

Evolution of salinity tolerance in the diving beetle tribe Hygrotini (Coleoptera, Dytiscidae)

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Some species of the diving beetle tribe Hygrotini (subfamily Hydroporinae) are among the few insects able to tolerate saline concentrations more than twice that of seawater. However, the phylogenetic relationships of the species of Hygrotini, and the origin and evolution of tolerance to salinity in this lineage, are unknown. In this work, we aim to reconstruct how many times salinity tolerance did evolve in Hygrotini, whether this evolution was gradual or if tolerance to hypersalinity could evolve directly from strictly freshwater (FW) species, and to estimate the probabilities of transition between habitats. We build a phylogeny with ca. 45% of the 137 species of Hygrotini, including all major lineages and almost all of the known halophile or tolerant species. We used sequence data of four mitochondrial (COI-5', COI-3', 16S + tRNA and NADH1) and three nuclear (28S, 18S and H3) gene fragments, plus ecological data to reconstruct the history of the salinity tolerance using Bayesian inference. Our results demonstrate multiple origins of the tolerance to salinity, although most saline and hypersaline species were concentrated in two lineages. The evolution of salinity was gradual, with no direct transitions from FW to hypersaline habitats, but with some reversals from tolerant to FW species. The oldest transition to saline tolerance, at the base of the clade with the highest number of saline species, was dated in the late Eocene-early Oligocene, a period with decreasing temperature and precipitation. This temporal coincidence suggests a link between increased aridity and the development of tolerance to saline waters, in agreement with recent research in other groups of aquatic Coleoptera.

1 | INTRODUCTION

Hydroporinae is the most diverse of the subfamilies of diving beetles (Dytiscidae) (Nilsson & Hájek, 2017a) and its species also display a large variety of ecologies and life habits (Miller & Bergsten, 2016). Many species of Hydroporinae live in extreme or unusual environments for diving beetles, such as subterranean aquifers, forest litter or hypersaline waters. While the origin and evolution of subterranean and terrestrial lifestyles have received recent attention (e.g., Leys & Watts, 2008; Tierney et al., 2015; Toussaint, Hendrich, Escalona, Porch, & Balke, 2016), the origin of the species of diving beetles able to sustain extreme salt concentration has never

been addressed in a phylogenetic context, whereas for other families of aquatic Coleoptera, such as Hydrophilidae or Hydraenidae, comparative studies on the evolution of saline tolerance are already available (Arribas et al., 2014; Sabatelli et al., 2016).

Within Hydroporinae, species which are exclusively halophile or which can tolerate saline or hypersaline waters have independently evolved in three tribes, Bidessini, Hydroporini and Hygrotini (Miller & Bergsten, 2016). The latter includes some of the most extreme examples of saline tolerance, with some species able to sustain concentrations above 70 g/L, twice that of seawater (Picazo, Moreno, & Millán, 2010; Timms & Hammer, 1988). The physiological mechanism of

salt tolerance of *Hygrotus (Coelambus) salinarius* (Wallis, 1924) has been studied by Tones (1978). However, the species' phylogenetic relationships are unknown, and in consequence whether it may be related to other saline tolerant species of *Hygrotus* Stephens, 1828 or what could have been the origin of its saline tolerance.

With this work we aim to investigate the phylogenetic relationships among salt tolerant species of Hygrotini, and their relationships with the rest of the species of the tribe. This will allow to answer some basic questions such as: (i) how many times did salinity tolerance evolve in Hygrotini? (ii) was this evolution gradual (i.e., from FW to intermediate salinities to hypersaline), or could tolerance to hypersalinity have evolved directly from strictly FW species? (iii) which were the probabilities of transitions between habitats in the evolution of Hygrotini? and (iv) are there any general patterns in the geographic and temporal origin of salinity tolerance in Hygrotini?

To answer these questions we built a molecular phylogeny with almost half of the 137 known species of the tribe, including all genera and recognised main species groups (Nilsson & Hájek, 2017a,b), and compiled data on the ecological tolerances of all described species from the literature and our own observations. In addition, our results demonstrated that two of the four currently recognised genera of Hygrotini and one subgenus are para- or polyphyletic, revealing the need of a new classification of the tribe, which will be presented in a separate paper (Villastrigo, Ribera, Manuel, Millán, & Fery, 2017).

2 | MATERIALS AND METHODS

2.1 | Molecular data

We obtained molecular data from 101 specimens of 64 species, including all four currently recognised genera of Hygrotini: *Heroceras* Guignot, 1950, *Herophydrus* Sharp, 1880, *Hygrotus* (with two subgenera, *Hygrotus* and *Coelambus* Thompson, 1860) and *Hyphoporus* Sharp, 1880 (Nilsson & Hájek, 2017a,b; see Table S1). We used as outgroups a selection of 12 species from four genera belonging to Hydroporini, shown to be related to Hygrotini (Ribera, Vogler, & Balke, 2008). Trees were rooted on *Laccornis* Gozis, 1914, considered to be outside Hydroporini and Hygrotini and in a basal position within Hydroporinae (Miller & Bergsten, 2014; Ribera et al., 2008).

2.2 | DNA extraction and sequencing

Specimens were collected in the field and preserved in absolute ethanol. DNA was extracted using commercial kits (mostly DNeasy Tissue Kit, Qiagen, Hilden, Germany) following the instructions of the manufacturers. Vouchers and

DNA samples are kept in the collections of the Institute of Evolutionary Biology (IBE, Barcelona) and Museo Nacional de Ciencias Naturales (MNCN, Madrid). We sequenced fragments of seven genes in six sequencing reactions, three mitochondrial: (i) 5' end of cytochrome *c* oxidase subunit I (COI-5, "barcode" fragment of Hebert, Ratnasingham, & De Waard, 2003), (ii) 3' end of cytochrome *c* oxidase subunit I (COI-3), (iii) 5' end of 16S RNA plus the Leucine tRNA plus 5' end of NADH dehydrogenase subunit I (16S); three nuclear fragments: (iv) an internal fragment of the large ribosomal unit 28S RNA (28S), (v) an internal fragment of the small ribosomal unit, 18S RNA (18S) and (vi) an internal fragment of Histone 3 (H3). Details on primers used and typical polymerase chain reaction (PCR) conditions are provided in Table S2. Sequences were assembled and edited with Geneious v6.0.6 (Kearse et al., 2012); new sequences have been submitted to the EMBL database with accession numbers LT882773-LT883126 (Table S1).

2.3 | Phylogenetic analyses

Edited sequences were aligned using the online version of MAFFT 7 with the G-INS-I algorithm (Katoh, Asimenos, & Toh, 2009). For one species (*Hygrotus (Coelambus) pedalis* (Fall, 1901)) we pooled sequences of two specimens in a chimera to complete the data set (Table S1), after testing their monophyly with COI-3. We used PartitionFinder v1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012) to estimate the best fitting evolutionary model initially using one partition for each gene fragment except for COI (split in COI-5 and COI-3) and 16S and tRNA (pooled in a single partition), and applied Akaike Information Criterion (AIC) scores as selected criteria.

