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ECOLOGICAL CONSTRAINTS FROM INCUMBENT CLADES DRIVE TRAIT EVOLUTION ACROSS THE TREE-OF-LIFE OF FRESHWATER MACROINVERTEBRATES

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Abstract

The rates of species and trait diversification vary across the Tree-of-Life and over time. Whereas species richness and clade age generally are decoupled, the correlation of accumulated trait diversity of clades (trait disparity) with clade age remains poorly explored. Total trait disparity may be coupled with clade age if the growth of disparity (disparification) within and across clades is continuous with time in an additive niche expansion process (linear-cumulative model), or alternatively if the rate of trait disparification varies over time and decreases as ecological space becomes gradually saturated (disparity-dependent model). Using a clock-calibrated phylogenetic tree for 143 freshwater macroinvertebrate families and richness and trait databases covering >6400 species, we measured trait disparity in 18 independent clades that successively transitioned to freshwater ecosystems and analyzed its relation with clade age. We found a positive correlation between clade age and total disparity within clades, but no relationship for most individual traits. Traits unique to freshwater lifestyle were highly variable within older clades, while disparity in younger clades shifted towards partially terrestrial lifestyles and saline tolerance to occupy habitats previously inaccessible or underutilized. These results argue that constraints from incumbent lineages limit trait disparity in younger clades that evolved for filling unoccupied regions of the trait space, which suggests that trait disparification may follow a disparity-dependent model. Overall, we provide an empirical pattern that reveals the potential of the disparity-dependent model for understanding fundamental processes shaping trait dynamics across the Tree-of-Life.

Key words: biological traits, trait disparity, functional traits, macroecology, niche filling, macroevolution, stream ecology

Introduction

Global biodiversity is not evenly distributed in space and time. Some areas on Earth are very rich in species whereas others are depauperate, and some deep lineages of the Tree-of-Life contain millions of species whereas others hold very few (Gaston and Blackburn 2000). Understanding this variation of global biodiversity and, particularly, the underlying processes that drive these patterns is a key challenge in macroecology and macroevolution (Swenson 2011). The simplest scenario of macroevolutionary diversification is a constant rate of species diversification through time (Magallón and Sanderson 2001; McPeck and Brown 2007). Under this model, species richness should be positively correlated with clade age because older clades have had more time for accumulating species than younger clades. However, clade-specific extinction may cause a lack of positive correlation between clade age and species diversity (Pyron and Burbrink, 2012) or the rate of diversification may vary during lineage evolution (Rabosky 2009). For instance, the diversification rate may be extraordinarily high early in the evolution of a lineage due to new ecological opportunities or the extinction of antagonists (Rabosky and Lovette 2008; Phillimore and Price 2008). As the number of species increases during a radiation, the progressive filling of ecological space is expected to gradually decrease the diversification rate (Jønsson et al. 2012), resulting in a diversity-dependent diversification process (Rabosky and Lovette 2008; Moen and Morlon, 2014).

Despite evidence for the role of functional traits on diversification rates (Adams et al. 2009), most studies of biodiversity patterns across the Tree-of-Life assess clade diversity based exclusively on species richness (Swenson 2011) and make inferences about the diversification of functional traits only indirectly from the dynamics of lineage growth. One might expect that trait divergence promotes diversification (Jønsson et al. 2012), but the accumulation of trait disparity (i.e., variance of traits for a given group of taxa) in a lineage driven by abiotic and biotic interactions may be decoupled from species richness (Adams et al. 2009; Burbrink et al. 2012; Bapst et al. 2012). Species diversification impacts the disparification in biological (e.g., life cycle duration, maximal body size, feeding habits, or reproduction) and ecological traits (e.g., traits reflecting associations with the environment, such as temperature, physical structure, or available nutrients). Therefore, in addition to assessing the accumulation of species richness, a holistic understanding of biodiversity should comprise other dimensions such as biological and ecological trait disparity (BTD and

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ETD) and their inter-relationships across the Tree-of-Life (Swenson 2011). To date, the majority of neontological studies that attempted to correlate the accumulation of trait diversity over time (disparification) and the rate of cladogenesis were limited to particular taxonomic lineages (Barraclough et al. 1999; Adams et al. 2009) and only a few biological traits, usually body size and body shape (Burbrink and Pyron 2010; Jönsson et al. 2012; Mahler et al. 2012). Tests of the null expectation that phenotypic disparity within a lineage increases gradually as a function of time since a common ancestor (Brownian Motion evolution; Harmon et al. 2010), resulted in contradictory outcomes (Harmon et al. 2010; Rabosky and Adams 2012; Burbrink et al. 2012). The inclusion of fossil data increased the fit of time-heterogeneous models of trait evolution (Slater et al. 2012), whereby early bursts of morphological disparity at low taxonomic diversity, which could enhance the probability of lineage establishment (Foote 1997; Erwin 2007), are followed by the slow, neutral accumulation of trait differentiation (Hunt 2007; Knope et al. 2015). However, patterns of extant trait disparity across the Tree-of-Life and its relation with clade age remain unexplored. Here, we test differences in extant BTD and ETD for multiple traits across numerous lineages that successively transitioned to freshwater ecosystems and thus differ in clade age. Such patterns may provide fundamental insights into the evolutionary path to accumulation of trait disparity of Earth's biotas.

Ecological transitions to novel habitats potentially promote trait disparity, species diversity, or both, by opening new adaptive zones (Simpson 1944, 1953; Burbrink and Pyron 2010), but phylogenetic studies on entire lineages have revealed that trait and species diversification are largely decoupled (Slater et al. 2010; Burbrink et al. 2012). One type of disparification model is characterized by an early rapid rise that abruptly increased disparity among lineages followed by subsequent rate decrease along with shrinking ecological opportunities (Harmon et al. 2003; Jönsson et al. 2012, Mahler et al. 2012), whereas other lineages showed continuous directional disparification to reach a particular trait value (Harmon et al. 2010). However, a more powerful test of the drivers of extant species diversity and trait disparity could be based on the analysis of multiple lineages that successively transitioned to the same habitat and whose interactions may affect the outcome of the diversification process in any of these lineages (Mahler et al. 2012). Many lineages of invertebrates have undergone transitions from terrestrial or marine habitats to freshwater, which can be used as a test case for the ecological and evolutionary processes following the successive transitions to novel

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environments. These lineages of various specific ages and presumably different ancestral life styles gradually accumulated in the modern freshwater ecosystems where they experienced competition or ecological constraints with incumbent lineages (Betancur-R et al. 2012).

