carried out with the R package APE (Paradis, Claude, & Strimmer, 2004) using a 1,000 random tree-set derived from the BEAST output and the consensus chronogram arranged for the ancestral state reconstruction, removing time by time the outgroup samples and unnecessary species of *Nesticella*.

## 2.5 | Ancestral range reconstruction

The biogeographic history of Nesticella and its ancestral distribution were inferred using two methods: statistical dispersal-vicariance analysis on S-DIVA (Yu, Harris, & He, 2010) and the likelihoodbased dispersal-extinction-cladogenesis model on LAGRANGE (Ree & Smith, 2008), both implemented in RASP v.3.2 (Yu, Harris, Blair, & He, 2015). S–DIVA allowed accounting for phylogenetic uncertainty in the node estimate, determining the probability of the supposed ancestral range for each node, while LAGRANGE inferred the rate of dispersal, local extinction and ancestral ranges. Ten operational regions (A-J) were defined based on the current distribution of extant species, known geological history of the areas and modern climate. We tested the assumption that specimens of N. mogera from Fiji were introduced recently (Lehtinen & Saaristo, 1980). A finding of little or no genetic difference from any other population served to support the hypothesis. If the hypothesis was not rejected, then specimens from Fiji were excluded because of a probable recent introduction of this species by anthropogenic activity. The S-DIVA analysis was performed under a range of

constraints using 1,000 random trees and a consensus tree derived from the BEAST results after removing the outgroup taxa. A maximum of three areas for each node was selected to reduce the presence of disjoint ancestral distributions upon considering that the modern range of extant species does not involve more than two or three regions. A further analysis used two maximal areas only for comparison. LAGRANGE was run with the same consensus tree as used in S-DIVA with and without range constraints and with two maximal areas for each node. The constrained version was preferentially selected because it excluded a priori improbable detached areas between distant zones and made the results more comparable with the S-DIVA analysis. The program also allowed the use of time-dispersal constraints to establish distinct dispersion capabilities between areas in a given timeframe. Therefore, three different time-slices were implemented and tested as constraints using five categories (1.0, 0.75, 0.5, 0.25, 0.1) to define increasing difficult dispersion between areas. The beginning of the first time slice was set at 37.6 Ma (estimated separation of Nesticella and Wraios), a tome of 30 Ma (start of the formation of the R.R.F.) was used to set the limit between the first and second timeframe. The boundary between the second and the third was set at 15 Ma to test the possible impact of the mid-Miocene climate change.

#### 2.6 Cave-adaptation reconstruction

To reconstruct the ancestral state and the time of adaptation to the subterranean environment by Nesticella, three different approaches were chosen and tested: Bayesian Inference (BI), Maximum Likelihood (ML) and Maximum Parsimony (MP). Following the classification of subterranean animals proposed by Sket (2008). we assigned Wraios longiembolus and each species of Nesticella to one of the four following categories based on their increasing bond to the cave habitat: epigean (A), species entirely not associated with the hypogean environment and can be found only outside caves, usually in the superficial layers of forest litter; subtroglophile (B), weakly related to the hypogean environment, can be found sometimes in caves (usually in the initial trait) but more commonly outside, under superficial stones, in forest litter and on ground vegetation; eutroglophile (C), highly related to the hypogean environment but lacking deep morphological adaptations to the subterranean life, can be found only in caves or in strict cave-like microhabitats with high humidity and a stable temperature; and troglobiont (D), strongly bound to the subterranean habitat and found exclusively in the deep part of the caves, show extensive morphological adaptation to the hypogean life such as eye reduction and depigmentation. For each species, ecological information was retrieved from the literature and carefully augmented with data from the abundant preserved material in the collections of the Chinese Academy of Sciences and our personal experience in the field.

