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Evolutionary scenarios for the origin of an Antarctic tardigrade species based on molecular clock analyses and biogeographic data

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Abstract

The origin of the Antarctic continental extant fauna is a highly debated topic, complicated by the paucity of organisms for which we have clear biogeographic distributions and understanding of their evolutionary timescale. To shed new light on this topic, we coupled molecular clock analyses with biogeographic studies on the heterotardigrade genus *Mopsechiniscus*. This taxon includes species with endemic distributions in Antarctica and other regions of the southern hemisphere. Molecular dating using different models and calibration priors retrieved similar divergence time for the split between the Antarctic and South American *Mopsechiniscus* lineages (32–48 Mya) and the estimated age of the Drake Passage opening that led to the separation of Antarctica and South America. Our divergence estimates are congruent with other independent studies in dating Gondwanan geological events. Although different analyses retrieved similar results for the internal relationships within the Heterotardigrada, our results indicated that the molecular dating of tardigrades using genes coding for ribosomal RNA (18S and 28S rDNA) is a complex task, revealed by a very wide range of posterior density and a relative difficulty in discriminating between competing models. Overall, our study indicates that *Mopsechiniscus* is an ancient genus with a clear Gondwanan distribution, in which speciation was probably directed by a co-occurrence of vicariance and glacial events.

Contents

Introduction

The biogeographic origins of the extant limno-terrestrial fauna of maritime and continental Antarctica, and the sub-Antarctic islands have been debated for more than a decade (e.g. Pugh and Scott, 2002; Barnes *et al.,* 2006; Convey *et al.,* 2007, 2008; Pugh and Convey, 2008; Convey, 2010; Vyverman *et al.,* 2010; Fraser *et al.,* 2012). The current biogeographic distributions of Antarctic endemic or circum-Antarctic taxa could be the result of recent, ongoing dispersal in Antarctic regions (re-colonisation hypothesis; see Pugh, 2004) and/or the consequence of relict Gondwanan taxa surviving the extreme, harsh conditions of Antarctica over the last 23 million years (My) (glacial refugia hypothesis; see Stevens *et al.,* 2006). In the latter case, the extant fauna would be represented by ancient endemic taxa or new more recent taxa that evolved *in situ* from populations that had survived in ice-free refugia.

Antarctica formed a major component of the Gondwanan continent with a rich fauna and flora before geological and climate evolution isolated the continent and successive ice ages eliminated most plant and animal life. The extant biota that might have been associated with that period has been restricted to cryptogams and terrestrial meiofauna (Convey, 2010). Tardigrades, rotifers, and nematodes are major components of terrestrial Antarctic meiofauna in terms of diversity, number of specimens, distribution, and colonised substrates. Antarctic terrestrial biota are often characterised by high endemism (Chown and Convey, 2007) and it has

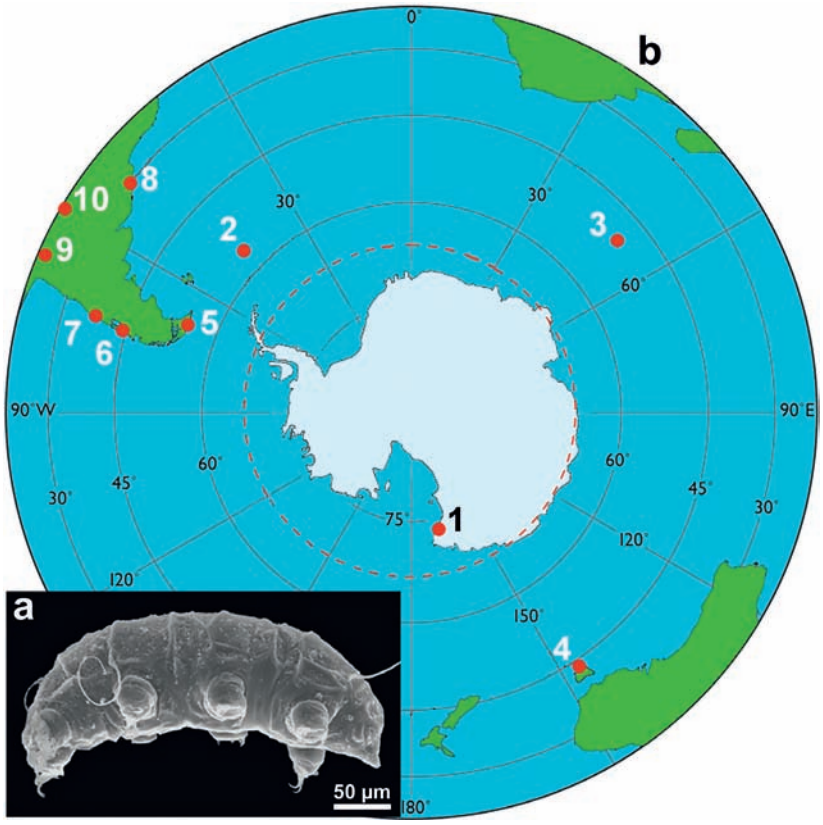


Fig 1. **a** Scanning electron micrograph of the heterotardigrade *Mopsechiniscus franciscae*. **b** Geographical distribution of the living species of *Mopsechiniscus* known to date (spots). 1 = *Mopsechiniscus franciscae*, Victoria Land, Antarctica; 2 = *Mopsechiniscus imberbis*, South Georgia, Sub-Antarctic Islands; 3 = *Mopsechiniscus frenoti*, Crozet Islands, Sub-Antarctic Islands; 4 = *Mopsechiniscus tasmanicus*, Tasmania, Australia; 5 = *Mopsechiniscus* sp., Chile; 6 = *Mopsechiniscus granulosus*, Argentina; 7 = *Mopsechiniscus granulosus*, Chile; 8 = *Mopsechiniscus* sp., Brazil; 9 = *Mopsechiniscus schusteri,* Venezuela; 10 = *Mopsechiniscus schusteri,* Costa Rica.

been hypothesised that long-term glacial habitat fragmentation and the consequent *in situ* isolation of biota was a likely cause (Stevens and Hogg, 2003, 2006).