Unlike the broad studies of correlates of species richness, a framework for testing the patterns of trait disparity over evolutionary time still remains to be developed (but see Betancur-R et al. 2012). Under a scenario of time dependence (Fig. 1A), a positive correlation between total trait disparity and clade age can be obtained if disparification rate is constant within and across clades over time following a “linear-cumulative model”. In this case, the amount of both total and individual trait disparity along a branch should be proportional to the corresponding branch length and therefore older lineages should have accumulated more BTD and ETD than younger lineages (Pagel 1999; Claramunt 2010). The same pattern of positive correlation for total trait disparity in time could be obtained also under a “disparity-dependent model”. In this case a slowdown in the rate of disparification within and across clades through time is predicted by saturation of ecological niche space at some (early) point in lineage evolution as disparity of already established species increases (i.e., clade-specific limits of individual-trait disparity) (Burbrink and Pyron 2010; Burbrink et al. 2012). Under this model, younger clades should have fewer ecological opportunities and reach lower disparification than older clades, but in addition may evolve new specific traits to adapt to ecological space underutilized by older clades (Burbrink and Pyron 2010; Jönsson et al. 2012; Mahler et al. 2012). If extant disparity of certain traits is higher in younger than older clades (Fig. 1A, central column) and younger clades diversified in traits for filling unoccupied regions of trait space (Fig 1A, right column), this pattern may support the disparity-dependent model. Alternatively, under a scenario of heterogeneous trait disparity across clades (Fig. 1B), which includes varying clade-specific disparification rates, evolutionary trait lability or limits to total maximum disparity, a weakened relationship between clade age and extant BTD and ETD within clades is expected, leading to age-independent disparification.

To investigate these hypotheses, we conducted an ecosystem-focused phylogenetic analysis for all families of freshwater macroinvertebrates in Europe and identified the most inclusive clades that have independently transitioned to the freshwater habitat at different times, thus providing a chronosequence of

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clade ages across the Tree-of-Life to test for the relationship between clade age and extant BTD and ETD. Each “clade” here refers to a lineage of any age derived from an ancestor that marks the independent transition to freshwater ecosystems, which provides an independent evolutionary replicate to test lineage disparification in the face of other such co-existing “clades”. These freshwater clades coincide with Linnaean categories at two taxonomic ranks: Order (e.g., Plecoptera, Trichoptera) and Infraorder or Superfamily (e.g., Culicomorpha, Tipulimorpha, and Empidoidea within Diptera; Planarioidea within Tricladida; Astacoidea within Decapoda; or Unionoidea and Veneroidea within Bivalvia) (Table 1; Supplementary material Table S1). The study was geographically limited to Europe because of the availability of high quality datasets: the entire taxonomical dataset included 8800 species and trait data at the genus level compiled from extensive literature sources included a total of 348 genera comprising >6400 species (>95% of the European diversity).

Freshwater macroinvertebrates are key organisms in rivers because they contribute to several important functional processes, such as litter decomposition, filtration and sediment retention (Tachet et al. 2010). In direct response to the flow constraints of rivers, freshwater macroinvertebrates acquired traits to limit downstream drifting or to cope with extraordinary hydrological events such as floods and droughts (Lytle and Poff 2004). River flow also produces a continuous change of habitat through erosion and deposition that increase habitat (e.g., riffle, pools) and substrate (e.g., boulders, gravel, sand) heterogeneity, promoting additional habitat specialization and greater trait disparity within clades (Resh et al. 1988; Lytle and Poff 2004). Freshwater macroinvertebrates thus display a myriad of traits affecting performance under various environmental conditions in the river ecosystem. These traits can be classified as BTD (related to life cycle duration, trophic guilds, respiration type) and ETD (reflecting niche associations with the physical environment: temperature, current velocity, salinity, pH, substrate). Major lineages exhibit great variation in most of these traits, but trait variation is not distributed uniformly among clades. In addition, phylogenetically distant species drawn from most of the Tree-of-Life co-occur in a single stream reach (Moss 1998), and hence several lineages may interact with one another, which allows us to test the disparity-dependent model. As many traits are highly variable and their character states (categories) overlap greatly in the various clades that comprise an ecosystem, we cannot simply study the increase of trait variation over time, in analogy to analyses of the accumulation of species numbers. Instead, establishing the disparity for all genera within each

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clade allows a possible expression of total trait disparity. This genus-level disparity can be expressed for each clade by summing the variance of genus-level trait variation, which provides a cumulative measure of disparity for each clade across the genera that compose the clade, as well as a measure of the evolutionary lability of traits. Given the inferred age of these clades, we link trait disparity within a clade to the time of the clade's origin. This provides the possibility to reconstruct the impact of co-existing lineages on each other's propensity to acquire adaptive trait disparity over evolutionary time.

Materials and methods

Phylogenetic tree and clade ages

A list of all freshwater macroinvertebrate families in Europe was built using information in Tachet et al. (2010) and DNA sequences of two or three European genera per family were compiled from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). The final dataset included molecular information for 238 genera distributed among 143 families for the mitochondrial Cytochrome *c* oxidase subunit I (COI, 1495 bp) and 16S rRNA genes (16S, 570 bp), and the nuclear 18S rRNA gene (18S, 3364 bp) (see GenBank accession numbers in Supplementary material Table S2). Molecular data were missing only for the family Dixidae, which was not included in the final dataset.