RASP (Yu et al., 2015) was used for BI analysis because it allowed for multistate Bayesian Inference of discrete states with

the function Bayesian Binary MCMC (BBM). The program was run using the consensus tree derived from the BEAST chronograms after convergence was reached. We used an estimated state frequency and a Gamma distribution for the site rate variation to run 10 million cycles, sampling every 1.000th tree and discharging the first 1,000 as burn-in. The maximum number of areas was limited to one to avoid mixed traits and for ease of comparison with the results of the other methods. ML analysis was performed with the RAYDISC command in the package CORHMM (Beaulieu et al., 2015) in R (Team, 2015) under the equal rates (ER) method. To account for phylogenetic uncertainty, 1,000 randomly selected trees derived from BEAST were used. The number of character categories was reduced to two to adapt the data to the binary system of the program; therefore, the species were grouped as being weakly related to the cave environment (epigean and subtroglophile = 0) or strongly related (eutroglophile and troglobiont = 1). The MP analysis was run in MESQUITE 3.04 (Maddison & Maddison, 2015) using the "trace character over trees" option with the same treeset and under the same categories of ML analysis with all changes weighted equally.

# 3 | RESULTS

#### 3.1 | Phylogeny of Nesticella

The final data sets with all the concatenated genes was composed of 6,141 nucleotides (*COI*: 1,197 bp; 16S: 465 bp; *H3*: 309 bp; *Act5c*: 723; 18S: 1,742; 28S: 1,705 bp) with 183 terminals. With the third codon position of *COI* removed the data set included 5,742 nucleotide positions.

Maximum Likelihood (ML) and Bayesian Inference (BI) analyses generated structurally identical phylogenetic trees (Figure 1b; see Appendix S1: Figures S1–S4). Internal nodes were generally highly supported by one or both methods and, as expected, some ML bootstrap values were slightly lower than corresponding BI posterior probabilities. Removing the third codon position of *COI* from the analysis led to identical results with a general slight improvement in the nodal support only. The same tree topology was also obtained from the BEAST analysis. This was the most complete phylogenetic reconstruction of *Nesticella* so far, and our results obtained the species groups established by Lin et al. (2016) on the basis of morphological characters.

Both ML and BI strongly supported the monophyly of *Nesticella* and resolved *Wraios* as its sister-group (ML bootstrap = 100, BI posterior probability = 1). These genera clearly separated from the other nesticids (ML = 100, BI = 1), in particular from *Hamus*, *Typhlonesticus* and the clade composed of the remaining members of the tribe Nesticini sensu Lehtinen and Saaristo (1980) (*Carpathonesticus, Cyclocarcina, Kryptonesticus, Nesticus* and *Speleoticus*). Thus, *Nesticella* and *Wraios* formed a distinct evolutionary lineage. *Nesticella* had two major lineages (NGR and SGR) with high support (ML = 100, BI = 1). These clades included the species geographically distributed almost entirely to the north and south of

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the R.R.F., approximately corresponding to the Asian tropical (SGR) and subtropical/temperate climate zones (NGR: Figure 1a). The SGR matched the nepalensis species group described by Lin et al. (2016) and showed a single origin despite the wide and diverse area of distribution, which extended from Africa to the whole of South and Southeast Asia. In contrast, the northern group (NGR) further separated into subclades NCLs I-IV, reflecting their morphological separation. These species groups were distributed in mainland China (NCL I = mogera-group and NCL II = brevipes-group), Indochina and Sumatra (NCL III = phami-group), and Taiwan and Korea (NCL IV = quelpartensis-group; Figure 1a). The monophyly of each clade was highly supported by both ML and BI, with the exception of NCL III, which consisted of N. phami and N. sumatrana. Although their range involved Southeast Asia, these species clearly shared a common ancestor with the northern rather than the southern clade. Furthermore, their position within the tree, although not directly well supported by all statistical methods used, remained constant in all tree typologies inferred by RAXML, MRBAYES and BEAST. Specimens of N. mogera from Fiji clearly clustered together with other individuals of the same species from mainland China, showing no genetic differences and, thus, did not reject the hypothesis of Lehtinen & Saaristo.