Identifying biogeographic patterns and hypothesising species evolutionary origin are difficult tasks that require a combination of scientific disciplines and appropriate models. Understanding the origin and evolution of specific Antarctic biota requires finding particular taxa with clear biogeographic distributions. A fundamental element for investigating evolutionary biogeographic patterns is the definition of a temporal framework. Recent advances in molecular studies have provided novel chronological approaches in the form of molecular dating (a.k.a. molecular palaeobiology, or molecular clocks), which uses molecular and palaeontological analyses to provide a temporal framework. This framework can be used to explain evolutionary and geological events (Rota-Stabelli *et al.,* 2013), and to test alternative biogeographic hypotheses.

Mopsechiniscus franciscae Guidetti *et al.,* 2014 was recently discovered in Victoria Land, Antarctica (Guidetti *et al.,* 2014; Fig. 1), and represents one of the only three heterotardigrade species found in continental Antarctica. This new discovery, led us to evaluate the utility of this genus as a model taxon to test evolutionary patterns and biogeographical history within the timeframe of key Antarctic geological events. *Mopsechiniscus* is one of the few Echiniscidae for which there are molecular studies for more than one species (Jørgensen *et al.,* 2011; Guidetti *et al.,* 2014). *Mopsechiniscus franciscae* possesses the capability of cryptobiosis, which enables most limnic and terrestrial tardigrades to tolerate harsh climate conditions, such as those present in Antarctica (for reviews see Guidetti *et al.,* 2011; Møbjerg *et al.,* 2011; Wełnicz *et al.,* 2011). This capability, coupled with their small body size (< 1 mm), may have provided tardigrades with a high advantage for dispersal and colonisation of Antarctica. However, until now, long distance dispersal has been demonstrated for a few terrestrial species only (Jørgensen *et al.*, 2007; Cesari *et al.,* 2009, 2016; Bertolani *et al.,* 2011; Guidetti *et al.*, 2016), and based on the limited distribution of most taxa, the tardigrade capability for long distance dispersal has been questioned (McInnes and Pugh, 1998; Pilato and Binda, 2001). Such as co-occurring micro-arthropods, Antarctic tar-

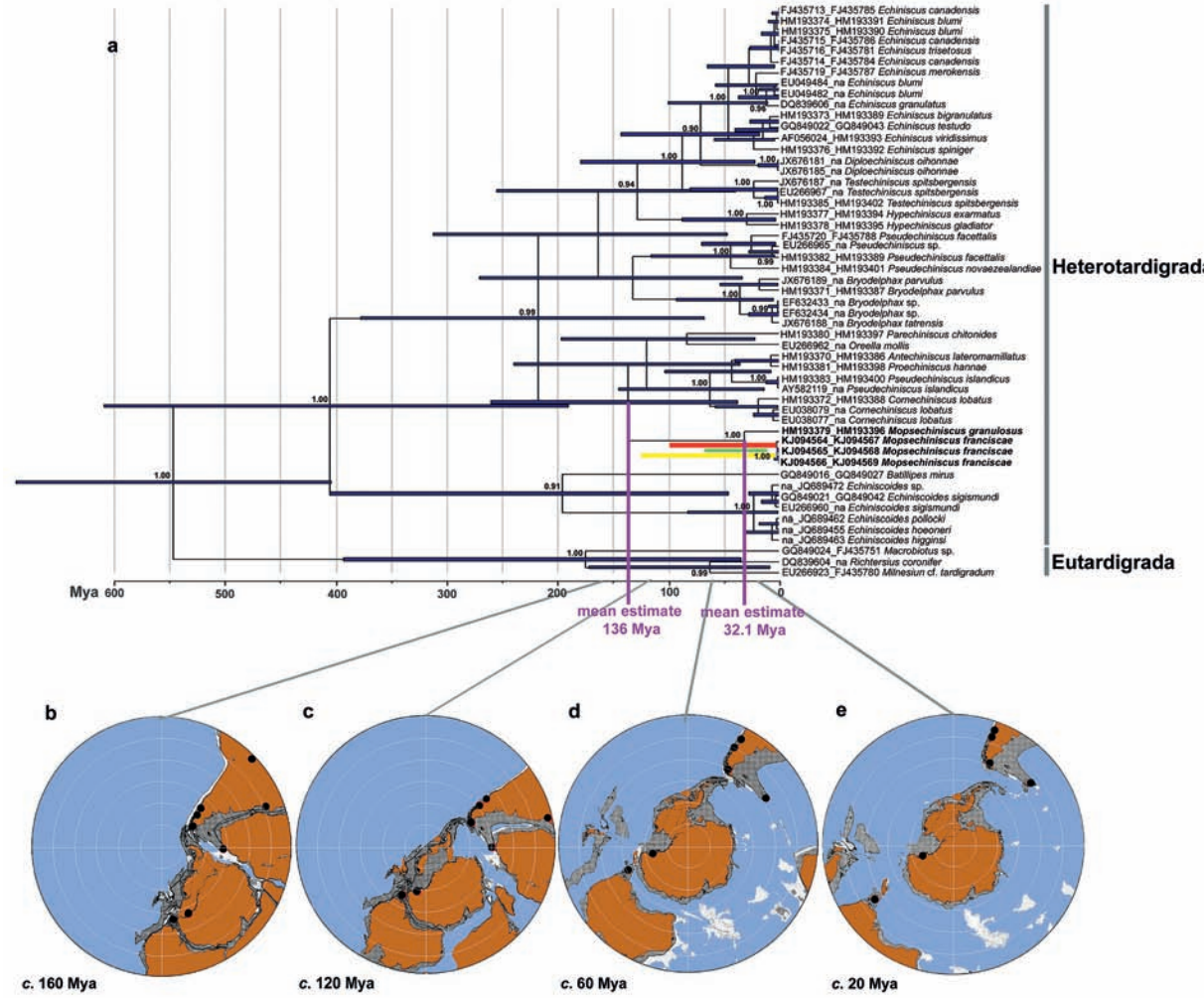


Fig 2. **a** Molecular clock analyses of the Heterotardigrada. Antarctic and South American *Mopsechiniscus* species (in bold) diverged approximately 32.1 My ago in a period highly compatible with the origin of the Drake Passage. Nodes are positioned at the mean divergence date, while bars indicate the 95% credibility intervals. The three coloured bars in the split between *M. franciscae* and *M. granulosus* indicate the 95% credibility interval from three independent analysis using different sets of evolutionary and palaeobiological priors. Red/grey-dark bars correspond to a clock analysis using the rate deduced from Rota-Stabelli *et al*. (2013). Green/grey-bar corresponds to a clock analyses using the rate deduced from Rota-Stabelli *et al*. (2013) and a Log-normal distribution clock analysis (for full tree of this analysis see Fig. 3a). Yellow/grey-light bar corresponds to the analysis using secondary node constrains from Regier *et al*. (2004) (for full tree of this analysis see Fig. 3b). Values close to branches indicate posterior probability values (> 0.9) of BI analysis. **b-e** Distribution of the extant *Mopsechiniscus* species (spots) mapped on the distribution of lands *ca*. 160 Mya (**b**), *ca*. 120 Mya (**c**), *ca*. 60 Mya (**d**), *ca*. 20 Mya (**e**). Figures of ancient land distributions (b-e) modified from Lawver *et al*. (1992). na = not available.