We used BEAST 1.8 (Drummond & Rambaut, 2007) for Bayesian phylogenetic analyses, using the partition and evolutionary models selected by PartitionFinder and a molecular-clock approach for estimating divergence times. We applied an uncorrelated lognormal relaxed clock to estimate substitution rates and a Yule speciation process as the tree prior. We calibrated the tree using rates estimated in Andújar, Serrano, and Gómez-Zurita (2012) for a genus of Carabidae (*Carabus*), in the same suborder Adephaga (rate of 0.0113 [95% confidence interval 0.0081–0.0147] substitutions per site per million years (subst/s/Ma) for COI-5; 0.0145 [0.01–0.0198] subst/s/Ma for COI-3 and 0.0016 [0.001–0.0022] subst/s/Ma for 16S + tRNA). Analyses were run for 100 million generations, assessing that convergence was correct and estimating the burn-in fraction with Tracer v1.6 (Drummond & Rambaut, 2007). We also used a fast maximum likelihood (ML) heuristic algorithm in RAxML-HPC2 (Stamatakis, 2006) in the CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010), using the same partition scheme as in BEAST with a GTR + G evolutionary model

independently estimated for each partition and assessing node support with 100 pseudoreplicas with a rapid bootstrapping algorithm (Stamatakis, Hoover, & Rougemont, 2008).

2.4 | Morphological data

To estimate the likely phylogenetic relationships of the species for which no molecular data could be obtained, we studied all described species of subgenera *Hygrotus* and *Coelambus* with the only exceptions of *H. (C.) artus* (Fall, 1919), known only from the holotype and considered to be possibly extinct (see Anderson, 1983), and *H. (C.) femoratus* (Fall, 1901), which is likely a junior synonym of *H. (C.) nubilus* (LeConte, 1855) (Anderson, 1983). We have also studied a large selection of species of *Herophydrus* and *Hyphoporus* (see Table S3 for the studied material).

The taxonomic classification of Hygrotini has suffered multiple changes, and some of the characters used to define genera (such as e.g., the morphology of the clypeus) are difficult to interpret and characterise (see e.g., Anderson, 1971; Balfour-Browne, 1934; Biström & Nilsson, 2002; Falkenström, 1933; Villastrigo et al., 2017). Species for which no molecular data were available were thus considered to be closely related to those showing a high morphological similarity, based both on external characters and on the female and male genitalia. We also recognised diagnostic characters or character combinations of the different clades of the molecular phylogeny and tested their presence in the species without molecular data (see Villastrigo et al., 2017 for more details). We used Mesquite v3.20 (Maddison & Maddison, 2017) to manually place all species in their most likely position in the phylogenetic tree, and collapsed uncertain nodes to create polytomies.

2.5 | Salinity tolerance data

We compiled ecological data on habitat preferences of all species of Hygrotini from bibliography and from our own observations (Table S3). Recent work on salinity tolerance in aquatic Coleoptera has recognised six different categories of habitat preferences: (i) FW (<0.5 g/L); (ii) mineralised (0.5–5 g/L); (iii) hyposaline (≥ 5 –20 g/L); (iv) mesosaline (≥ 20 –40 g/L); (v) hypersaline (≥ 40 –80 g/L); (vi) extreme

hypersaline (>80 g/L) (Arribas et al., 2014). As quantitative observations in species of Hygrotini were very scarce we reduced these categories to three: (i) species strictly bounded to FW environments (approximately <0.5 g/L), corresponding to category (i) above; (ii) species that can tolerate a wide range of salinities (approximately 0.5–40 g/L), corresponding to categories (ii–iv) above; and (iii) hypersaline species (approximately >40 g/L), corresponding to categories (v) and (vi) above. To reconstruct the evolution of saline tolerance we pruned the data set to one specimen per species and deleted the outgroups, using salinity tolerance as a qualitative trait. This reduced matrix was analysed in BEAST using the same settings as for the phylogenetic reconstruction, with an asymmetric substitution model for the trait reconstruction and dating the ancestral node according to the results of the previous analysis (with a Gamma distribution with shape 30 and scale 2.227). We also reconstructed the evolution of saline tolerance in the extended phylogeny, including species for which no molecular data were available, using parsimony in Mesquite.

3 | RESULTS

3.1 | Molecular phylogeny

The best partition schemes selected by PartitionFinder pooled the two fragments of COI and several of the nuclear genes, and favoured the most complex evolutionary models for most partitions (Table 1). The topological differences between the Bayesian analysis and the ML searches were minimal and always affected poorly supported nodes (Figures 1, S1), associated with the position of three species: *Hygrotus (Coelambus) fumatus* (Sharp, 1882), *Hygrotus (Coelambus) urgensis* (Jakovlev, 1899) and *Hygrotus (Hygrotus) hydropicus* (LeConte, 1852). In all cases, the monophyly of Hygrotini was recovered with strong support, as were most of the internal nodes (Figures 1, S1).

In all analyses, Hygrotini was divided into two lineages (posterior probability [pp] = 1; bootstrap support [BS] = 98): (A) three Nearctic and one Palaearctic species of subgenera *Hygrotus* and *Coelambus* and (B) the remaining species of the tribe (Fig. 1). The latter was in turn divided into four clades: (B1) a group of Palaearctic species of subgenus

TABLE 1 Best partition schemes and optimal evolutionary models as estimated with PartitionFinder

Complete data set			Reduced data set		
P	Genes	Model	P	Genes	Model
#1	COI-5 + COI-3	GTR + I + G	#1	COI-5 + COI-3	GTR + I + G
#2	16S + NAD1	GTR + I + G	#2	16S + NADH	GTR + I + G
#3	18S + 28S + H3	GTR + I + G	#3	18S + 28S	GTR + I
			#4	H3	GTR + I + G

P, partition.