The COI sequences were aligned in MAFFT v.6 (Katoh et al. 2005), using the G-INS-i strategy. The 16S and 18S sequences were aligned individually using ProbconsRNA 1.1 (Do et al. 2005) and ambiguously aligned regions were removed using Gblocks (Castresana 2000), with the minimum length of block set to 5 and the 'With Half' option for gap allowance. To minimize the possibility of utilizing non-orthologous loci such as numts (nuclear copies of mtDNA), the COI sequences were carefully checked for stop codons and tree topologies of 16S and COI gene trees were compared for incongruences. The best-fit model of nucleotide substitution for each gene was selected using the Akaike Information Criterion in jModelTest (Posada 2008). A test of substitution saturation (Xia et al. 2003; Xia and Lemey 2009) was performed for each gene in DAMBE5 (Xia, 2013). Bayesian phylogenetic analyses were performed in BEAST v. 1.6.2 (Drummond and Rambaut 2007), using an uncorrelated lognormal relaxed clock to account for rate heterogeneity among lineages and a Yule tree prior. The mean substitution rate of the three gene fragments was fixed arbitrarily to

1, while a separate clock model and a separate substitution model was applied to each gene partition. Given that subsequent analyses did not require absolute ages, and taking into account the limitations of calibrating a phylogeny using fossils in the absence of a comprehensive fossil record for many of the major clades, we opted to use relative times. Alternative methods for relaxing the molecular clock hypothesis were not evaluated due to the large size of the dataset. The convergence and mixing of the MCMC chains was assessed using Tracer 1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>). Samples from three independent runs (50 million generations each, sampling every 5,000th generation) were pooled after removing a 10% burn-in using LogCombiner v1.6.2 and the means of the node heights were summarized on a 'maximum clade credibility' tree using TreeAnnotator v.1.6.2 (Drummond and Rambaut 2007).

Some of the 143 freshwater families used in the phylogenetic analysis are members of clades that also include genera confined to terrestrial and marine ecosystems. For the analysis of disparity of freshwater communities, we only used those monophyletic lineages at the Order and Infraorder/Superfamily levels comprised of genera that were confined exclusively to freshwater ecosystems (Table 1). This selection resulted in a subset of 18 clades that included 98 families, 759 genera, and 6451 species. Relative clade age was used instead of absolute time because of the lack of calibration points for molecular clock estimations across the entire tree. For those clades in which the whole Order was confined to freshwater ecosystems (e.g., Ephemeroptera, Odonata), stem age was estimated, whereas for those clades at Infraorder/Superfamily level that secondarily colonized freshwater habitats and their close relatives in the Order were terrestrial and not considered in this phylogeny, the mean distance between stem and crown age was used because stem age alone would overestimate aquatic-clade ages (e.g., Decapoda, Diptera) (Fig. 2, Table 1). Finally, clade age was standardized between 0 and 1 by $y = 0.01 + ((x - \min(x)) / (\max(x) - \min(x))) \times 0.09$ with value of “0.01” representing the youngest origin and value of “1” corresponding to the oldest group.

Taxonomic richness and trait disparity

A taxonomic list of the number of genera and species per family was acquired from www.freshwaterecology.info, which gathers biological information on more than 8,800 European macroinvertebrate species. Due to high variation in number of families among clades, which can bias the

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comparison of total species richness among clades (e.g., Planariidae included 2 families whereas Trichoptera included 23), taxonomic richness was measured as the mean number of genera or species per family within a clade.

Trait data were extracted from Tachet et al. (2010), Stötzner et al. (2007) and Bonada and Dolédec (2011). These databases include information on biological traits (e.g., morphology, life history, behavior) and habitat preferences (e.g., substrate, current velocity, temperature preferences) of European freshwater macroinvertebrates genera. Trait information was organized into 11 biological traits (61 trait categories) and 9 ecological traits (41 trait categories) that synthesized all ranges of variation within each trait (Supplementary material Table S3). For example, the biological trait “aquatic stage” has four categories: egg, larva, nymph, imago; and the ecological trait “temperature” has three categories: cold (<15°C), warm (>15°C), eurythermic. For each genus and trait category, a quantification (known as fuzzy coding; Chevenet et al. 1994) of the affinity (a_{ij}^k) of the considered species belonging to each genus i ($1 < i < t$) for each category k ($1 < k < h$) of a given trait j ($1 < j < v$) was assigned, (i.e., from 0 if the species belonging to the given genus has no affinity for the category to 5 if the species has a high affinity for the category), as a measure of species variability within genera. This information was further treated as frequency distribution (q_{ij}^k):

$$q_{ij}^k = \frac{a_{ij}^k}{\sum_{k=1}^h a_{ij}^k} \text{ with } q_{ij}^k \geq 0 \text{ and } \sum_{k=1}^h q_{ij}^k = 1 \quad (1)$$

Traits of these databases at genus level were used to compute BTD and ETD except for dipteran clades for which trait information was available only at the family level. Information on biological and ecological traits was finally obtained for 348 and 330 genera belonging to 98 families (Supplementary material Table S4 and S5).

Since traits originated independently within each clade from different ancestral states in successive transitions to freshwater habitats (i.e., traits are not homologous across clades), the dynamics of trait changes cannot be simulated directly on the tree, unlike in analyses applying phylogenetic comparative methods (Pagel 1999; Harmon et al. 2010). Instead, the form and strength of the relationship between extant within-

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clade trait disparity for all individual traits and clade age should confirm or reject our predictions. Within-clade BTM was estimated by the sum of the trait variance obtained separately for each clade on the array crossing traits and genera. From equation (1), let denote \bar{q}_j^k , the average trait category profile. Let $q_{ij}^{k*} = q_{ij}^k - \frac{1}{v} \times \bar{q}_j^k$ be the centered profile of genus i ($1 < i < t$) for category k ($1 < k < h$) of trait j ($1 < j < v$) in a given clade. Trait variance was obtained by:

$$TVar_j = \frac{1}{h} \frac{1}{t} \sum_{k=1}^h \sum_{i=1}^t q_{ij}^{k*} \times q_{ij}^{k*} \quad (2)$$