digrade distribution could represent a fauna comprising disjunct relicts (Marshall and Pugh, 1996; Pugh and Convey, 2000) or be the products of post-glacial speciation (Stevens and Hogg, 2003).

In order to shed new light on the origin and geochronology of the Antarctic continental fauna, we explored possible evolutionary scenarios for the origin of a component of the Antarctic meiofauna (i.e. the tardigrade *M. franciscae*) using molecular dating analyses and historical biogeography.

Material and methods

Literature data were used to map the known distribution of *Mopsechiniscus* species on current and past

Table 1. Tree and clock prior selection using Calibration set 1. For each of the combinations of priors (Model 1 to 7), the mean estimates in mill model using Calibration set 1.

	Model specification		Posterior Age estimates (with 95% Highest Posterior Density)		
	Clock	Tree prior	Tardigrada (root): Hetero-Eutardigrada split	First Heterotardigrada split	Origin of the line leading to *Mopsechiniscus*
Model 1	strict	Coalescent	557 (692, 417)	500 (644, 361)	151 (205, 105)
Model 2	relaxed random	Coalescent	558 (700, 422)	502 (649, 361)	135 (187, 80)
Model 3	relaxed log-normal	Coalescent	557 (696, 418)	431 (617, 236)	102 (190, 41)
Model 4	relaxed exponential	Coalescent	558 (692, 411)	339 (569, 110)	59 (149, 12)
Model 5	relaxed exponential	Incomplete Birth-Death	549 (687, 408)	416 (605, 212)	139 (265,42)
Model 6	**relaxed exponential**	**Birth-Death**	**547 (689, 405)**	**405 (609, 191)**	**136 (260, 38)**
Model 7	relaxed exponential	Yule process	549 (689, 404)	464 (628, 296)	158 (288, 70)

BF = Bayes factors

landmass maps to define their distribution patterns (Richters, 1907, 1920; Du Bois-Reymond Marcus, 1944; Ramazzotti, 1962; Mihelčič, 1967, 1971/72; Grigarick *et al.,* 1983; Binda and Kristensen, 1986; Kristensen, 1987; Rossi and Claps, 1989; Ottesen and Meier, 1990; Rossi *et al.,* 2009; Dastych and Moscal, 1992; Dastych, 1999a, b c, 2000, 2001; Kaczmarek *et al.,* 2014; Guidetti *et al.,* 2014; Roszkowska *et al.,* 2016).

For molecular phylogenetics and dating analyses, we assembled a concatenated dataset of 18S (SSU, 686 bp corresponding to positions 1213-1899 of the *Echiniscus canadensis* 18S complete sequence, Genbank Acc. No. FJ435714) and 28S (LSU, 968 bp corresponding to positions 1194-2192 of the *E. canadensis* 28S complete sequence, Genbank Acc. No. FJ435784) rRNA, as they are the only gene fragments available for *Mopsechiniscus* species. Sequences from three *M. franciscae* specimens, one *Mopsechiniscus granulosus* Mihelčič, 1967 specimen, 46 other Heterotardigrada (belonging to 32 recognised species), and three Eutardigrada used as outgroups, were retrieved and downloaded from GenBank (accession numbers are provided in Fig. 2). Sequences of 18S and 28S were aligned individually with MUSCLE 3.8.31 (Edgar, 2004) using a default run followed by two refinement runs (using option –refine), and concatenated using an in house PERL script, then they were checked by visual inspection. This resulted in a dataset (main dataset) of 53 sequences belonging to 36 taxa and 1655 positions, and 51% of missing data. To account for alignment quality and the effect of missing data, we generated a second dataset with the same taxon sampling but customised to exclude various positional gaps, which are present in all Heterotardigrada (insertions in the distantly related Eutardigrada) or poorly represented along the alignment. This dataset (reduced-dataset) comprises 53 sequences belonging to 36 taxa and 824 positions, and 20% of missing data. Analyses were conducted in BEAST v1.8 (Drummond and Rambaut, 2007) using a homogenous GTR model of nucleotide replacement coupled with a gamma distribution with four discrete categories. Best fitting model evaluations were performed taking into account the Akaike information criterion and Bayes information criterion (jModelTest 0.0.1; Posada, 2008). All BEAST analyses were run twice for 10 million generations each, sampling every 1000 generations, and using a starting random tree. Convergence of the most relevant parameters was checked with Tracer v1.6 and a consensus divergence tree was calculated with TreeAnnotator v1.8.2 using a burning-in of 1000 sampled trees.

Since time estimates are sensitive to tree and clock priors settings, we performed a model selection using Bayes factors based on the log marginal likelihoods, which were estimated using the smoothed harmonic mean method (Suchard *et al.,* 2001) on 100 bootstrap replicates. Various combinations of priors were tested by running the main dataset with calibration set 1 (see below) in BEAST and varying one tree/clock prior at the time (Table 1). The strict, the relaxed random, the relaxed lognormal, and the relaxed exponential clocks were all tested as clock priors; the coalescent, the Birth Death, the incomplete Birth Death, and the Yule process were tested as tree priors. According to the test, positive values of 2lnBF difference from different runs indicated the better fit of a model combination over a previously ranking model combination. Significance of the Bayes factor was assessed in accordance with Kass and Raftery's (1995) table.