#### 3.2 | Divergence time

The final chronogram (Figure 2b), with the 3rd codon position of COI excluded, was dated using all five calibration points. The common ancestor of Nesticella and Wraios was estimated to have existed during the Eocene, before 37.6 Ma (95% HPD: 47.3-28.5 Ma), when the two sister genera first diverged. The origin of Nesticella and the split between the northern and southern lineages was predicted to have occurred around 28.3 Ma (95% HPD: 35.5-21.4 Ma) during mid-Oligocene, a period of great local geological changes in Asia. The SGR (nepalensis-group) was derived from a common ancestor around 20.8 Ma (95% HPD: 27.2-14.8 Ma), from which the group gradually diversified into the species currently distributed in Africa, South and Southeast Asia during the second half of the Miocene and throughout the Pliocene. Within this species group, the most recent speciation occurred in Madagascar, with local species emerging around 3.8-2.5 Ma during the late Pliocene and early Pleistocene. The origin of all the northern subclades (NCLs I-IV) involved less time during the late Oligocene and the boundary with Miocene. NCL IV (quelpartensis-group) first diverged from the others around 25.5 Ma (95% HPD: 32.4-19.4 Ma), followed by the NCL I (mogera-group) at 24 Ma (95% HPD: 30.5-18.4 Ma) and finally by the NCL II (brevipes-group) and NCL III (phami-group) at 22.8 Ma (95% HPD: 29-17.3 Ma). All species belonging to NCL II, including cave-adapted Chinese Nesticella, emerged from a common ancestor about 18.8 Ma (95% HPD: 24-14.3 Ma) in the early Miocene and gradually diversified during the second half of that epoch. A late and greater diversification involving both NCLs I and II began at the end of the Miocene with the further establishment of most of the modern Chinese species



between 7 and 1.5 Ma during the Pliocene and early Pleistocene Epoch. The alternative chronogram made with only three calibration points estimated a very similar age for the upper nodes of the tree

(Appendix S1: Figure S5). The age of basal nodes was slightly older but still highly compatible with the time-estimation inferred from the full analysis.

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**FIGURE 1** Summary of the phylogenetic analysis of the genus *Nesticella* and its current distribution in Asia and Madagascar. (a) Geographic distribution of the genus *Wraios* and the major lineages of *Nesticella*. Each dot represents one locality. Different colours refer to different groups/clades: North clade I (red), North clade II (blue), North clade III (yellow), North clade IV (orange), South group (violet) and *Wraios* (green). Black dashed line indicates the approximate limit of permanently cave-adapted *Nesticella* in Asia. Red and blue lines show the location of the Ailao Shan-Red River Fault zone (R.R.F.) and the Yangtze River. (b). Combined results of the phylogenetic analysis based on six gene fragments. Dots at the nodes refer to the RAxML Maximum Likelihood bootstrap value (left half) and MrBayes Bayesian Inference posterior probability (right half); white, grey or black colours indicate increasing values as reported in the related legend. Branch lengths are scaled in relation to the number of substitutions per site [Colour figure can be viewed at wileyonlinelibrary.com]



**FIGURE 2** Diversification of *Nesticella* through time. (a) Lineages-through-time (LTT) plot with 95% confidence interval of the genus *Nesticella* along the last 28 Myr. (b) Time-calibrated phylogeny of the reduced data set from the BEAST analysis. The data set includes species of *Nesticella* and related genera of the family Nesticidae, Linyphildae and Pimoidae. Stars refer to the five time-calibration points used in the analysis: geological events (red) and fossils/literature (yellow) [Colour figure can be viewed at wileyonlinelibrary.com]

# 3.3 | Diversification rate through time

The LTTs differed among groups. The complete reconstructed LTT for *Nesticella* (Figure 2a) showed a roughly constant diversification rate during the late Eocene–early Miocene with a reduction in the mid-Miocene, around 15–14 Ma, followed by a gradual recovery in the last 11–9 Myr. The LTT of the NGR (Figure 4b) had approximately the same pattern, but with a much more accentuated shift, when diversification almost halted during the same time interval (14.5–9 Ma). A slow growth from 9 Ma followed this, but increased substantially in the last 5.5 Myr. Almost opposite results were displayed in the LTT of the SGR (Figure 4b), where a more uniform and almost constant trend occurred throughout the Miocene Epoch,

followed by a slight slowdown only in the last 7.5 Myr, starting from the end of the Miocene.