Within-clade BTM was then obtained by summing the individual trait variance obtained in equation (2) across all traits:

$$BTM = \sum_{j=1}^v TVar_j \quad (3)$$

A high BTM value for a clade (i.e., high variance among genera) would reflect a high functional divergence of its constituent genera. To determine the contribution of disparity of each individual biological trait to disparity of all traits within clades and to distinguish between linear-cumulative and disparity-dependent models (Fig. 1, central column), we used equation (2) for each biological trait separately for each clade. The resulting trait variance across the genera of a clade was used as a proxy of within-clade trait disparity. Then the individual within-clade trait disparity was regressed against clade age. A linear-cumulative model of disparification should be accepted if trait disparity increases with time and the disparity in trait values collectively show a positive correlation with clade age; whereas in a disparity-dependent model some traits would be uncorrelated or show negative correlation with clade age. The within-clade trait disparity values were further put into the form of a contingency table crossing individual traits and clades. Correspondence Analysis (COA) was performed on this table to derive scores that best depict the association between clades and individual trait disparity across genera. We selected the first two dimensions of COA since they represented the main variability patterns with 54.5% and 59.7% of the biological and ecological trait variation respectively. Finally, to assess whether the variance of each individual trait disparity within a clade was related to clade age, the COA scores were regressed against clade age. The same approach was used for the ecological traits to measure ETD.

To demonstrate a possible role of competition with incumbent clades as disparity increases, it was necessary to show that young clades are filling unoccupied regions of trait space. Using the trait-by-genera array, trait similarity across genera was estimated using fuzzy correspondence analysis (FCA; Chevenet et al. 1994), a multivariate technique that allowed the joint ordination of trait categories and genera. To identify those traits contributing to the highest difference across genera, the proportion of variance explained by each trait was computed. The great majority of clades were found either close to the lower or to the upper limit of the distribution of “clade age” (Table 1), therefore allowing to divide the clades into two distinct categories, namely “young clades” (<0.3, 11 clades) and “old clades” (>0.7, 5 clades). However, two clades fell in the middle of the distribution, and thus we had to create a third group of “medium-age clades” (0.5-0.6, 2 clades). We further computed the proportion of variance in the trait-by-genera array explained by clade age grouping, and the observed variance between groups was tested using a Monte-Carlo procedure, which yielded a simulated probability obtained after 9,999 permutations of the genera. The analysis was performed separately for biological and ecological traits.

Correlations between clade age and taxonomic richness, BTD and ETD were computed at three levels considering: 1) all freshwater macroinvertebrates, 2) insects only, because they are the most diverse group in the Tree-of-Life of freshwater macroinvertebrates and may show a different pattern due to high interaction among major lineages, and 3) insects without Diptera, because this was the only Order for which traits were available up to the family level, but not genera. Correlations between clade age and COA-axis scores were computed considering all macroinvertebrates. Given that related clades may have similar BTD and ETD, the effect on trait values of phylogenetic non-independence and shared evolutionary history between taxa was removed using phylogenetic generalized linear models (pGLS). Various models of trait evolution and their associated covariance structure were considered, which included Brownian motion, Martins and Hansen (1997), Grafen (1989), Freckleton, Harvey and Pagel (2002), and Blomberg, Garland and Ives (2003) models available in the ‘ape’ package of the R software (R Development Core Team 2011). The best model was selected using the AIC criterion. The above analyses were performed using the maximum clade credibility tree of the Bayesian analyses. Additionally, to account for uncertainty in tree topology, 10 trees from the posterior distribution were sampled randomly and pGLS models were performed on within-

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clade variance of BTM for all macroinvertebrates against clade age, using the same best-fit model of trait evolution as selected for the maximum clade credibility tree. All statistical analyses were performed with the ‘ade4’ (Dray and Dufour 2007), ‘stats’ (R Development Core Team 2011), ‘ape’ (Paradis et al. 2004), and ‘nlme’ (Pinheiro et al. 2013) packages of the R software (R Development Core Team 2011).

Results

The relative-time calibrated tree under the assumption of an uncorrelated relaxed molecular clock was rooted on freshwater Turbellaria, Hydrozoa, and Demospongiae (Fig. 2), showing certain incongruence with the animal Tree-of-Life with respect to the position of Turbellaria (Dunn et al. 2014). A major deep split between the lophotrochozoan (Bivalvia, Gastropoda, and Clitellata) and arthropod (Insecta and Malacostraca) groups was in agreement with the animal Tree-of-Life (Dunn et al. 2014), while the phylogenetic relationships within Insecta were highly concordant with recent phylogenomic studies (Misof et al. 2014). Overall, the topology was highly concordant with previously published phylogenetic trees for each specific clade (Supplementary material Table S1) and most basal nodes were highly supported (posterior probabilities >0.9; Supplementary material Fig. S1), representing the first molecular phylogeny to include all known European freshwater macroinvertebrate families. The observed incongruence at the root of the tree, which may be due to substitution saturation in the two mitochondrial genes (see Supplementary material Table S6) or to the fact that only freshwater families were included in the dataset for phylogenetic reconstruction, did not affect the estimation of relative clade ages used for subsequent analyses. At the family level, Insecta had the greatest taxonomic diversity and the most recent diversification compared to the other phyla. The monophyly of 18 clades representing independent freshwater radiations was highly supported (posterior probabilities >0.9), except for Nepomorpha (posterior probabilities=0.56). Within-clade nodal support was generally high except for Diptera (Supplementary material Fig. S1).