With no fossil or sub-fossil records to constrain the Heterotardigrada, the clock was calibrated using two

ions of years for four nodes of interest are provided; the heights of the 95% Highest Posterior Density are in parenthesis. In bold the most fitting

	Model selection				
M. franciscae-M. granulosus split	ln BF	ln BF to highest	2 ln BF to previous	rank	significance
34.9 (59, 15)	-6112.7	0.1	0.3	6	not significant
35.1 (60, 16)	-6112.8	lowest	lowest	7	-
29.1 (66, 7)	-6097.1	15.8	31.2	5	very strong
19 (60, 2)	-6092.3	20.5	9.5	4	strong
32.7 (91, 4)	-6091.5	21.3	1.6	3	not significant
32.1 (99, 3)	**-6089.4**	**23.4**	**2.4**	**BEST**	**positive**
61.2 (147, 14)	-6090.6	22.2	1.8	2	not significant

distinct calibration-sets based on replacement rates and posterior estimates for the split of the Eutardigrada derived from previous studies (Regier *et al.,* 2004; Rota-Stabelli *et al.,* 2013). For calibration-set-1, the clock was calibrated using the replacement rate of 0.001564 mutations per site per million years (mut/site/My), with standard deviation (SD) of 0.001012 mut/site/My, based on the analysis of 18S and 28S rDNA genes from a wide range of ecdysozoans (including the tardigrade clade), together with a permissive root prior of 579 Mya, SD 70 My (allowing sampling 95% of quantiles from 463 to 716 Mya) (Rota-Stabelli *et al.,* 2013). An alternative calibration strategy (calibration-set 2) was employed using, as a root prior, a more restrictive distribution (compared to the first) centred on 659 Mya (SD 20 My; to allow 95% of sampling between 626 and 691 Mya) and by constraining two nodes within the eutardigrade outgroup (Macrobiotidae 144 Mya, SD 15 My; Eutardigrada 453 Mya, SD 12 My); this substantially different approach is based on the results of Regier *et al.* (2004), who build a posterior time estimate for some tardigrade clades. To check for the effect of the dataset on divergence times, we repeated the molecular clock analysis on the reduced-dataset using both calibration-set 1 and calibration-set 2.

Results and discussion

Molecular clock analyses

Test of model fit

Results of the model selection based on Bayes factors (BF, Table 1) indicated that the best fitting model employed a combination of a relaxed exponential clock and a Birth-Death model of tree diversification. We used this combination of priors for subsequent analyses (Tables 2, 3). The difference in BF to other model combinations was quite low (BF 2.4 over the second ranking model), but enough to set this model as favoured. In general, model combinations involving a relaxed exponential were "strongly" favoured over the relaxed lognormal, the latter being in turn "very strongly" favoured over the strict and the random clock models. As for the tree priors, the difference between the Coalescent and the three Birth-Death type processes we tested was very low.

Because the difference of the marginal likelihood using different priors was extremely low, we advocated great care in interpreting the result of our model selection: Table 1 is however a good indication for future tardigrade clock studies using RNA sequences, rather than as decision maker to discriminate among competing time estimates.

Molecular dating

Our results for the tardigrade phylogenetic relationships, based on the best fitting priors deduced from Table 1 and using calibration-set 1 (Fig. 2), were congruent with previous studies (Jørgensen *et al.,* 2011; Guil and Giribet, 2012; Guil *et al.,* 2013; Vicente *et al.,* 2013; Guidetti *et al.,* 2014). By employing different calibration sets (Table 2), and testing the effect of site selection (Table 3), we have attempted to account for the uncertainty imbedded in molecular clock studies.

The analyses conducted using two different calibration sets (Table 2) provided different estimates. Nonetheless, from the results, it was possible to infer the

Table 2. Molecular dating using two different sets of calibration priors. Analyses were conducted using the complete dataset and the most fitting vide the mean estimates in millions of years for four nodes of interest; the heights of the 95% Highest Posterior Density are in parenthesis.

	Calibration priors			Posterior Age estimates (with 95% Highest Posterior Density)	
	Root prior	Costrains	Rate prior	Tardigrada (root): Hetero-Eutardigrada split	First Heterotardigrada split
Calibration-set 1	579 (716, 463)	none	0.00156	547 (689, 405)	405 (609, 191)
Calibration-set 2	659 (691, 626)	Eutard. (453 SD 12) Macro. (144 SD 15)	no prior	659 (697, 619)	521 (677, 314)
Average				603 (693, 512)	563 (643, 253)
Most estimates falling in:				Cryogenian/ Ediacaran	Cambrian (Paleozoic)

Eutard. = Eutardigrada origin. Macro. = Macrobiotidae origin. BF = Bayes factors

Table 3. Molecular clock analyses using reduced-datasets. Analyses were conducted using the most fitting relaxed clock and tree prior as defined nodes of interest are provided; the heights of the 95% Highest Posterior Density are in parenthesis.

	Calibration priors			Posterior Age estimates (with 95% Highest Posterior Density)	
	Root prior	Costrains	Rate prior	Tardigrada (root): Hetero-Eutardigrada split	First Heterotardigrada split
Calibration-set 1	579 (716, 463)	none	0.00156	548 (689, 405)	498 (592, 170)
Calibration-set 2	659 (691, 626)	Eutard. (453, SD 12) Macro. (144, SD 15)	no prior	662 (699, 622)	482 (657, 300)
Average				605 (694, 514)	490 (625, 235)
Most estimates falling in:				Cryogenian/ Ediacaran	Cambrian (Paleozoic)

Eutard. = Eutardigrada origin. Macro. = Macrobiotidae origin. BF = Bayes factors

root of the tree, i.e. when the Heterotardigrada and Eutardigrada lines split apart, showing to be at 659 and 547 Mya, which was before the Cambrian Period (Table 2). The two methods also produced similar mean estimates, 146 and 136 Mya (early Cretaceous), for the divergence of the lineage that led to *Mopsechiniscus*, though the 95% Highest Posterior Density (95% HPD) for this node was very wide (ranging from 294 to 38 Mya). Both methods placed the split between the phylogenetic lines of the *Mopsechiniscus* species (*M. franciscae*: Antarctica; Guidetti *et al.,* 2014 and *M. granulosus*: Chile; Jørgensen *et al.,* 2011) between 47.8 and 32.1 Mya (Paleogene, during late Eocene early Oligocene), with a 95% HPD of 131–3 Mya (Table 2; Fig. 2). Analyses conducted on the reduced-dataset produced similar estimates (Table 3; Fig. 3) with average data in line with: a possible pre-Cambrian origin of the Tardigrada; an early Cretaceous-Eocene origin for the lineage that led to *Mopsechiniscus*; a late Paleogene split between *M. franciscae* and *M. granulosus*.