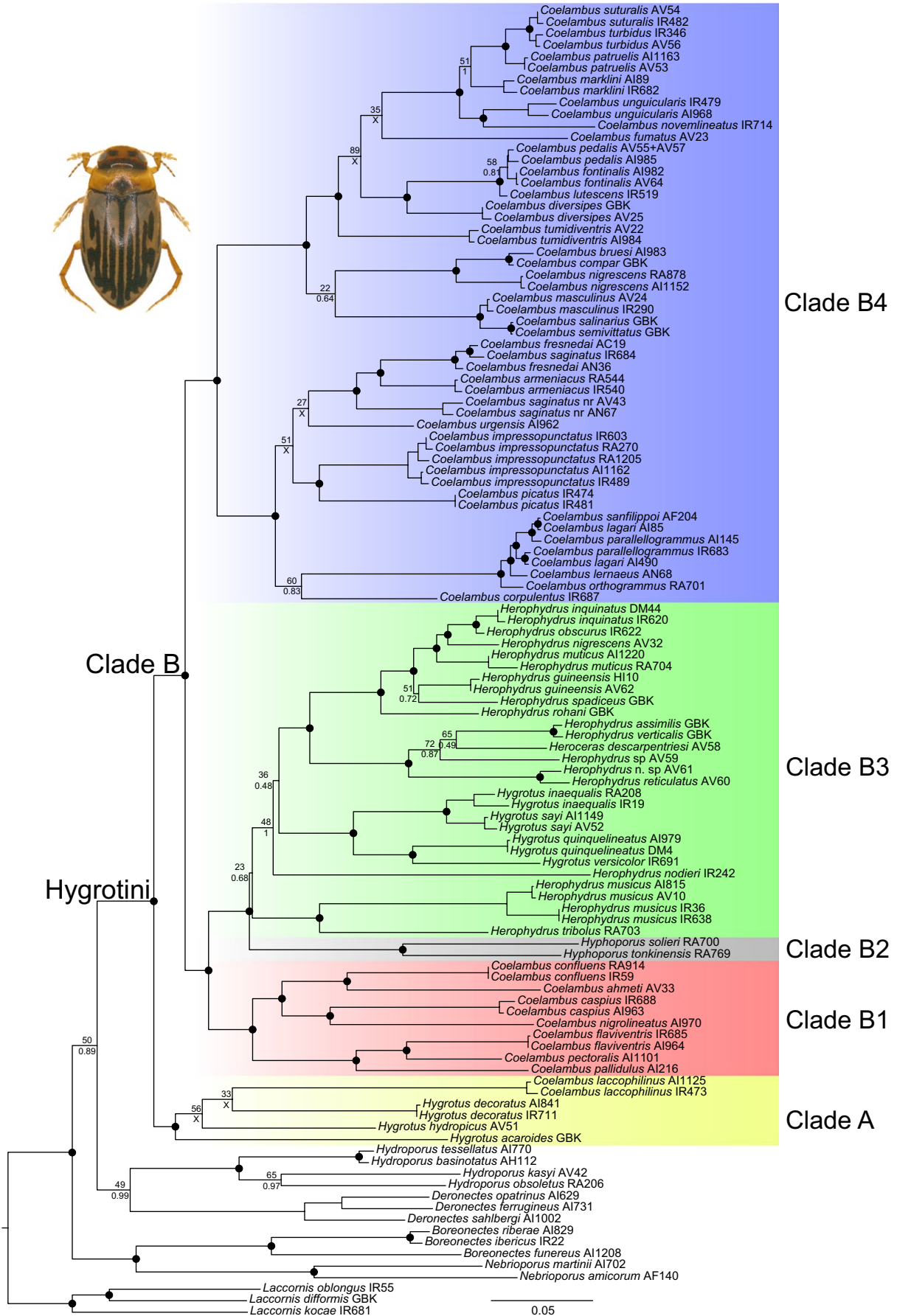


FIGURE 1 Best maximum likelihood (ML) phylogram obtained in RAxML with the extended dataset of Hygrotini (including outgroups and multiple terminals per species). Black circles, nodes with bootstrap support in RAxML (BS) >70 and posterior probability (pp) in BEAST >0.95; when support values were lower: numbers above nodes, BS; numbers below nodes, pp. X, nodes not recovered in the BEAST analyses (Fig. S1). See Table S1 for details on the specimens. For clarity, only the subgenus name is given in *Hygrotus* s.str. and *Coelambus*. Habitus photograph: *Hygrotus* (*Coelambus*) *lagari* (Fery, 1992) (from Millán et al., 2014)

Coelambus, (B2) the two sampled species of *Hyphoporus*, (B3) a large group of species including *Heroceras*, all sampled *Herophydrus* and most species of *Hygrotus* s. str., and (B4) the remaining species of subgenus *Coelambus*, in turn divided into two sister clades, one with mostly Palearctic species and a second with mostly Nearctic species.

The monophyly of all clades had strong support in both Bayesian and ML analyses except for clade B3 (Fig. 1). This clade included the genus *Herophydrus* as paraphyletic with respect to *Heroceras* and most species of the subgenus *Hygrotus*, the latter grouped in a monophyletic lineage. *Heroceras* and the sampled species of *Herophydrus* from Madagascar with the exception of *H. spadiceus* Sharp, 1882 formed a strongly supported clade (pp = 1, BS = 100), in turn placed (with lower support) inside a lineage with most of the remaining sampled African *Herophydrus* (Fig. 1).

The internal phylogeny of the main clades was generally in good agreement with the recognised species groups among subgenera *Hygrotus* and *Coelambus* based on morphology (see e.g., Anderson, 1971, 1976, 1983; Fery, 1992, 1995, 2003), although not with the phylogeny of *Herophydrus* obtained by Biström and Nilsson (2002).

3.2 | Evolution of tolerance to salinity

Differences between the analyses of the complete and reduced data set referred mostly to the position of *Hyphoporus* (clade B2). In the complete data set it was placed as sister to clade B3, and both sister to clade B1, with very strong support (Fig. 1). In the reduced data set, without outgroups and with only one terminal per species, it was placed as sister to clade B4, but with low support (Fig. 2). However, the uncertainty in the phylogenetic position of *Hyphoporus* (clade B2) did not have any effect on the reconstruction, as the two possible sister clades, B3 (Fig. 1) or B4 (Figures 2, S2), were reconstructed to have a FW ancestor, and thus, their common ancestor was also reconstructed as living in FW habitats.

According to the Bayesian reconstruction in the reduced data set, tolerance to salinity emerged independently from a FW ancestor at least ten times within three of the main lineages of Hygrotini: B1, B3 and B4 (Fig. 2). There were five subsequent independent transitions from tolerant to hypersaline (mainly in the Nearctic lineage of clade B4), although in some cases these transitions affected only the terminal branches in our phylogeny. Four of the transitions from FW to salinity tolerant and one of the transitions to hypersalinity led to clades with more than one species, and in only one clade

(B4) there have been three reversal transitions from tolerant to FW. No direct transitions from FW to hypersaline habitats were found in the reconstruction (Fig. 2).

The oldest transition to salinity tolerance, at the base of the clade with the highest number of saline species, was dated to the late Eocene-early Oligocene. Three of the transitions to hypersalinity tolerance were estimated to have occurred during the Oligocene, one of them with a further diversification in the early Pleistocene, including *H. (C.) salinarius* and *Hygrotus* (*Coelambus*) *masculinus* (Crotch, 1874; Fig. 2). Other transitions to hypersalinity tolerance affecting single species occurred in the middle Miocene (*Hygrotus* (*Coelambus*) *diversipes* Leech, 1966) and the Pleistocene (*Hygrotus* (*Coelambus*) *fontinalis* Leech, 1966; see Fig. 2).

The highest estimated transition rate was from FW to tolerant (1.97 transitions/branch/My [t/b/My]), followed by tolerant to hypersaline (1.45 t/b/My, Table 2). Direct transition from FW to hypersaline waters had the lowest rate (0.23 t/b/My), even lower than that estimated for reversal from hypersaline tolerance to FW (0.37 t/b/My, Table 2).