# 3.4 | Biogeography and distribution

The results of the ancestral area reconstruction analysis were summarized in Figure 3b and portrayed graphically in Figure 3c,d. The outcomes of the S-DIVA and LAGRANGE tested under alternative restrictions were placed in the (Appendix S1: Figures S6–S9). General concordance occurred for the two methods and different constraints. However, in some cases S–DIVA obtained approximately the same probability for more than one ancestral area. The estimated centre of origin of the genus covered southeast China and North Indochina,



**FIGURE 3** Biogeography of *Nesticella* and probable dispersion routes in Asia. (a) Distribution map of the 10 delimited areas (A–J) used herein. Black dotted circle indicates the most probable area of origin of the genus. Red dashed line shows the location of the Ailao Shan-Red River Fault zone (R.R.F.) (b) Combined results of the ancestral area reconstructions: coloured pie charts at the nodes indicate the probability of ancestral areas inferred using a parsimony-based statistical dispersal–vicariance method (S–DIVA), coloured squares on the stems show the most likely ancestral range reconstructed using the likelihood-based divergence–extinction–cladogenesis (DEC) model. (c) Potential dispersion routes (arrows) and major diversification areas (sunburst) of clades of *Nesticella*: South group (violet), North clade I (red), North clade II (blue), North clade III (yellow) and North clade IV (orange). Dashed arrows show possible dispersions according to the current distributions of the extant species [Colour figure can be viewed at wileyonlinelibrary.com]

probably including the Hengduan Mountains in eastern Tibet (Figure 3a), as suggested by the S–DIVA analysis (node 1). The unconstrained LAGRANGE analysis yielded an enlarged ancestral area and considered also northern China as a potential birth region for the genus (see Appendix S1: Figure S9), although the other analyses did reject this hypothesis. A mixture of vicariance and dispersal events seemed to have shaped the current distribution of *Nesticella*.

After the separation to the north and south of the R.R.F. (node 2), both major clades clearly followed different evolutionary histories. Several scattering episodes, including long-distance dispersions, were inferred for the SGR, the oldest of which started at approximately 21–13.5 Ma (nodes 3–5) and drove the species from Indochina to the Himalayan area through one or possibly two separate dispersion routes. During the second half of the Miocene, between 13.5 and

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**FIGURE 4** Cave adaptation in *Nesticella*. (a) Combined results of the ancestral state reconstructions for *Nesticella*. Coloured pie charts on each node represent the probability of each ancestor being in one of the potential levels of cave adaptation as inferred by the Bayesian Inference (upper pie) and Maximum Likelihood/Maximum Parsimony (lower pie). Different colours of the pies refer to different levels of cave adaptation as reported in the legend. The grass symbols indicate the presence of a strong bond with the epigean environment within lineages of *Nesticella* while the black cave symbols refer to the appearance of a strong relationship with the cave environment. The red arrow indicates the appearance of true troglobiont species. (b) Lineages-through-time (LTT) plot with 95% confidence interval of *Nesticella* occurring in the North (blue line) and South (violet line) groups in relation to the global temperature trend from Oligocene to present (red line). Black arrow shows the probable beginning of the habitat shift in the North group of *Nesticella*. Modified from (Zachos et al., 2001) and (Zachos et al., 2008) [Colour figure can be viewed at wileyonlinelibrary.com]

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5 Ma, the SGR species gradually spread throughout Indochina. The complexity of the process and the heterogeneity of the involved areas did not allow precise envisioning of the events. Regardless, a probable dispersal of *Nesticella* was from North Indochina to the south-southeast, which resulted in the colonization of Sundaland and the Philippines first (13.5–12 Ma, nodes 5–6) and subsequently Sulawesi Island (>10.6 Ma, node 7). More recent scattering events involved the spreading of species from their original Indochinese distribution area to South Asia. Both S–DIVA and LAGRANGE predicted that spiders arrived in Sri Lanka sometime between 9 Ma (node 8) and 7.5 Ma (node 9). The arrival of *Nesticella* in Madagascar was the most recent event. It happened approximately between 7.5 Ma (node 9) and 6 Ma (node 10), with local species being present around 3.8 Ma.