Although the mean estimates were concordant for various nodes, particularly those describing the origin of *M. franciscae*, the range of visited posterior estimates was extremely large in all the analyses (see Tables 2 and 3 for a breakdown of the 95% HPD): overall, this indicated a large uncertainty in the precise estimation of tardigrade radiation using RNA 18S and 28S makers. This may be caused by a variety of reasons. First, the paucity of calibration priors currently available for tardigrades forced us in relying mostly on prior replacement rates with relatively high standard deviations: this likely reflected in highly uncertain posterior estimates. Second, the relatively short length of the alignment and the high amount of missing data may have inserted a high stochastic effect. This possibility was reinforced by the range of the HPD being slightly smaller when using a reduced (less missing data) dataset (compare Tables 2 and 3). Finally, the fast evolving nature of tardigrade genes (Campbell *et al.,* 2011) may have complicated the correct estimation of their divergence; this possibility is compatible with a study of Ecdysozoan evolution showing that tardigrade

relaxed clock and tree prior as defined in Table 1 (relaxed exponential clock plus Birth Death process). For each of the calibration sets, we pro-

		Model selection		
Origin of the line Leading to *Mopsechiniscus*	*M. franciscae-M. granulosus* split	ln BF	2 ln BF to previous	rank & significance
136 (260, 38)	32.1 (99, 3)	-6089.42	-	-
146 (294,43)	47.8 (131,3)	-6088.46	1.93	BEST, not significant
141 (277, 41)	40.0 (115, 3)			
Jurassic (Mesozoic)	Paleogene (Cenozoic)			

in Table 1 (relaxed exponential clock plus Birth Death process). For each of the calibration-sets, the mean estimates in millions of years for four

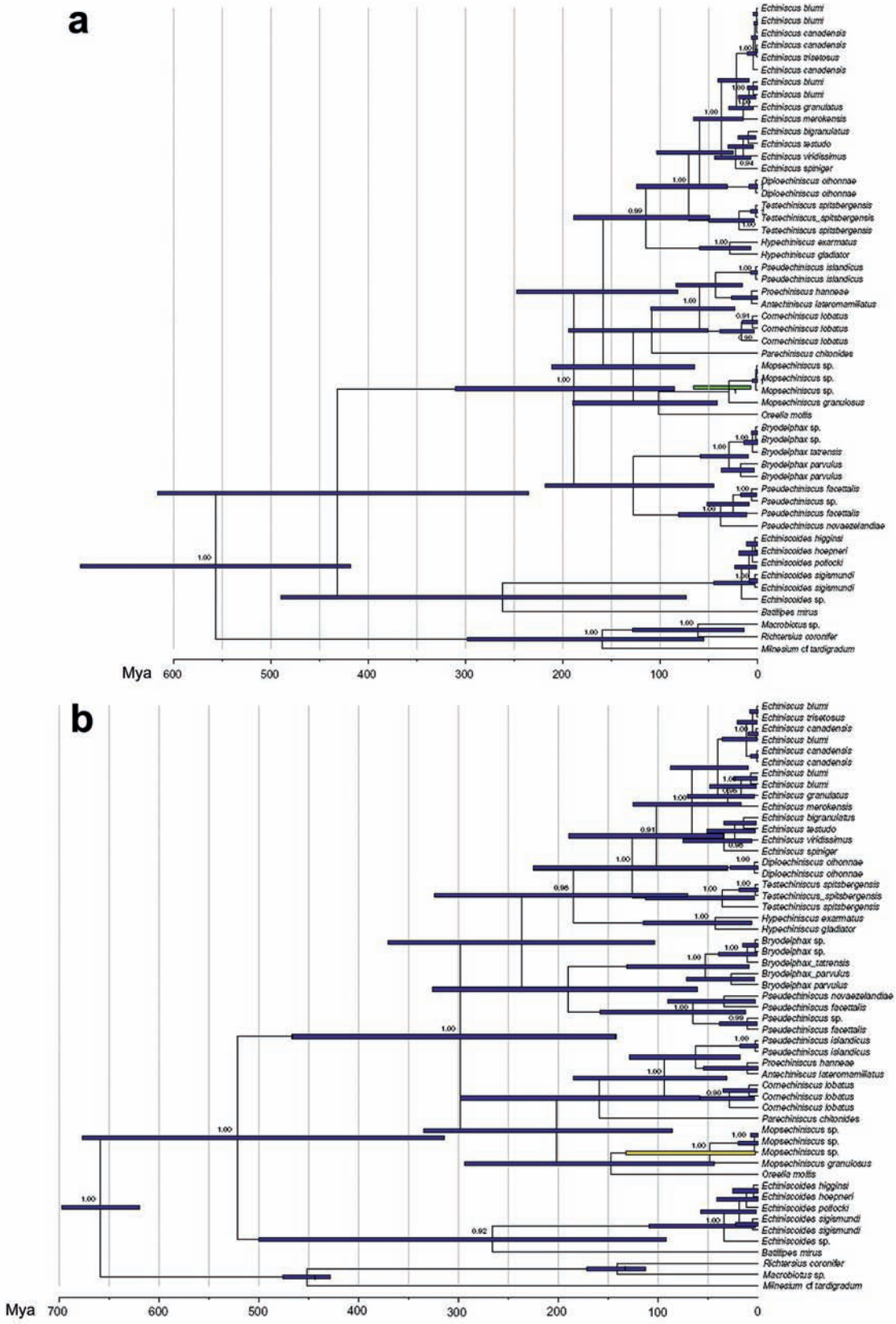
		Model comparison			
Origin of the line leading to *Mopsechiniscus*	*M. franciscae-M. granulosus* split	ln BF	ln BF to highest	2 ln BF to previous	rank & significance
124 (241, 36)	29.7 (91, 3)	-4281.27	lowest	lowest	3
178 (294, 66)	43.7 (124, 9)	-4277.19	4.07	8.15	2, strong
151 (268, 51)	36.7 (108, 6)				
Jurassic (Mesozoic)	Paleogene (Cenozoic)				

divergence was the most unstable within the sample, with estimates strongly varying with parameter variations (Rota-Stabelli *et al.,* 2013). Overall, our molecular dating of the Heterotardigrada indicated that their divergence estimate is a complex issue which should be tackled in the future by employing more markers and possibly outgroups to the tardigrades in order to allow external calibrations.