For most species without molecular data a close relative included in the phylogeny could be identified based on similarities in the genitalia or the external morphology, although in a few cases no obvious relatives could be identified, and the species were placed in an unresolved polytomy in the less inclusive clade to which they could be ascribed (Fig. S2). The parsimony reconstruction of salinity tolerance in this extended phylogeny gave similar results to the reconstruction using only the molecular data. The last common ancestor of Hygrotini was a FW species, as was the reconstructed ancestors of clades A, B2 and B3. For nodes including species with the three states of the trait, such as in clades B1 and B4, the ancestral reconstruction was ambiguous in the parsimony analysis (Fig. S2). On the contrary, in the Bayesian analysis of the reduced data set they were reconstructed as FW with a high probability (Fig. 2). Most of the tolerant or hypersaline species were included within clades with at least some other tolerant species, in agreement with the results obtained with the phylogeny using only species with molecular data (Figures 2, S2).

4 | DISCUSSION

4.1 | Molecular phylogeny

We obtained a robust phylogeny of *Hygrotini*, with good support for most internal nodes except for the monophyly of

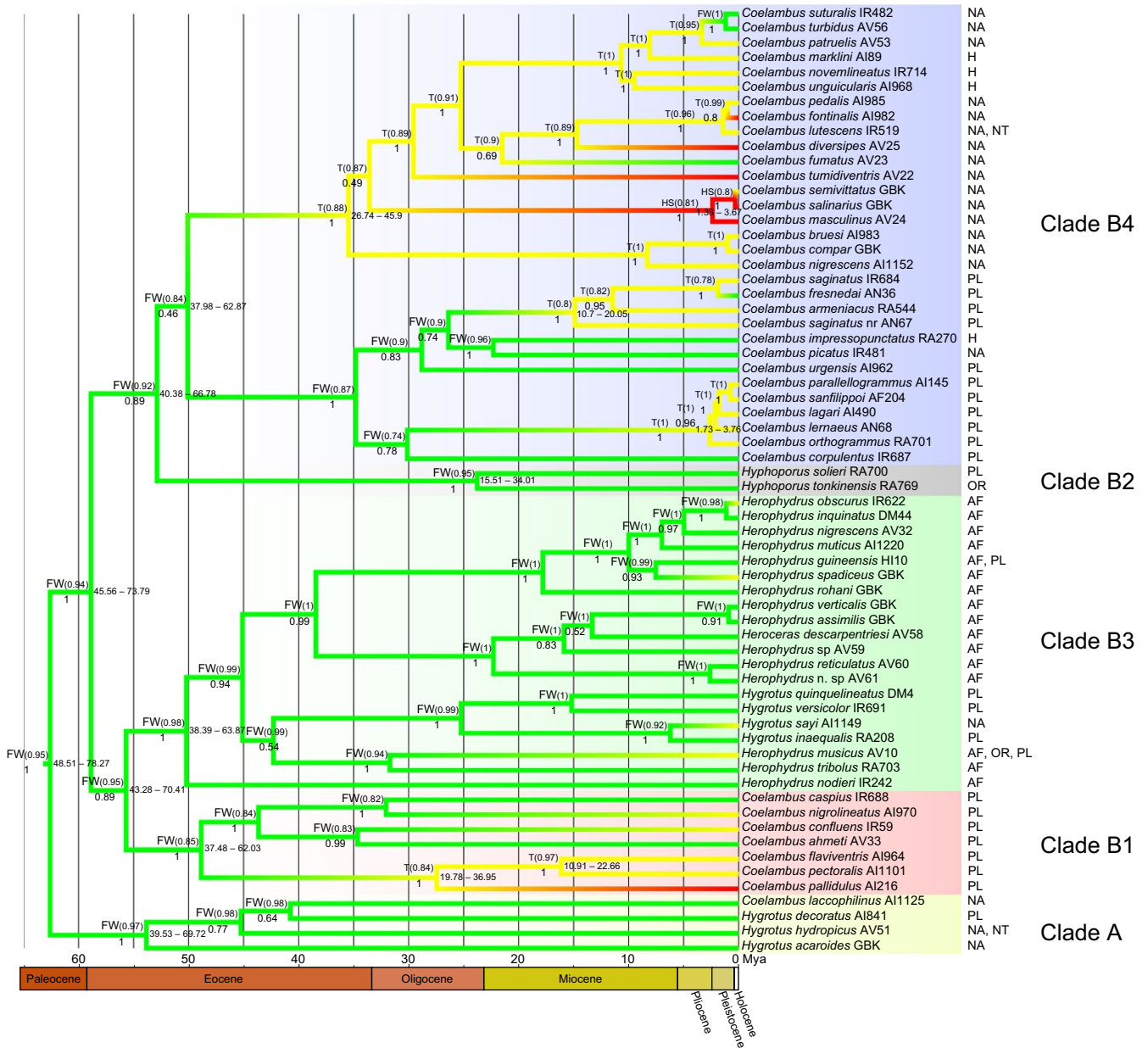


FIGURE 2 Reconstruction of the evolution of tolerance to salinity in the reduced data set of Hygotrini (excluding outgroups and with only one terminal per species) in BEAST. Letters above nodes: reconstructed state of the trait (FW, freshwater, green; T, tolerant, yellow; HS, hypersaline, red); numbers inside parentheses: posterior probability of the reconstructed discrete state; numbers below nodes: node support (pp); numbers inside nodes: 95% interval of the reconstructed age of selected nodes. For clarity, only the subgenus name is given in *Hygotrus* s.str. and *Coelambus*. See Tables S1 and S3 for details on the specimens and the ecological typification of the species, respectively. Geographic distribution: AF: Afrotropical; H: Holarctic; NA: Nearctic; NT: Neotropical (northern Mexico); OR: Oriental; PL: Palearctic

	Freshwater	Tolerant	Hypersaline
Freshwater	–	1.97 (0.33–3.99)	0.23 (<0.001–0.72)
Tolerant	1.23 (0.09–2.83)	–	1.4 (0.03–3.02)
Hypersaline	0.37 (<0.001–1.17)	0.73 (<0.001–1.9)	–

In brackets, 95% confidence interval.

TABLE 2 Estimated transition rates in BEAST between the discrete states of tolerance to salinity

clade B3 and its relationship with *Hyphoporus*. Our species sampling was also very dense except for *Herophydrus* and for *Hyphoporus*, of which we could obtain fresh material of

only two species. These two species, however, belong to the two different morphological groups recognised in the revision of the genus by Vazirani (1969).