Differing from the SGR, the NGR had more probable vicariance events and less clear long-distance dispersals. Most dispersal occurred only at the beginning of this clade's evolutionary history, during late Oligocene and early Miocene Epochs (25.5-17.5 Ma). These early episodes involved all the subclades starting from ancestral populations living in southern China and northern Indochina. Although precise determination of the timing and mode of these events was imprecise, NCLs IV and I probably reached eastern and northern China by 25.5 Ma (node 11) and 24 Ma (node 12), respectively, followed by the settlement of NCL III in southern Indochina and Indonesia around 22.8 Ma (node 13) and 17.5 Ma (node 14), respectively. Later dispersals of NCL II (nodes 15-16) dated to around 18.8–17.5 Ma from southern China to the southwest along the R.R.F. and Hengduan Mountains. No further recent dispersions were inferred clearly; instead, general stagnations and local diversification were recorded in NCLs II and I in east-central China during the last 16 Myr (node 17) and 7 Myr (node 18), respectively.

# 3.5 Cave adaptation

The ancestral state reconstruction (Figure 4a) inferred using RASP, CORHMM and MESQUITE was concordant and void of significant discrepancies between the three different approaches (Appendix S1: Figures S10-S12). The strict hypogean environment was not the original habitat of Nesticella and Wraios. The ancestors of Nesticella were probably surface dwellers with a weak affinity to the subterranean environment. The same general habitat preference was shared between the forebears and all the derived species belonging to the SGR currently distributed in tropical habitats south and west of the R.R.F. Likewise, the NGR also originated from surface-adapted ancestors and developed a stronger bond with the subterranean environment only starting from the mid-Miocene. The shift to cave dwelling was estimated to have occurred independently four or five times within this group and in all the subclades currently located north of the R.R.F. (NCLs I, II and IV). This habit did not evolve in NCL III in Southeast Asia. In the NCL II, the adaptation occurred at least two or three times during the second half of the Miocene, probably sometime between 15 and 11 Ma and in a similar or slightly recent period of time for NCL I. The last habitat shift happened in NCL IV, possibly at the end of the Pliocene or during the early Pleistocene. In contrast, despite their close relationship and common origin with the other neighbouring cave-adapted *Nesticella*, a transition from epigean to hypogean habitats did not occur in the species of NCL II living on Hainan Island (*N. qiongensis*) or along the R.R.F. and the Hengduan Mountains (*N. arcuata, N. dazhuangensis*, *N. hongheensis, N. jingpo*, region D in the phylogeographic reconstruction). The RASP analysis suggested that real troglobitic species evolved only once, during the Pliocene Epoch (4.23.3 Ma), and it involved a small group of species (*N. caeca, N. gazuida, Nesticella* sp.) within NCL II.

# 4 | DISCUSSION

### 4.1 | Origin of Nesticella and the role of the R.R.F

Nesticella has been proposed to be a rather ancient group (Zhang & Li, 2013), and our study does not reject this hypothesis. The origin of the genus tracks back to the Eocene, approximately at the end of this Epoch (37.6 Ma), from ancestors living in an area adjacent to the Tropic of Cancer (23.5°N) and covering approximately modern southern and south-western China and North Indochina (Figure 3a, b). Ancestral state reconstruction predicts that early *Nesticella* were essentially surface dwellers and had only a weak association with the subterranean environment (Figure 4a). Therefore, the predominantly epigean lifestyle, which occurs nowadays in most of the extant species, appears to be the primitive state of these spiders.