The relationship between taxonomic richness at species and genus levels with clade age was not significant when considering all macroinvertebrates or only insects (Fig. 3; Supplementary material Table S7). Despite this, the highest richness at both levels was found for young clades of Diptera (Culicimorpha, Tipulomorpha, and Empidoidea), and Coleoptera (Adephaga and Hydrophiloidea). In contrast, as predicted

under a time dependence scenario (Fig. 1A), significant and positive relationships were observed for total within-clade BTD and ETD and clade age, if calculated for all macroinvertebrates or only insects, and whether Diptera were included or not (Fig. 3; Supplementary Material Table S7). The best evolutionary model that correlated total within-clade BTD and clade age was Brownian motion [i.e., trait covariance between any pair of taxa decreases linearly with time (branch length) since their divergence], whereas the best model for ETD was Martins and Hansen [Martins and Hansen (1997) used Ornstein-Uhlenbeck (OU) models, which incorporate both selection and drift and are thus qualitatively different from, and more general than, pure drift models based on Brownian motion; trait that evolve under OU models cannot cross certain threshold values and vary around a certain optimum (stabilizing selection)]. Older clades (e.g., Planarioidea, Odonata) had a greater total BTD and ETD at the genus level than more recent clades (e.g., Byrrhoidea, Empidoidea). Similar results were obtained from the 10 randomly sampled trees from the posterior distribution of the Bayesian analysis, which supports the robustness of our results to uncertainty in tree topology and clade age (Supplementary material Table S8 and S9).

The most variable biological traits within clades (i.e., high disparity among constituent genera) included life duration of adults, number of reproduction cycles per year (voltinism), potential maximum body size and resistance forms, which together contributed 2/3 of the total variability among genera within clades (Supplementary material Table S10a). The first COA-axis score associated to BTD was positively correlated to clade age (pGLS, Martins and Hansen model, $P=0.021$), indicating that these variable traits had a higher disparity in older than in younger clades (Fig. 4A left side on the axis 1), whereas other traits (aquatic stages, respiration) showed the opposite trend (Fig. 4A positive values on axis 1). These patterns illustrated that the mode and time of disparification of individual traits were variable (Fig. 5; Supplementary material Fig. S2; Table S10a). For example, the variance of the life duration of adults (Fig. 5A) and resistance forms ($P<0.05$), and the number of reproduction cycles per year and locomotion ($0.05<P<0.09$) were significantly higher in older clades (Planarioidea, Odonata, Plecoptera). In contrast the variance of the number of aquatic stages was generally higher ($0.05<P<0.09$) in the younger clades (Fig. 5C). Trait disparity for the remaining biological traits was not related to clade age (see example in Fig. 5B; Supplementary material Fig. S2). The joint ordination of biological traits and genera showed a weak but significant difference between groups of

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“old”, “medium” and “young” clades [variance explained Axis1=0.319, $P < 0.001$ (no difference between medium and old); variance explained Axis2=0.414, $P < 0.001$ (difference between all ages); and variance explained in a global permutation test for all axes=0.104, simulated $P=0.001$] (Fig. 4C; Supplementary material Table S11), suggesting that younger clades are filling regions of the biological trait space unoccupied by incumbent lineages. These results show that disparity of most biological traits did not increase with time in an unbounded fashion, as would be expected under the linear-cumulative model.

The most variable ecological traits included sensitivity to nutrient inputs (trophic and saprobity status), water temperature, and current velocity preferences, which together contributed 2/3 of the total variability among genera within clades (Supplementary material Table S10b). Only the second COA-axis score associated to ETD was positively correlated to clade age (pGLS, Martins and Hansen model, $P=0.046$), suggesting a weaker pattern of distribution of ETD across the Tree-of-Life compared to the pattern detected for BTD (Fig. 4B). The analysis of the contribution of each individual trait to total ETD exhibited a higher value in older clades for substrate preferences (Fig. 5D), elevation and nutrient levels ($0.05 < P < 0.09$) (Supplementary material Fig. S3; Table S10b). In contrast, the contribution to total trait disparity of salinity preference was higher in younger clades, but this trend was only marginally significant ($0.05 < P < 0.1$) (Fig. 5F). Trait disparity of the remaining ecological traits, which are the majority, was not related to clade age (see example in Fig. 5E; Supplementary material Fig. S3). Again, groups of “old”, “medium” and “young” clades were weakly but significantly different when the similarity in ecological traits across genera was tested [variance explained Axis1=0.242, $P < 0.001$ (no difference between medium and old); variance explained Axis2=0.028, $P < 0.007$ (difference between all ages); and variance explained in a global permutation test for all axes=0.085, simulated $P=0.001$] (Fig. 4C; Supplementary material Table S11), suggesting clades of varying age are filling differentiated segments of the ecological trait space.

Discussion

Age dependence of clade biological and ecological trait disparity

Placing species numbers of freshwater macroinvertebrates on the phylogenetic tree showed that there is no relationship between taxonomic richness and clade age, which conforms to findings reported for the whole

eukaryotic Tree-of-Life (Rabosky et al. 2012). In contrast, consistent with the scenario of time dependence, our results showed that older clades had a higher total BTD and ETD than younger ones and, therefore, species richness and trait disparity were uncorrelated, in agreement with other studies (Burbrink et al. 2012; Bapst et al. 2012; Knope et al. 2015). This is the first evidence showing that the time since a clade transitioned to freshwater ecosystems determines its accumulated biological and ecological trait disparity. Genera within older clades are generally present in a greater number of distinct freshwater habitats and are more biologically diverse than genera within younger clades. The analysis of trait disparity among genera within Orders and Infraorders/Superfamilies allowed us to estimate disparity for each independent freshwater clade despite the fact that many of these traits are present in all of the major clades and that age ranges of the genera in a clade are highly variable. This analysis shows that the total variance in a clade is limited and hence, while each genus within the older clades does not necessarily exhibit greater total trait disparity, the sum of trait disparity within a clade increases with age. In addition, the variability in trait disparity with clade age among individual traits indicated that regardless of the mechanism and rate of evolution, some traits are more evolutionary labile than others and not all traits follow the same evolutionary model (Hunt 2007; Harmon et al. 2010).