The most unexpected result of our phylogeny was the deep division of Hygrotini in two clades, one of them formed by a small number of species. Despite the lack of clear synapomorphies, these species share some presumably plesiomorphic characters of body shape, colouration and male genitalia, the latter being in general simpler and more similar to those of other related tribes of Hydrophorinae. The internal relationships within clade A are still poorly defined, and would need further morphological and molecular studies to be clarified. Within clade B3, the close relationships of *Hygrotus* s. str., *Herophydrus* and *Heroceras* had been previously noted by many authors (Abellán et al., 2013; Alarie & Michat, 2007; Biström & Nilsson, 2002; Miller & Bergsten, 2014; Ribera, Hogan, & Vogler, 2002; Ribera et al., 2008), and suggested by the (incomplete) molecular and morphological phylogenies available prior to our study. Species of *Hyphoporus* have always been considered to be related to the species of *Herophydrus*, of which they differ mostly in the male genital shape (e.g., Biström & Nilsson, 2002; Miller & Bergsten, 2016). The non-monophyly of genera *Hygrotus* and *Herophydrus* and subgenera *Hygrotus* and *Coelambus* requires a revised classification of the tribe, which will be formalised in a separate paper (Villastrigo et al., 2017).

4.2 | Evolution of the tolerance to salinity in Hygrotini

We found several independent origins for the salinity tolerance in the tribe Hygrotini, with at least 10 transitions from FW to saline ecosystems both in the Palaearctic and the Nearctic. The multiple origin of saline species was expected, as they were included in different morphologically well characterised subgenera or species groups, but the number of transitions was lower than anticipated, as some of the saline species in clade B4 that were not thought to be related (as e.g., *Hygrotus* (*Coelambus*) *marklini* (Gyllenhall, 1813) and *H. (C.) salinarius*) were grouped in the same monophyletic radiation. In other families of aquatic Coleoptera, species that were apparently not closely related were also found to have a common origin of their tolerance to salinity, as for example in the genera *Ochthebius* Leach, 1815 (Hydraenidae; see Sabatelli et al., 2016) and *Enochrus* Thomson, 1859 (Hydrophilidae; see Arribas et al., 2014).

We did not find any direct transition from FW to hypersaline habitats, a transition that had the lowest estimated probability, suggesting that in Hygrotini the adaptation to salinity has been a gradual process. The only possible exception was *Hygrotus* (*Coelambus*) *pallidulus* (Aubé, 1850), sister to a group of tolerant species, but the clade was reconstructed as having a FW ancestor with high probability. However, *H. (C.) pallidulus* together with its tolerant sisters are included in a wider lineage with several other tolerant species of which no molecular data could be obtained, rendering the

condition of their common ancestor ambiguous (as can be seen in Fig. S2). This gradual evolution is in contrast to the direct transitions from FW to hypersaline tolerance found in a group of Mediterranean species of *Enochrus* (Arribas et al., 2014). These transitions were associated with periods of aridification of the climate, leading to the hypothesis that saline tolerance may have been a by-product (an exaptation) of adaptation to desiccation (Arribas et al., 2014; Pallarés, Velasco, Millán, Bilton, & Arribas, 2016). The capability to produce hyperosmotic excreta is a plesiomorphic character in insects, likely linked to the necessary adaptations to a terrestrial environment (Bradley, 2008; Bradley et al., 2009; Cloudsley-Thompson, 2001). In the only species for which the salinity tolerance mechanism is known, *H. (C.) salinarius*, adults maintain a hyposmotic haemolymph also with hyperosmotic excreta (Tones, 1978), with no evidence of any additional mechanism particular to this species. However, larvae of *H. (C.) salinarius* maintain a hyperosmotic haemolymph even at high saline concentrations (Tones, 1978), suggesting a different mechanism to that of adults. Although there is an increasing knowledge of the physiological basis of salinity tolerance in adult Coleoptera (e.g., Céspedes, Pallarés, Arribas, Millán, & Velasco, 2013; Pallarés, Arribas, Bilton, Millán, & Velasco, 2015; Pallarés, Arribas, Céspedes, Millán, & Velasco, 2012), the physiology of larval tolerance to salinity is still unknown. However, both the different characteristics of the cuticle (less sclerotised and without the protection of the elytra) and the biology (usually more strictly linked to the aquatic environment) suggest that the existence of different mechanisms to salinity tolerance in adults and larvae may be frequent.

In any case, it must be noted that we have data only on the ecological preferences of the species of Hygrotini, not on their physiological tolerances. If tolerance to salinity is an exaptation derived from a plesiomorphic adaptation to terrestrial environments (as hypothesised by Arribas et al., 2014; see Pallarés, Botella-Cruz, Arribas, Millán, & Velasco, 2017 for an experimental confirmation of the link between salinity and desiccation tolerance), tolerance to at least certain degree of salinity may be widespread even in species commonly found in FW habitats, as has been demonstrated to be the case in other groups of aquatic Coleoptera (Céspedes et al., 2013; Pallarés et al., 2015). Our ecological typification was also in most cases based on qualitative descriptions, without quantitative data. For some species there are few, if any, reports on their habitat, and in some cases we have assumed a FW habitat when the information was not very precise, as when species are found in saline or hypersaline habitats this is usually reported. In some cases qualitative reports can be ambiguous, such as for example when species are considered “halophile” or “halobiont” when they occur in slightly mineralised waters in a landscape otherwise lacking any true saline or hypersaline habitat, such as for example *Hygrotus*

(*Hygrotus*) *inaequalis* (Fabricius, 1777) or *H. (Coelambus) impressopunctatus* (Schaller, 1783) in some areas in central and northern Europe (e.g., Bellstedt, 2008).

In Hygrotini, the transitions to saline habitats were estimated to have occurred in different periods, from the late Eocene to the Plio- and Pleistocene, without a clear pattern of associations to arid periods, in contrast to what happened in the genus *Enochrus* in the Mediterranean region (Arribas et al., 2014). However, the oldest transition to saline habitats in Hygrotini, and the one leading to the higher number of saline species, occurred at the end of the Eocene in clade B4 in the Nearctic region, coincident with a global decrease in temperatures and the onset of the first Oligocene glaciations (Liu et al., 2009; Zachos, Pagani, Sloan, Thomas, & Billups, 2001). In North America, this decrease in temperature was associated with a decrease in precipitation (Retallack, 2007), leaving open the possibility that this transition to saline habitats was also a response to an increased aridification in this lineage.

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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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Table S2:

A) Primers used in the amplifying and sequencing reactions.

gene	primer	sequence	ref.
COI-3'	Jerry (5')	CAACATTTATTTTGATTTTTTGG	6
	Pat (3')	TCCAATGCACTAATCTGCCATATTA	6
	Chy (5')	T(A/T)GTAGCCCA(T/C)TTTCATTA(T/C)GT	4
	Tom (3')	AC(A/G)TAATGAAA(A/G)TGGGCTAC(T/A)A	4
COI-5'	Uni	TAATACGACTCACTATAGGGATTCAACCAATCATAAAGATATTGGAAC	2
	LepF1b		
	Uni	ATTAACCCTCACTAAAGTAACTTCTGGATGTCCAAAAAATCA	2
	LepR1		
16S+trnL+nad1 / 16S	16SaR (5')	CGCCTGTTTAACAAAAACAT	6
	ND1 (3')	GGTCCCTTACGAATTTGAATATATCCT	6
	16Sb	CCGGTCTGAACTCAGATCATGT	6
18S	18S 5'	GACAACCTGGTTGATCCTGCCAGT(1)	5
	18S b5.0	TAACCGCAACAACCTTTAAT(1)	5
H3	H3aF (5')	ATGGCTCGTACCAAGCAGACRCG	1
	H3aR (3')	ATATCCTTRGGCATRATRGTGAC	1
28S	ka	ACACGGACCAAGGAGTCTAGCATG	3
	kb	CGTCTGCTGTCTTAAGTTAC	3

B) Standard PCR conditions for the amplification of the studied fragments.

step	time	temperature
1	3'	96°
2	30''	94°
3	30''-1'	47-50° *
4	1'	72°
5	Go to step 2 and repeat 34-40 x	
6	10'	72°

* Depending on the annealing temperatures of the primers pair used

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Evolution of salinity tolerance in the diving beetle tribe Hygrotini (Coleoptera, Dytiscidae)

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Table S3

Checklist of the species of Hygrotini (following Nilsson & Hájek 2017a,b) with the typical habitat and the species for which material could be studied.