Around the mid-Oligocene (~28 Ma), Nesticella apparently underwent its first, important split into two major clades, NGR and SGR (Figure 2b). These groups currently have distinct, well-defined areas of distribution, covering eastern Asia (NGR) and South and Southeast Asia and Africa (SGR; Figure 1a). This distinct geographical segregation suggests a vicariant origin related to a geological rather than a climatic event. The subclades of the NGR (NCLs I-IV) appear to have originated shortly after the group's formation, along the Oligocene-Miocene boundary, between 25.5 and 22.8 Ma. This implies that the same driver of the initial split also affected the NGR. We associate these cladogenic events with a well-known geological process that occurred in Asia during the predicted period and geographical area: the south-eastern lateral extrusion of the continental landmass under the pressure of the colliding Asian and Indian plates (Leloup et al., 1995; Tapponnier et al., 1990). These tectonic movements occurred during Oligocene and early Miocene starting approximately 34-30 Ma (Gilley et al., 2003; Tapponnier et al., 1990) with further, consistent lateral extrusion and shearing between 26-17 Ma (Leloup et al., 2001) provoking extensive faulting and folding of the upper crust in southern China and north-western Vietnam. The event eventually generated the Ailao Shan-Red River shear zone (R.R.F.) (Hallet & Molnar, 2001; Lacassin et al., 1997; Phan Trong et al., 2012). This major discontinuity impacted the local biota (Averyanov, Loc, Hiep, & Harder, 2003; Bain & Hurley, 2011; Che et al., 2010; Yuan et al., 2016; Zhao, Xia, Cannon, Kress, & Li, 2016), possibly including Nesticella (Zhang & Li, 2013). Our results, which were inferred using a

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more comprehensive range of species, support the key role this geological event played in shaping the early phylogeny of these spiders. Our analyses also reject the hypothesis of the rift formation having been the main driver for subterranean adaptation in *Nesticella*; the estimated distribution of the cave-restricted species does not match the location of the gorge (Figure 1a) and both main clades appear to have maintained their original epigean lifestyles during and after the cladogenesis (Figure 4a). Nevertheless, the separation into distinct groups that have disjointed geographical distributions forced the northern and southern lineages to follow separate evolutionary lines. This indirectly provided the conditions for their subsequent ecological differentiation during the Miocene.

# 4.2 | Cave adaptation in subtropical and temperate latitudes

In spite of disparities in habitat preference, the vast majority of modern Nesticella, including surface-dwelling species living in tropics, are essentially troglophilic organisms. They clearly prefer to live in caves, litter in tropical rainforests or similar habitats with high and constant levels of humidity and temperature. Nesticella share this distinctive trait with the other nesticid spiders. Biospeleologists generally agree that true cave-dwelling invertebrates are derived from epigean progenitors with some grade of exaptation to the subterranean ecosystems (Holsinger, 2000). Therefore, primitive Nesticella probably had an inclination for living in sheltered microhabitats (including the entrances of caves). This preference appears to have persisted throughout the history of Nesticella, making these spiders potentially susceptible to surface environmental perturbations and predisposed to permanent shifts to being troglobites. Our ancestral state reconstruction support this possibility by predicting the establishment of the hypogean clades of Nesticella during the mid-Miocene (approximately 15-14 Ma) (Figure 4a), which coincides with the decreased global temperatures (Figure 4b) and renewal of the general climate that characterizes this Epoch.