We also observed significantly higher BTD within older than younger clades in traits related to full freshwater life styles such as life cycle duration and resistance. For instance, BTD within older clades ranges from small species with short and multivoltine life cycles to large species with long and semivoltine life cycles (multiple years for completion, e.g. up to 10 years in bivalves). This suggests that despite the instability of river habitats, species with long life cycles persisted because of the accumulating disparity in resistance forms (e.g., diapause in Odonata to cope with river harshness and seasonality; Corbet et al. 2006) across sublineages within older clades. Similarly, genera within older clades showed increased disparity in individual ecological traits that enabled multiple substrate and current velocity preferences, which corroborates their diverse adaptations to the range of freshwater conditions prevailing in rivers.

In contrast to older clades, genera within the younger clades had high disparity in individual traits that enabled coping with ecologically harsh environments (e.g., brackish habitats) and a partially terrestrial

life style. For example, almost all genera within younger clades retained non-aquatic respiration and active flight dispersal in the adults. In addition, holometabolous insects retained aquatic nymphs from their terrestrial ancestors, a trait that avoids intraspecific competition between larvae and adults (Tachet et al. 2010). Such terrestrial-related traits are advantageous for escaping the aquatic environment during seasonal floods and droughts (Lytle 2001; Lytle and Poff 2004) and thus indicate a benefit for lineages associated with peripheral freshwater environments for clades that made the transition to freshwater life style only after most niche space was already filled. In addition, younger clades such as Diptera or Coleoptera also include genera showing disparity in tolerance to salinity not typically found in freshwater macroinvertebrates (Arribas et al. 2014). This provides evidence for disparification in traits partly missing from the incumbent, older clades, indicating key evolutionary innovations that probably allowed the radiation of young clades in underutilized adaptive zones.

Comparing extant patterns of trait disparity over the successive colonization events of freshwater habitats, only a few individual traits increased disparity according to the predictions of a linear-cumulative model, whereas the overall pattern of trait disparity across freshwater lineages supports our expectations based on the hypothetical disparity-dependent model. The pattern suggests that disparification in older clades progressively decreased the ecological opportunities available to newly colonizing lineages, whose disparification then involved traits for existence in extreme or peripheral habitats less accessible to a fully aquatic life style, as suggested during adaptive radiations (Burbrink and Pyron 2010; Mahler et al. 2010; Harmon et al. 2010). These findings are consistent, for example, with studies on catfishes during the repeated ecological transitions from marine to freshwater habitats (Betancur-R et al. 2012). They also match trends in the fossil records that show an early morphological disparification followed by a declining rate because of decreased ecological opportunities for subsequent arrivals (Foote 1997; Bapst et al. 2012; Knope et al. 2015).

Phylogenetic signature of ecological and biological traits disparification

The phylogenetic signature for ETD was less clear in comparison to BTD. Most categories of ecological traits were evenly distributed among genera within older and younger clades, except for substrate, elevation and nutrient preferences that were higher within older than younger clades. Substrate together with

current velocity are intimately related to harsh flow forces and flow variation in freshwater lotic systems (Resh et al. 1988; Lytle 2001), and apparently genera within older clades were more successful in acquiring a high disparity in these traits, which permits their occupancy of a broader range of suitable freshwater habitats. These results indicate that high flow forces and flow variation in rivers are selective regimes affecting trait disparification. Apart from direct selection on adaptive traits, flow forces and flow variation limit population growth and abundance (Resh et al. 1988; Lytle 2001), which indirectly diminish intense biotic interactions and extinction due to competition (Huston 1979; Resh et al. 1988).

The drivers of trait disparification in lotic systems probably contrast with the more stable terrestrial environments where communities can result in tighter biotic interactions that have favored diversification through coevolution (Emerson and Kolm 2005). Yet, freshwater ecosystems are still characterized by the co-occurrence and interaction of phylogenetically distant species drawn from diverse parts of the Tree-of-Life in a single stream reach and a high overlap of ecological niches that apparently did affect the trait disparification, although the adaptive significance of disparification for co-existence of species is not actually measured here. We can also not be sure if the disparification represents primarily the outcome of competitive interactions in historical communities that were driving the disparity of clades during the early stages of the transition to aquatic habitats, or if it is driven by contemporary adaptive forces in extant communities. Equally, we cannot be sure about the geographic framework in which the disparity originated; while the study was geographically limited to Europe, many lineages (genera) under study are more widespread and in addition may have undergone biogeographic range shifts, and thus evolutionarily the interactions among them may have played out elsewhere. However, our study does not focus on the local communities, but instead only attempts to capture the evolutionary outcome of such competitive interactions for diversification of deep clades, across larger scales of space and time.

Trait disparification and species diversification against clade age

An important finding is that BTB and ETD are broadly correlated with clade age, while species diversification is not, and hence suggesting that processes underlying species diversification and trait disparification are not mechanistically linked, which is in accordance with other studies (Barraclough,

Hogan and Vogler 1999; Burbrink et al. 2012). Although transitions to a new habitat should promote both species and trait diversification (Harmon et al. 2003; Rabosky and Lovette 2008; Jönsson et al. 2012), speciation is not necessarily accompanied by changes in ecological and biological traits of the kind studied here, and hence species diversity varies tremendously among clades and evolutionary ages. In particular, the high level of taxonomic diversity in certain young, species-rich clades, such as Culicimorpha, Tipulomorpha, Empidoidea, Aephaga and Hydrophiloidea, was not correlated with either BTD or ETD. Several mechanisms can explain the decoupling of species diversity and trait disparity. Young clades may achieve a high net diversification rate without concomitant change in the rate of trait diversification, as described for the super-radiations of beetles (Hunt et al. 2007) and flies (Wiegmann et al. 2011) whose high species richness may be explained primarily by low extinction rates. In contrast, in clades characterized by low taxonomical diversity and high trait disparity, e.g. Planarioidea and Odonata, species numbers may be driven by high extinction that exceeds speciation, which is consistent with findings for the marine invasion of cetaceans (Slater et al. 2010); or diversification can occur with little trait change if speciation occurs primarily through other factors such as geographic isolation (Adams et al. 2009). Regardless of the exact mechanisms, trait disparity retains the evolutionary signature associated with interactions between early and late lineages undergoing the transition to freshwater ecosystems, whereas species diversification is intrinsic to each particular clade.