AF, Afrotropical; H, Holarctic; NA, Nearctic; NT, Neotropical; OR, Oriental; PL, Palaearctic

ID	Genus	Subgenus	Species	Authors	Distribution	habitat	data type studied
1	<i>Heroceras</i>		<i>descarpentriasi</i>	(Peschet, 1923)	AF	?	morphological & molecular
2	<i>Herophydrus</i>		<i>assimilis</i>	Régimbart, 1895	AF	?	morphological & molecular
3	<i>Herophydrus</i>		<i>bilardoi</i>	Biström & Nilsson, 2002	AF	?	only literature
4	<i>Herophydrus</i>		<i>capensis</i>	Régimbart, 1895	AF	?	only literature
5	<i>Herophydrus</i>		<i>cleopatrae</i>	(Peyron, 1858)	PL	?	morphological
6	<i>Herophydrus</i>		<i>confusus</i>	Régimbart, 1895	AF	?	only literature
7	<i>Herophydrus</i>		<i>discrepatus</i>	Guignot, 1954	AF	Tolerant	only literature
8	<i>Herophydrus</i>		<i>endroedyi</i>	Biström & Nilsson, 2002	AF	Freshwater	only literature
9	<i>Herophydrus</i>		<i>gigantoides</i>	Biström & Nilsson, 2002	AF	?	only literature
10	<i>Herophydrus</i>		<i>gigas</i>	Régimbart, 1895	AF	Freshwater	morphological
11	<i>Herophydrus</i>		<i>goldschmidti</i>	Pedernazi & Rocchi, 2009	AF	?	only literature
12	<i>Herophydrus</i>		<i>gschwendtneri</i>	Omer-Cooper, 1957	AF	?	only literature
13	<i>Herophydrus</i>		<i>guineensis</i>	(Aubé, 1838)	AF, PL	Freshwater	morphological & molecular
14	<i>Herophydrus</i>		<i>heros</i>	Sharp, 1882	AF	Freshwater	morphological
15	<i>Herophydrus</i>		<i>hyphoporoides</i>	Régimbart, 1895	AF	?	only literature
16	<i>Herophydrus</i>		<i>ignoratus</i>	Gschwendtner, 1933	AF	?	only literature
17	<i>Herophydrus</i>		<i>inquinatus</i>	(Boheman, 1848)	AF	Freshwater	morphological & molecular
18	<i>Herophydrus</i>		<i>janssensi</i>	Guignot, 1952	AF	?	only literature
19	<i>Herophydrus</i>		<i>kalaharii</i>	Gschwendtner, 1935	AF	?	only literature
20	<i>Herophydrus</i>		<i>morandi</i>	Guignot, 1952	OR	?	only literature
21	<i>Herophydrus</i>		<i>musicus</i>	(Klug, 1834)	AF, OR, PL	Tolerant	morphological & molecular
22	<i>Herophydrus</i>		<i>muticus</i>	(Sharp, 1882)	AF	Freshwater	morphological & molecular
23	<i>Herophydrus</i>		<i>natator</i>	Biström & Nilsson, 2002	AF	?	only literature
24	<i>Herophydrus</i>		<i>nigrescens</i>	Biström & Nilsson, 2002	AF	Tolerant	morphological & molecular
25	<i>Herophydrus</i>		<i>nodieri</i>	(Régimbart, 1895)	AF	Freshwater	morphological & molecular
26	<i>Herophydrus</i>		<i>obscurus</i>	Sharp, 1882	AF	Tolerant	morphological & molecular
27	<i>Herophydrus</i>		<i>obsoletus</i>	Régimbart, 1895	AF	?	only literature
28	<i>Herophydrus</i>		<i>ovalis</i>	Gschwendtner, 1932	AF	?	only literature
29	<i>Herophydrus</i>		<i>pallidus</i>	Omer-Cooper, 1931	AF	?	only literature
30	<i>Herophydrus</i>		<i>pauliani</i>	Guignot, 1950	AF	?	only literature
31	<i>Herophydrus</i>		<i>quadrilineatus</i>	Régimbart, 1895	AF	?	morphological
32	<i>Herophydrus</i>		<i>reticulatus</i>	Pederzani & Rocchi, 2009	AF	Freshwater	morphological & molecular
33	<i>Herophydrus</i>		<i>ritsemae</i>	Régimbart, 1889	AF	Freshwater	only literature
34	<i>Herophydrus</i>		<i>rohani</i>	Peschet, 1924	AF	Freshwater	molecular
35	<i>Herophydrus</i>		<i>rufus</i>	(Clark, 1863)	AF	?	morphological
36	<i>Herophydrus</i>		<i>sjostedti</i>	Régimbart, 1908	AF	?	only literature
37	<i>Herophydrus</i>		<i>spadiceus</i>	Sharp, 1882	AF	Tolerant	molecular
38	<i>Herophydrus</i>		<i>sudanensis</i>	Guignot, 1952	AF	Freshwater	only literature
39	<i>Herophydrus</i>		<i>travniceki</i>	Štátný, 2012	AF	?	morphological
40	<i>Herophydrus</i>		<i>tribolus</i>	Guignot, 1953	AF	Freshwater	morphological & molecular