Origin and distribution of *Mopsechiniscus*

Biogeographical distribution and ecology of Mopsechiniscus *species*

Reports of *Mopsechiniscus* species are largely confined to the Southern Hemisphere, with the most northern reference to be found for Costa Rica, and the majority of references from South America (Venezuela, Brazil, Argentina, and Chile), southern Australia, sub-Antarctic, and continental Antarctica (Fig. 1). In particular, four of the six described species are considered endemic and have been found only in the type localities: *Mopsechiniscus imberbis* (Richters, 1907) from South Georgia, sub-Antarctic (Richters, 1907, 1920; Ottesen and Meier, 1990); *Mopsechiniscus franciscae* from Victoria Land, Antarctica (Guidetti *et al.,* 2014); *Mopsechiniscus tasmanicus* Dastych and Moscal, 1992 from Tasmania, Australia (Dastych and Moscal, 1992); and *Mopsechiniscus frenoti* Dastych, 1999, from the Île de la Possession, Crozet Islands, sub-Antarctic (Dastych, 1999c). *Mopsechiniscus schusteri* Dastych, 1999 has been reported from Venezuela (Grigarick *et al.,* 1983; Dastych, 1999a) and Costa Rica (Kaczmarek *et al.,* 2014); and *Mopsechiniscus granulosus* Mihelčič, 1967 has been reported from several Argentinean localities (Mihelčič, 1967, 1971/72; Binda and Kristensen, 1986; Kristensen, 1987; Rossi and Claps, 1989; Dastych, 2000; Rossi *et al.,* 2009; Roszkowska *et al.,* 2016) and Chile (Ramazzotti, 1962; Dastych and Moscal, 1992; Dastych, 2000). According to Dastych (1999a, 2001), there are two


a
Echiniscus blumi
Echiniscus blumi
Echiniscus canadensis
Echiniscus canadensis
Echiniscus trisetosus
Echiniscus canadensis
Echiniscus blumi
Echiniscus blumi
Echiniscus granulatus
Echiniscus merokensis
Echiniscus bigranulatus
Echiniscus testudo
Echiniscus viridissimus
Echiniscus spiniger
Diploechiniscus othonnae
Diploechiniscus othonnae
Testechiniscus spitsbergensis
Testechiniscus_spitsbergensis
Testechiniscus spitsbergensis
Hypechiniscus exarmatus
Hypechiniscus gladiator
Pseudechiniscus islandicus
Pseudechiniscus islandicus
Proechiniscus hanneae
Antechiniscus lateromamillatus
Comechiniscus lobatus
Comechiniscus lobatus
Comechiniscus lobatus
Parechiniscus chitonides
Mopsechiniscus sp.
Mopsechiniscus sp.
Mopsechiniscus sp.
Mopsechiniscus granulosus
Oreella mollis
Bryodelphax sp.
Bryodelphax sp.
Bryodelphax tatrensis
Bryodelphax parvulus
Bryodelphax parvulus
Pseudechiniscus facettalis
Pseudechiniscus sp.
Pseudechiniscus facettalis
Pseudechiniscus novaezelandiae
Echiniscoides higginsi
Echiniscoides hoepneri
Echiniscoides pollocki
Echiniscoides sigismundi
Echiniscoides sigismundi
Echiniscoides sp.
Batillipes mirus
Macrobiotus sp.
Richtersius coronifer
Milnesium cf tardigradum
Mya 600 500 400 300 200 100 0
b
Echiniscus blumi
Echiniscus trisetosus
Echiniscus canadensis
Echiniscus blumi
Echiniscus canadensis
Echiniscus canadensis
Echiniscus blumi
Echiniscus blumi
Echiniscus granulatus
Echiniscus merokensis
Echiniscus bigranulatus
Echiniscus testudo
Echiniscus viridissimus
Echiniscus spiniger
Diploechiniscus othonnae
Diploechiniscus othonnae
Testechiniscus spitsbergensis
Testechiniscus_spitsbergensis
Testechiniscus spitsbergensis
Hypechiniscus exarmatus
Hypechiniscus gladiator
Bryodelphax sp.
Bryodelphax sp.
Bryodelphax_tatrensis
Bryodelphax_parvulus
Bryodelphax parvulus
Pseudechiniscus novaezelandiae
Pseudechiniscus facettalis
Pseudechiniscus sp.
Pseudechiniscus facettalis
Pseudechiniscus islandicus
Pseudechiniscus islandicus
Proechiniscus hanneae
Antechiniscus lateromamillatus
Comechiniscus lobatus
Comechiniscus lobatus
Comechiniscus lobatus
Parechiniscus chitonides
Mopsechiniscus sp.
Mopsechiniscus sp.
Mopsechiniscus sp.
Mopsechiniscus granulosus
Oreella mollis
Echiniscoides higginsi
Echiniscoides hoepneri
Echiniscoides pollocki
Echiniscoides sigismundi
Echiniscoides sigismundi
Echiniscoides sp.
Batillipes mirus
Richtersius coronifer
Macrobiotus sp.
Milnesium cf tardigradum
Mya 700 600 500 400 300 200 100 0

more undescribed new species from Brazil (Du Bois-Reymond Marcus, 1944), and Chile. The distribution of *Mopsechiniscus* species point to a Gondwanan distribution for the genus, as has already been hypothesized (Dastych and Moscal, 1992; Dastych, 2001).

However, ecological data on *Mopsechiniscus* species are very limited (see Dastych, 2001). The species of this genus have been found in moss and lichens, rarely liverworts, at different altitudes. An association with environments rich in *Nothofagus* forests has also been noted (Ramazzotti, 1962; Kristensen, 1987; Dastych & Moscal, 1992). In sub-Antarctic islands, *Mospechiniscus* was found at 70-700 m a.s.l., in Tierra del Fuego at *ca*. 1000 m (above the tree line), in subtropical and/or cloud forest from 50 m up to 2000 m, suggesting a mountain and/or subpolar character of the genus (Dastych, 2001). The Antarctic species (*M. franciscae*) used in this study was found in a large patch of moss growing on gravel soil in a very small valley at Crater Cirque (Guidetti *et al*., 2014), while the South American species (*M. granulosus*) was collected at Angol in Chile, though the original substrate was not reported (Jørgensen *et al*., 2011).