Following the "climate optimum" of around 17–15 Ma (Wan, Kürschner, Clift, Li, & Li, 2009; Zachos, Dickens, & Zeebe, 2008), a general cooling gradually replaced Earth's warm and humid climate of the early Miocene (Zachos, Pagani, Sloan, Thomas, & Billups, 2001). In Asia, temperature shifts were particularly intense at high and middle latitudes where winter monsoons significantly intensified (Jacques et al., 2013; Tang et al., 2015) and summer monsoons possibly declined (Jiang & Ding, 2008). Thus, local seasonality involving a lowering the winter mean temperature and humidity appeared. Simultaneously, an arid belt involving deserts and dry, open habitats expanded progressively in Central Asia (Miao et al., 2012, 2013). The climate change strongly affected the vegetation cover, particularly in middle latitudes. This involved a slow, constant reduction of once widely distributed warm-temperate evergreen forests with their gradual shift to the coasts and lower latitudes, which boreal forests, grasslands and savannas replaced (Cerling et al., 1997; Jacques et al., 2013; Pound et al., 2012). These events, the increasing seasonality and the gradual vegetational substitution that occurred in eastern Asia during the second half of the Miocene, likely progressively created new superficial conditions that would not have favoured species adapted to tropical habitats such as Nesticella. Because climate can strongly influence species diversification, particularly extinctions (Ezard, Aze, Pearson, & Purvis, 2011), it is plausible that Asian clades in middle latitudes experienced temporary reductions in diversity and local mass extinctions owing to habitat loss. The sudden decrease in the diversification rate of the NGR around 14.5 Ma and its continuous stagnation between 14.5 and 9 Ma (Figure 4b) are consistent with this hypothesis since, generally, a plateau in an LTT plot indicates a mass extinction event (Crisp & Cook, 2009). Nonetheless, groups of individuals can escape extinction by changing their habitat preference and successfully adapting to a new environment. In particular, troglophilic organisms, which are expected to live in shallow, moist habitats, may successfully retreat to caves to survive unfavourable periods because of the presence of constant internal temperature and humidity in those environments (Bryson et al., 2014; Holsinger, 2000). Our analyses suggest that eastern Asian Nesticella experienced this. Increasing sedentariness observed in the NGR starting from the middle of the Miocene (>17-16 Ma; Figure 3b-d) corroborates this hypothesis. Accordingly, the southern China karst region, which is extremely rich in ancient gorges and large cave systems (Schindler, 1982), could have offered ideal refugia for local species, and, thus, promoted the gradual hypogean settlement while their epigean counterparts progressively disappeared. Therefore, Chinese species of Nesticella would represent a clade of "climate relicts," with the mid-Miocene climate change having been the main driver of their adaptation to caves. Further, species currently living in the south-westernmost part of China (e.g. N. arcuata, N. dazhuangensis, N. hongheensis and N. jingpo), did not develop a strict subterranean lifestyle despite being phylogenetically and geographically closely related to the other cave-adapted lineages (Figure 4a). The southernmost area of China apparently retained high humidity and temperature and a diffuse tropical vegetation throughout the Neogene, as palaeovegetational studies indicate (Sun et al., 2011; Yao, Bruch, Mosbrugger, & Li, 2011). Therefore, and differing from the other northern populations, it is likely that the contemporary climatic change only marginally influenced these spider communities, which survived stably on the surface until present.

### 4.3 Recent diversification burst

A boost in the rate of diversification usually follows a habitat shift (Hou, Sket, Fiser, & Li, 2011). The LTT of the NGR only has a constant increase during the late Miocene (Figure 4b) but a sudden increase occurs in the rate starting at the beginning of the Pliocene Epoch, suggesting a recent, fast diversification in lineages of Chinese *Nesticella* (Zhang & Li, 2013). This pattern is not easy to explain because it may involve numerous factors. Although future comprehensive studies are necessary to clarify this phenomenon, the climate shift during the Miocene Epoch may be the driver of the general gradual change. Differing from Pleistocene glaciations or other recent climatic drivers that operated within relatively short WILEY— Global Change Biology

timeframes (Ehlers, Gibbard, & Hughes, 2011), the evolution of climate during the Miocene progressed over several million years. The slowness of the change could have influenced cave adaptation in Nesticella by circumventing a fast and sudden passage to the subterranean lifestyle in favour of a more gradual one. Such a transitional stage would have allowed species to dwell temporarily on the surface during specific life stages or favourable periods of the year. This behaviour is known to occur in extant cave-dwelling spiders with a low grade of cave adaptation (Smithers, 2005). Therefore, some extent of gene flow could have continued for long periods of time between the local cave communities during the second half of the Miocene. Such would have slowed down speciation despite the increasing deterioration of surface conditions. Exacerbation of the external conditions during the early Pliocene (Figure 4b) and the following glacial periods may have reduced progressively genetic contacts, driving allopatric speciation and the high level of diversity that currently characterizes Nesticella in China.