41	<i>Herophydrus</i>		<i>variabilis secundus</i>	Régimbart, 1906	AF	?	only literature
42	<i>Herophydrus</i>		<i>variabilis variabilis</i>	Guignot, 1954	AF	?	only literature
43	<i>Herophydrus</i>		<i>vazirani</i>	(Nilsson, 1999)	PL	?	only literature
44	<i>Herophydrus</i>		<i>verticalis</i>	Sharp, 1882	AF	Freshwater	morphological & molecular
45	<i>Herophydrus</i>		<i>vittatus</i>	Régimbart, 1895	AF	?	only literature
46	<i>Herophydrus</i>		<i>wewalkai</i>	Biström & Nilsson, 2002	AF	?	only literature
47	<i>Hygrotus</i>	<i>Coelambus</i>	<i>ahmeti</i>	Hájek, Fery & Erman, 2005	PL	Freshwater	morphological & molecular
48	<i>Hygrotus</i>	<i>Coelambus</i>	<i>armeniacus</i>	(Zaitzev, 1927)	PL	Tolerant	morphological & molecular
49	<i>Hygrotus</i>	<i>Coelambus</i>	<i>artus</i>	(Fall, 1919)	NA	Tolerant (2)	only literature
50	<i>Hygrotus</i>	<i>Coelambus</i>	<i>berneri</i>	Young & Wolfe, 1984	NA	?	morphological
51	<i>Hygrotus</i>	<i>Coelambus</i>	<i>bruesi</i>	(Fall, 1928)	NA	Tolerant	morphological & molecular
52	<i>Hygrotus</i>	<i>Coelambus</i>	<i>caspius</i>	(Wehncke, 1875)	PL	Freshwater	morphological & molecular
53	<i>Hygrotus</i>	<i>Coelambus</i>	<i>chinensis</i>	(Sharp, 1882)	PL	?	morphological
54	<i>Hygrotus</i>	<i>Coelambus</i>	<i>collatus</i>	(Fall, 1919)	NA	?	morphological
55	<i>Hygrotus</i>	<i>Coelambus</i>	<i>compar</i>	(Fall, 1919)	NA	Tolerant	morphological & molecular
56	<i>Hygrotus</i>	<i>Coelambus</i>	<i>confluens</i>	(Fabricius, 1787)	PL	Tolerant	morphological & molecular
57	<i>Hygrotus</i>	<i>Coelambus</i>	<i>corpulentus</i>	(Schaum, 1864)	PL	?	morphological & molecular
58	<i>Hygrotus</i>	<i>Coelambus</i>	<i>curvilobus</i>	Fery, Sadeghi & Hosseini, 2005	PL	?	morphological
59	<i>Hygrotus</i>	<i>Coelambus</i>	<i>curvipes</i>	(Leech, 1938)	NA	?	morphological
60	<i>Hygrotus</i>	<i>Coelambus</i>	<i>dissimilis</i>	(Geminger & Harold, 1868)	NA	?	morphological
61	<i>Hygrotus</i>	<i>Coelambus</i>	<i>diversipes</i>	Leech, 1966	NA	Hypersaline	morphological & molecular
62	<i>Hygrotus</i>	<i>Coelambus</i>	<i>enneagrammus</i>	(Ahrens, 1833)	PL	Tolerant	morphological
63	<i>Hygrotus</i>	<i>Coelambus</i>	<i>falli</i>	(Wallis, 1924)	NA	?	morphological
64	<i>Hygrotus</i>	<i>Coelambus</i>	<i>femoratus</i>	(Fall, 1901)	NA	?	only literature
65	<i>Hygrotus</i>	<i>Coelambus</i>	<i>flaviventris</i>	(Motschulsky, 1860)	PL	Tolerant	morphological & molecular
66	<i>Hygrotus</i>	<i>Coelambus</i>	<i>fontinalis</i>	Leech, 1966	NA	Hypersaline	morphological & molecular
67	<i>Hygrotus</i>	<i>Coelambus</i>	<i>fraternus</i>	(LeConte, 1852)	NA	?	morphological
68	<i>Hygrotus</i>	<i>Coelambus</i>	<i>fresnedai</i>	(Fery, 1992)	PL	Freshwater (3)	morphological & molecular
69	<i>Hygrotus</i>	<i>Coelambus</i>	<i>fumatus</i>	(Sharp, 1882)	NA	Freshwater	morphological & molecular
70	<i>Hygrotus</i>	<i>Coelambus</i>	<i>impressopunctatus</i>	(Schaller, 1783)	H	Freshwater (3)	morphological & molecular
71	<i>Hygrotus</i>	<i>Coelambus</i>	<i>infuscatus</i>	(Sharp, 1882)	NA	Tolerant	morphological
72	<i>Hygrotus</i>	<i>Coelambus</i>	<i>inscriptus</i>	(Sharp, 1882)	PL	Hypersaline	morphological
73	<i>Hygrotus</i>	<i>Coelambus</i>	<i>laccophilinus</i>	(LeConte, 1878)	NA	Freshwater	morphological & molecular
74	<i>Hygrotus</i>	<i>Coelambus</i>	<i>lagari</i>	(Fery, 1992)	PL	Tolerant	morphological & molecular
75	<i>Hygrotus</i>	<i>Coelambus</i>	<i>lernaeus</i>	(Schaum, 1857)	PL	Tolerant	morphological & molecular
76	<i>Hygrotus</i>	<i>Coelambus</i>	<i>lutescens</i>	(LeConte, 1852)	NA	Tolerant	morphological & molecular