Mopsechiniscus *in Antarctica*

Three scenarios could account for the presence of *Mopsechiniscus* in Antarctica, in addition to South America: 1) an early ancestor and speciation occurring early during the initial break-up of Gondwana (*ca*. 150 Mya); 2) speciation occurring after the break between Antarctica from South America (*ca*. 30 Mya); 3) recent colonisation via zoogenic introduction (e.g. penguins or sea birds), wind dispersal, and/or anthropogenic activities.

At the species level, modern molecular methods have revealed a remarkable level of endemism of the Antarctic biota, questioning the widespread assumption that small organisms are likely to be ubiquitous and the taxa to which they belong species poor (Chown and Convey, 2007). Recent molecular analyses showed that most Antarctic tardigrades appear to be locally endemic, with a greater diversity than had previously been considered. There were also potentially undescribed species, and a lack of connectivity between most Operational Taxonomic Units from continental Antarctica and those from other Antarctic geographical zones (Velasco-Castrillón *et al*., 2015). Endemism is very high for maritime and continental Antarctic tardigrade species (more than 80%), with less than 3% of the species in common with taxa from South America, and even fewer when comparing only continental Antarctic species (McInnes, 1994; Velasco-Castillo *et al*., 2014; Kaczmarek *et al*., 2015). Some species, e.g. *Acutuncus antarcticus* (Richters, 1904) and *Milnesium antarcticum* Tumanov, 2006, are widespread across continental Antarctica, while others have a very limited distribution within this continent (Sohlenius and Boström, 2005; Velasco-Castrillón *et al*., 2015; Cesari *et al*., 2016).

This situation is not limited to tardigrades, but occurs also in other representatives of the terrestrial meiofauna with cryptobiotic adaptations, like nematodes and rotifers. These animals show a very similar distribution pattern, e.g. all Victoria Land nematodes are endemic to Antarctica, and many are common and widely distributed at landscape scale (Adams *et al*., 2014). For rotifers, the level of endemism in Antarctica is 95% of the species, higher than any other continent, with many bdelloid species occurring only in maritime or continental Antarctica (Iakovenko *et al*., 2007). Molecular studies showed a widespread range for some rotifers in continental Antarctica, but only one bdelloid lineage from continental Antarctica was also present in maritime Antarctica, and no close similarities were found with worldwide locations, or between Antarctic Peninsula and Tierra del Fuego (Argentina) (Velasco-Castrillón *et al*., 2014).

Based on these findings, anhydrobiotic metazoans living in Antarctica (i.e. tardigrades, nematodes, and rotifers) show a restricted distribution, with high percentage of endemic Antarctic taxa, in spite of their potential for high dispersal. Therefore, it is extremely unlikely that the high number (33) of endemic tardigrade species in Antarctica (including two endemic genera) are the result of speciation after recent colonisation. The literature portrays a very limited and/ or endemic distribution for *Mopsechiniscus* species, and for continental Antarctica a very reduced presence of echiniscids. This information implies a relatively low dispersal capability for these taxa and, therefore, few possibilities for recent colonisation events. Although,

◀ *Fig 3*. **a** Molecular clock analysis of the Heterotardigrada using the rate deduced from Rota-Stabelli *et al*. (2013) and a Log-normal distribution clock analysis. **b** Molecular clock analysis of the Heterotardigrada using secondary node constraints from Regier *et al*. (2004). Values close to branches indicate posterior probability values (> 0.9) of BI analysis while bars indicate the 95% credibility intervals.

it cannot be excluded, the third scenario, a recent colonisation of Antarctica, is very unlikely for *Mopsechiniscus* species. If the presence of *M. franciscae* in Antarctica was the result of a recent colonization, this event would have to be considered extremely rare as no other tardigrade species reported from continental Antarctica have been recorded elsewhere in the world, the exception being a single record (to be confirmed) of *A. antarcticus* (the most common and widespread Antarctic tardigrade species) in Tierra del Fuego (Claps *et al*., 2008).

According to the molecular clock, the origin of the genus *Mopsechiniscus* can be placed somewhere between the origin of its phylogenetic line (i.e. when it separated from its sister taxon; 146–136 Mya) and the split between *M. franciscae* and *M. granulosus* (47.8–32.1 Mya) (Table 2). This is after Gondwana separated from Laurasia (*ca*. 200–180 Mya), but before the complete breakup of Gondwana (*ca*. 50–30 Mya). This time frame for the origin of the genus and the current distribution of *Mopsechiniscus* species (Fig. 1; see above) indicate a Gondwanan presence of the genus during the Mesozoic period.

If a more anterior time for the origin of *Mopsechiniscus* is chosen, e.g. close to the lower limit of the 95% HPD (294 Mya), then the genus would have been extant during the period of existence of the super-continent Pangea (from about 300 Mya until its breakup about 180 Mya). In this scenario, the distribution of the genus would have to have been confined to the Gondwana region of the Pangea throughout the 120 My of super-continent's duration. A later time for the origin of *Mopsechiniscus*, e.g. close to the upper limit of the 95% HPD (3 Mya), would require a very late colonisation of three continents and a subsequent rapid speciation within the genus. Again, not the most parsimonious option. According to Dastych (2001), and as discussed above, *Mopsechiniscus* represents a Gondwanan faunal element with a distribution pattern that is a result of historical factors and subsequent speciation, rather than purely dispersal events.

Our two main analyses (Tables 2, 3) and those used for model comparison (Table 1) returned congruent mean estimates, falling within a time range of 48-32 Mya, for the split between the Antarctic and the South American *Mopsechiniscus* lineages. This is compatible with the estimated separation of Antarctica and South America (e.g. Livermore *et al*., 2005), in which the opening of the Drake Passage prevented exchanges of organisms by land and reduced the dispersal ranges of species. The strongly debated estimate for the origin of this passage ranges approximately from 50 to 20 Mya, but in most cases not earlier than the cooling period that followed the Eocene/Oligocene boundary (33.7 Mya; Livermore *et al*., 2005). Recent geological studies also indicate that the Drake Passage opening is older than 28.5 Mya (Dalziel *et al*., 2013). Estimates for the general break-up of Australia, Antarctica and South America derived from a molecular clock analysis on *Nothofagus*, the southern beech with a Gondwanan distribution (Zhang, 2011), provided a range of 50–30 Mya (Cook and Crisp, 2005). All these independent studies on the dating of the geological events that separated Antarctica from South America indicate a time similar to our results for the separation of the respective *Mopsechiniscus* lineages.