#### 4.4 | Epigean evolution at tropical latitudes

Comparisons of the biogeographic histories of adjacent biomes can reveal dissimilar patterns (Favre et al., 2015). In our study, the inferred evolutionary history of the NGR contrasts with the SGR. Reconstructed ancestral states reveal that the SGR and the other species distributed in Southeast Asia (NCL III), evolved in a way clearly different from NGR. The former groups did not experience a permanent habitat shift, and they maintained the primitive, mostly epigean lifestyle throughout the entire Neogene (Figure 4a). These results are consistent with the rather constant rate of lineage accumulation observed in the SGR along the same period (Figure 4b), which often typifies species evolving in tropical environments (Couvreur, Forest, & Baker, 2011) and indicates a lack of any possible adaptive radiations (Crisp & Cook, 2009) usually associated with habitat shifts (Schluter, 2000). In addition, active dispersions are typical of the SGR since its appearance in the early Miocene. The scattering of species continued unchanged during the following epochs, gradually spreading the boundary of the group along the entire Asian tropical belt (Figure 3b,c). This wide and surprisingly effective expansion through distant territories once again assumes that the species involved possessed a strong inclination for erratic, epigean-related behaviour rather than a subterranean, sedentary lifestyle. Considering all evidence, we assert that the SGR evolved in an environment that differed from that of the NGR, and probably with relatively uniform climatic conditions that void of distinct changes in habitat, temperature and humidity. This scenario fits well with the classical concept of the tropical environment preserving ecologically stable conditions over millions of years. Even if modern research resolves a higher level of dynamism of the tropical belt, rainforests appear to have persisted in low latitudes during unfavourable climatic times by experiencing relatively less environmental changes than the deep climatic and vegetational shifts of temperate and subtropical latitudes (Morley, 2000). During the late Neogene, tropical evergreen and warm-temperate jungles became increasingly fragmented and they

were replaced locally by tropical savannas and mixed forests. However, they continued to be the dominant biome in South and Southeast Asia, gradually reaching their modern composition (Pound et al., 2011, 2012). Stable environmental and climatic conditions would have encouraged troglophilic organisms such as *Nesticella* to live on the surface. Moreover, the distributed cover of tropical forests along the equatorial region could have boosted surface dispersions, which would have favoured colonization of new territories rather than promoting local isolation. In the absence of any external climatic trigger strong enough to act as an evolutionary force, a permanent shift to a subterranean habitat would have been unnecessary in the *Nesticella* SGR. This would have limited the appearance of strict hypogean-adapted lineages and promoted instead the mostly epigean lifestyle of the extant species.

In conclusion, our analyses resolve a direct correlation between the climatic and vegetational fluctuations that occurred in Asia during the second half of the Miocene Epoch and the development of a strict subterranean lifestyle in Nesticella. Therefore, we propose a "climatic relict" origin for the extant cave-dwelling lineages living at Asian middle latitudes and suggest that the mid-Miocene climate change was the key factor of their adaptation and their ecological differences from their tropical counterparts. Accordingly, we cautiously infer that similar drastic changes in the habitat preference could have affected other Asian troglophilic organisms. Further parallel studies comparing the evolutionary history of closely related spider families (e.g. Linyphiidae, Pimoidae or Theridiidae) or other organisms possessing similar habitat preferences and geographical distributions to Nesticella, as well as an old origin, could shed further light on the impact of the ancient climate changes on the development of the Asian invertebrate subterranean fauna.

#### DATA ACCESSIBILITY

DNA sequences have been deposited in GenBank. See Appendix S1: Table S1 for the complete list.

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# SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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