77	<i>Hygrotus</i>	<i>Coelambus</i>	<i>marklini</i>	(Gyllenhal, 1813)	H	Tolerant (4)	morphological & molecular
78	<i>Hygrotus</i>	<i>Coelambus</i>	<i>masculinus</i>	(Crotch, 1874)	NA	Hypersaline	morphological & molecular
79	<i>Hygrotus</i>	<i>Coelambus</i>	<i>nigrescens</i>	(Fall, 1919)	NA	Freshwater	morphological & molecular
80	<i>Hygrotus</i>	<i>Coelambus</i>	<i>nigrolineatus</i>	(Steven, 1808)	PL	Tolerant	morphological & molecular
81	<i>Hygrotus</i>	<i>Coelambus</i>	<i>novemlineatus</i>	(Stephens, 1829)	H	Tolerant	morphological & molecular
82	<i>Hygrotus</i>	<i>Coelambus</i>	<i>nubilus</i>	(LeConte, 1855)	NA, NT	?	morphological
83	<i>Hygrotus</i>	<i>Coelambus</i>	<i>obscuriplagiatus</i>	(Fall, 1919)	NA	?	morphological
84	<i>Hygrotus</i>	<i>Coelambus</i>	<i>orthogrammus</i>	(Sharp, 1882)	PL	Tolerant	morphological & molecular
85	<i>Hygrotus</i>	<i>Coelambus</i>	<i>pallidulus</i>	(Aubé, 1850)	PL	Hypersaline (1)	morphological & molecular
86	<i>Hygrotus</i>	<i>Coelambus</i>	<i>parallelogrammus</i>	(Ahrens, 1812)	PL	Tolerant	morphological & molecular
87	<i>Hygrotus</i>	<i>Coelambus</i>	<i>patruelis</i>	(LeConte, 1855)	NA	Tolerant	morphological & molecular
88	<i>Hygrotus</i>	<i>Coelambus</i>	<i>pectoralis</i>	(Motschulsky, 1860)	PL	Tolerant	morphological & molecular
89	<i>Hygrotus</i>	<i>Coelambus</i>	<i>pedalis</i>	(Fall, 1901)	NA	Tolerant	morphological & molecular
90	<i>Hygrotus</i>	<i>Coelambus</i>	<i>picatus</i>	(Kirby, 1837)	NA	Freshwater	morphological & molecular
91	<i>Hygrotus</i>	<i>Coelambus</i>	<i>polonicus polonicus</i>	(Aubé, 1842)	PL	Tolerant	morphological
92	<i>Hygrotus</i>	<i>Coelambus</i>	<i>polonicus sahlbergi</i>	(Sharp, 1882)	PL	Tolerant	morphological
93	<i>Hygrotus</i>	<i>Coelambus</i>	<i>punctilineatus</i>	(Fall, 1919)	NA	Tolerant	morphological
94	<i>Hygrotus</i>	<i>Coelambus</i>	<i>saginitus</i>	(Schaum, 1857)	PL	Tolerant	morphological & molecular
95	<i>Hygrotus</i>	<i>Coelambus</i>	<i>salinarius</i>	(Wallis, 1924)	NA	Hypersaline	morphological & molecular
96	<i>Hygrotus</i>	<i>Coelambus</i>	<i>sanfilippoi</i>	(Fery, 1992)	PL	Tolerant	morphological & molecular
97	<i>Hygrotus</i>	<i>Coelambus</i>	<i>sellatus</i>	(LeConte, 1866)	NA	Tolerant	morphological
98	<i>Hygrotus</i>	<i>Coelambus</i>	<i>semenowi</i>	(Jakovlev, 1899)	PL	Freshwater	morphological
99	<i>Hygrotus</i>	<i>Coelambus</i>	<i>semivittatus</i>	(Fall, 1919)	NA	Tolerant	morphological & molecular
100	<i>Hygrotus</i>	<i>Coelambus</i>	<i>stefanschoedli</i>	Fery, Sadeghi & Hosseinie, 2005	PL	?	morphological
101	<i>Hygrotus</i>	<i>Coelambus</i>	<i>suturalis</i>	(LeConte, 1850)	NA	Freshwater	morphological & molecular
102	<i>Hygrotus</i>	<i>Coelambus</i>	<i>sylvanus</i>	(Fall, 1917)	NA	Freshwater	morphological
103	<i>Hygrotus</i>	<i>Coelambus</i>	<i>thermarum</i>	(Darlington, 1928)	NA	?	morphological
104	<i>Hygrotus</i>	<i>Coelambus</i>	<i>tumidiventris</i>	(Fall, 1919)	NA	Hypersaline	morphological & molecular
105	<i>Hygrotus</i>	<i>Coelambus</i>	<i>turbidus</i>	(LeConte, 1855)	NA	Freshwater	morphological & molecular
106	<i>Hygrotus</i>	<i>Coelambus</i>	<i>unguicularis</i>	(Crotch, 1874)	H	Tolerant	morphological & molecular
107	<i>Hygrotus</i>	<i>Coelambus</i>	<i>urgensis</i>	(Jakovlev, 1899)	PL	Freshwater	morphological & molecular
108	<i>Hygrotus</i>	<i>Coelambus</i>	<i>wardii</i>	(Clark, 1862)	NA, NT	?	morphological
109	<i>Hygrotus</i>	<i>Coelambus</i>	<i>zigetangco</i>	Fery, 2003	PL	?	morphological
110	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>acaroides</i>	(LeConte, 1855)	NA	Freshwater	morphological & molecular
111	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>aequalis</i>	Falkenström, 1932	PL	Freshwater	morphological
112	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>decoratus</i>	(Gyllenhal, 1810)	PL	Freshwater	morphological & molecular
113	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>farctus</i>	(LeConte, 1855)	NA	?	morphological
114	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>hydropicus</i>	(LeConte, 1852)	NA	?	morphological & molecular
115	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>inaequalis</i>	(Fabricius, 1777)	PL	Freshwater	morphological & molecular
116	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>intermedius</i>	(Fall, 1919)	NA	Freshwater	morphological
117	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>marginipennis</i>	(Blatchley, 1912)	NA	Tolerant	morphological
118	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>quinquelineatus</i>	(Zetterstedt, 1828)	PL	Freshwater	morphological & molecular
119	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>sayi</i>	J. Balfour-Browne, 1944	NA	Tolerant	morphological & molecular
120	<i>Hygrotus</i>	<i>Hygrotus</i>	<i>versicolor</i>	(Schaller, 1783)	PL	Freshwater	morphological & molecular
121	<i>Hyphoporus</i>		<i>anitae</i>	Vazirani, 1969	PL	Freshwater	only literature
122	<i>Hyphoporus</i>		<i>aper</i>	Sharp, 1882	OR, PL	?	only literature
123	<i>Hyphoporus</i>		<i>bengalensis</i>	Severin, 1890	OR	?	morphological
124	<i>Hyphoporus</i>		<i>bertrandi</i>	Vazirani, 1969	PL	Freshwater	only literature
125	<i>Hyphoporus</i>		<i>caliginosus</i>	Régimbart, 1899	OR	?	only literature
126	<i>Hyphoporus</i>		<i>dehraduni</i>	Vazirani, 1969	PL	?	only literature
127	<i>Hyphoporus</i>		<i>elevatus</i>	Sharp, 1882	OR, PL	?	morphological
128	<i>Hyphoporus</i>		<i>geetae</i>	Vazirani, 1969	OR	?	only literature
129	<i>Hyphoporus</i>		<i>josephi</i>	Vazirani, 1969	OR	?	only literature
130	<i>Hyphoporus</i>		<i>kempi</i>	Gschwendtner, 1936	OR, PL	Tolerant	only literature
131	<i>Hyphoporus</i>		<i>montanus</i>	Régimbart, 1899	OR	?	only literature
132	<i>Hyphoporus</i>		<i>nilghiricus</i>	Régimbart, 1903	OR, PL	Freshwater	only literature
133	<i>Hyphoporus</i>		<i>oudomxai</i>	Brancucci & Biström, 2013	OR	?	only literature
134	<i>Hyphoporus</i>		<i>pacistanus</i>	Guignot, 1959	PL	?	only literature
135	<i>Hyphoporus</i>		<i>pugnator</i>	Sharp, 1890	OR	?	only literature
136	<i>Hyphoporus</i>		<i>severini</i>	Régimbart, 1892	OR, PL	Tolerant	morphological
137	<i>Hyphoporus</i>		<i>solieri</i>	(Aubé, 1838)	PL	Freshwater	morphological & molecular
138	<i>Hyphoporus</i>		<i>subaequalis</i>	Vazirani, 1969	OR	?	only literature
139	<i>Hyphoporus</i>		<i>tonkinensis</i>	Régimbart, 1899	OR	?	morphological & molecular

Notes

- 1 Although regularly found in low mineralized environments, it can tolerate hypersaline waters, in which it can be found in large numbers
- 2 Characterized as tolerant based on data of the type locality (the only known locality of the species)
- 3 Occasionally can be found in more mineralized environments, but their typical habitat is freshwater
- 4 In south Europe only found in freshwater habitats

Fig. S2. Reconstructed evolution of salinity tolerance in the tribe Hygrotini, using parsimony with the estimated relationships of species for which no molecular data were available.