Therefore, our molecular clock analyses support vicariance for the *M. franciscae* speciation caused by the separation of Antarctica and South America. The scenario of recent dispersal and re-colonisation is very unlikely, as stated previously, but due to the high 95% HPD range alternative scenarios cannot be excluded.

Interestingly, the proposed permanent glaciation of Antarctica at 34 Mya (Scher and Martin, 2006) could have accelerated the geographic isolation of *Mopsechiniscus* populations; the reduction of the available habitats increased the distance between, and reduced the number of, populations, with clear consequences for the subsequent speciation process. Therefore, the allopatric speciation of Antarctic tardigrades could be the consequence of two geographical separation/ isolation promoting events: the separation between Antarctica and South America and the glaciation of Antarctica.

If our hypothesis is correct, the vicariance events have separated the *Mopsechiniscus* lineages and isolated *M. franciscae* (and possibly other tardigrade endemic species) in Antarctica, implying that this species represents a relict faunal fragment that survived the Last Glacial Maximum (LGM; *ca*. 20,000 years ago). Even the lower 95% HPD limit (3 Mya) for the origin of the Antarctic *Mopsechiniscus* lineage would indicate a long presence in Antarctica, and the consequent survival through the extreme conditions present during the LGM.

Other possible scenarios for the presence of *M. franciscae* in Antarctica cannot be excluded, although we consider them unlikely for the reasons explained above. As *M. franciscae* is the only *Mopsechiniscus* species identified in Antarctica, other speciation processes (e.g. sympatric or parapatric speciation) are

very improbable. In principle, a recent colonization of Antarctica by *M. franciscae* either from unexplored regions or by another *Mopsechiniscus* species followed by speciation is possible. Both scenarios would imply a high dispersal capability and a wide distribution outside Antarctica; two characteristics for which there is currently no evidence, and therefore should be considered improbable. The presence of *M. franciscae* in Antarctica could represent the result of regional extinctions outside Antarctica, or of other *Mopsechiniscus* species (e.g. *M. granulosus*) within Antarctica. This scenario would suggest the coexistence of more than one *Mopsechiniscus* species in an area, a situation that has never been reported.

Mopsechiniscus *in the sub-Antarctic islands*

The presence of *Mopsechiniscus* on two sub-Antarctic islands is intriguing. South Georgia, where *M. imberbis* is found, is recognised as a micro-continent that has geological links with the Patagonian Andean Mountains, situated to the east of the Beagle Channel and attached to the southern margin of the Burdwood Bank (Dalziel *et al.,* 1975). There is some debate over the positioning and subsequent movement of this micro-continental plate during the late Cretaceous-early Paleogene period (Dalziel *et al.,* 1975; Barker, 2001; Thomson, 2004). Lawver and Gahagan (2003) provide a computerised continental break-up history of whole Antarctica, placing South Georgia to the south of Burdwood Bank and in close proximity to Tierra del Fuego. With the addition of our results for the estimated molecular clock division between two phylogenetic lineages (the Antarctic *M. franciscae* and the South American *M. granulosus*), the latter scenario is congruent with the hypothesis that *Mopsechiniscus* was present on South Georgia before it broke away from Tierra del Fuego.

Mopsechiniscus frenoti of the Crozet Islands is morphologically very similar to *M. franciscae* from Antarctica (Guidetti *et al.,* 2014) and, based on the available data, we can infer that the two species represent sister taxa within the genus. The volcanic rocks of the sub-Antarctic Crozet archipelago have a relatively recent origin (*ca.* 8.8 Mya; Giret *et al.,* 2003). However, the origins of the islands on the Crozet Plateau are debated (see Craig, 2003). Suggested interpretations of palaeogeographic data put these islands near India, close to Madagascar or off Antarctica over a period between late Cretaceous (*ca.* 70 Mya) to the Palaeocene (60–63 Mya) (Craig, 2003). The Crozet Islands host a number of indigenous species including plants, carabid beetles, and black flies (Chown *et al.,* 1998; Vernon *et al.,* 1999; Craig *et al.,* 2003). How these indigenous flora and fauna, and indeed *Mopsechiniscus*, reached the islands is unknown. Winds and birds are potential vectors, especially as the Crozet Islands would have been formed 1,000 km closer to Africa during the Palaeocene (Schlich *et al.,* 1974), but there is no evidence that passive long distance dispersal is common for *Mopsechiniscus*.

It is entirely possible that *Mopsechiniscus* species indigenous of South Georgia and the Crozet Islands could also be present in localities we have not yet discovered. Alternatively, they may have become isolated on the islands by traversing land/island bridges that have by now vanished.

Conclusions

A species' geographical distribution is the result of a variety of spatio-temporal events, including among others vicariant speciation, diffusion, jump dispersal, extinction, and colonisation. Although we were not able to give a definitive evidence, *Mopsechiniscus* was shown to be an ancient tardigrade genus with a Gondwanan distribution that has been further articulated at species level by geological events. Our molecular dating, with all its uncertainty, provided an estimate of when two *Mopsechiniscus* phylogenetic lineages separated. According to these results, the allopatric speciation of *M. franciscae* and *M. granulosus* could coincide with two geographical separation/ isolation promoting events: the break-up of Gondwana and the glaciation of Antarctica; our divergence estimates are indeed congruent with other independent studies in dating Gondwanan geological events. From a methodological point of view, our molecular clock results indicated that the molecular dating of tardigrades (using genes coding for ribosomal RNA) is extremely complicated and accompanied by a high degree of uncertainty revealed by: a very wide % HPD, a variable estimate when varying priors, and a relative difficulty in discriminating between competing models. Future studies could increase the number of genes and the taxa considered in the analyses, which should reduce the HPD values.

Overall, our results indicate that *Mopsechiniscus* can be used for biogeographical and temporal studies, paving the way to further studies to better understand the origin of Antarctic microfauna.

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