Concluding remarks

Our results are a step towards the overdue synthesis of community ecology and studies of clade evolution. We showed a decreasing within-clade trait disparity over evolutionary time, which suggests that multiple freshwater macroinvertebrate clades have radiated during successive transitions to freshwater ecosystems exploiting novel ways for reaching previously inaccessible ecological space that was already partially filled. As within-clade variance of extant trait disparity is associated with clade age, this has consequences for applied studies of the relationship between diversity and ecosystem function (Cadotte et al. 2013) and the effects of climate change on biodiversity (Thuiller et al. 2011). Ecological responses and vulnerability of organisms from across the Tree-of-Life may depend on the clade age because of its correlation with BTD and ETD. For instance, species within older clades may have a wider ecological niche

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and larger geographical range because of full ecosystem adaptation and a longer history of movement, whereas younger clades should be more vulnerable because of ecological specialization and limited distribution, except if this ecological specialization renders them more fit for a given niche. We were able to conduct the broadest assessment to date of the relationship between clade age and trait disparity, but several aspects of our approach have to be considered before making further generalizations. For example, our expectations for the relationship between clade age and trait disparity (Fig. 1) capture model behaviour at the extremes, and may not account adequately for patterns generated by intermediate processes. A better understanding of the mechanisms behind disparification should explore trait dynamics by simulations within and across clades varying the clade specific rate of evolution and clade maximum disparity (i.e., limits to total disparity). In addition, a fully sampled tree would be more conducive to mathematically rigorous analyses of disparification through time, but this is the first study considering a whole ecosystem-based group of organisms to quantify clade age and trait disparity, therefore providing a starting point to stimulate further discussion and research on this important topic. The genus level used to obtain values of disparity is not entirely arbitrary, as genera are usually well-defined radiations composed of species that differ mainly in the type of biological and ecological traits under investigation here. Their ages may differ and the dynamics of disparification within genera (e.g. disparification early or late) could also differ, and thus the regressions of trait disparity with clade age may be simplistic without considering genus age, but these details cannot be addressed with the currently available phylogenetic data. Overall, large-scale phylogenies integrated with extensive environmental, biological and ecological trait data should provide a framework for a better understanding of biodiversity patterns over evolutionary time and give new insights into the evolution and fundamental processes shaping trait dynamics across the Tree-of-Life.

Author contributions: C.M., A.P.V. and N.B. conceived the study, S.D. and N.B. performed taxonomical and trait diversity compilations, C.M. performed sequence compilation, A.P. performed phylogenetic analysis and S.D. performed statistical analyses. C.M., A.P.V. and N.B. wrote a draft of the paper, and all authors contributed substantially to the revision. We declare no competing interests.

Acknowledgements

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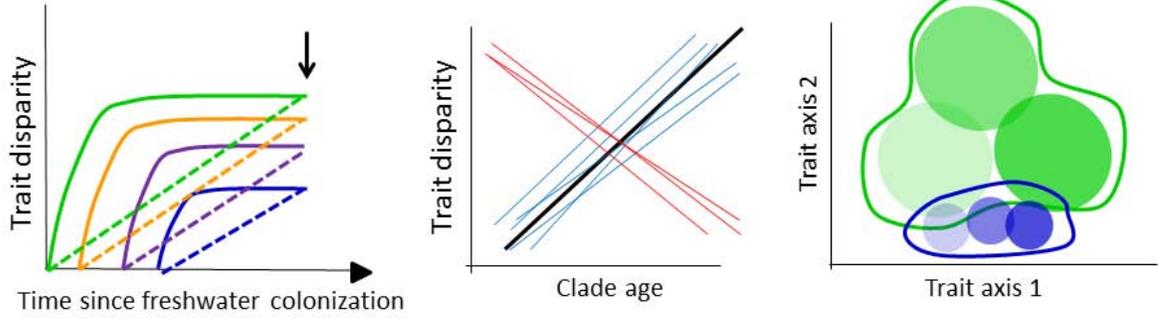
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Figure Legends

Fig. 1 Two scenarios of the correlation between clade age and extant within-clade trait disparity assuming (A) time dependence or (B) time independence of trait disparification of clades. For both scenarios, total trait disparity can be attained by linear-cumulative disparification (dashed lines) or clade-specific disparification with limits to total maximum disparity (plain lines). In left column, colors represent different freshwater clades that vary in age. For the time dependent scenario (A), expected total trait disparity within-clades should be positively correlated with clade age (central column), whereas this relation is expected to be decoupled for the alternative time independence scenario (B, central column). Under the linear-accumulative model assuming time dependence (A), trait disparity for an individual trait would be positively correlated with clade age, similarly to the Brownian Motion model of gradual evolution for homologous traits (blue lines in central column), and the variance of trait disparity would be higher in older than younger clades (right column). In contrast, the evolution of individual traits under the disparity-dependent model would result in higher trait disparity for some specific trait in younger than older clades (A, red lines in central column), but also in smaller and non-overlapped variance of trait disparity between older and younger clades (A, right column). Alternatively, in the time independence scenario (B), variance of disparity among traits would be unrelated to clade age.

(A) Time dependence of accumulated trait disparity of clades



(B) Time independence of accumulated trait disparity of clades

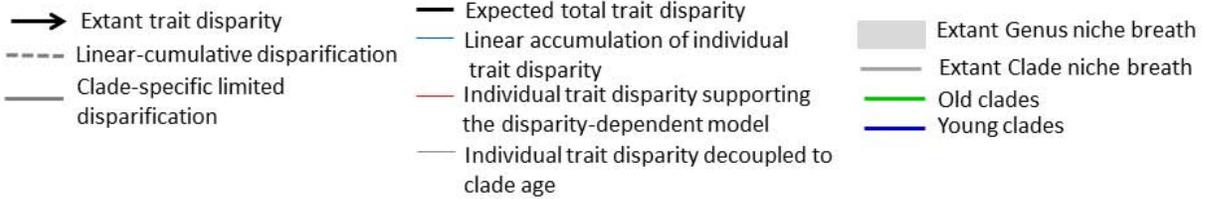
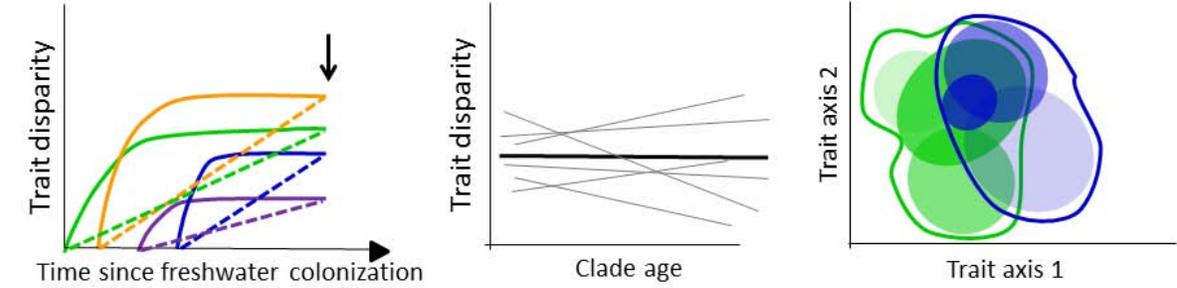


Fig. 2 Relatively clock-calibrated 3-gene phylogenetic tree representing 143 aquatic macroinvertebrate families generated using an uncorrelated lognormal relaxed clock in BEAST. Orange rectangles indicate clades corresponding to Orders that exclusively diversified in freshwater ecosystems. Blue rectangles indicate clades that secondarily colonized freshwater habitats, while their close relatives within the same Order were terrestrials. Orders names are indicated, and classes names were shortened to the initial letter: T, Turbellaria; H, Hydrozoa; D, Demospongiae; I, Insecta; M, Malacostraca; B, Bivalvia; G, Gastropoda; C, Clitellata.

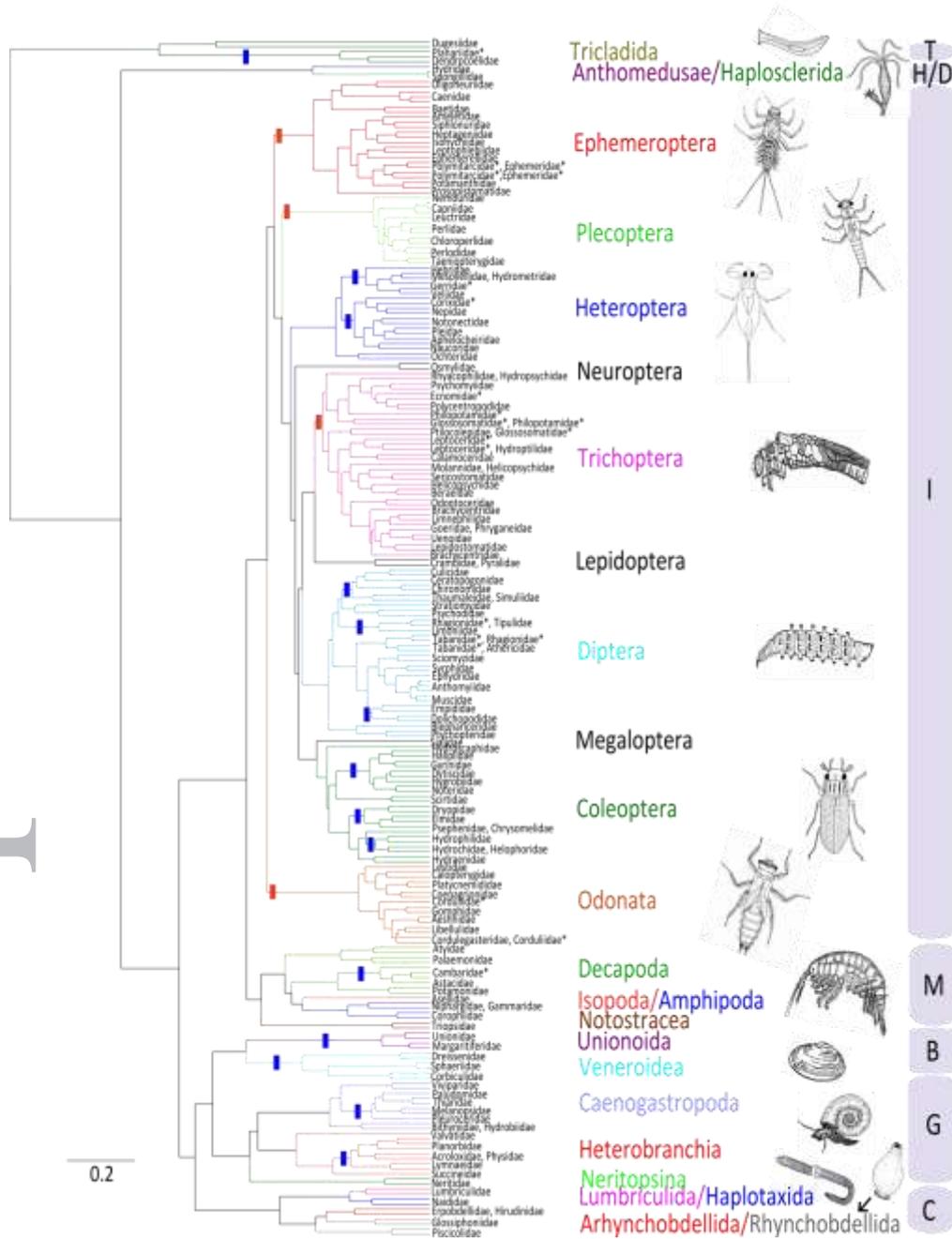


Fig 3 Relationship between the age of clade (relative time) and (A) species richness values per family and clade; (B) genera richness values per family and clade; (C) biological traits disparity (BTD); and (D) ecological trait disparity (ETD) (in red non-insects and in blue insects). Plain thick lines of the pGLS regressions correspond to the model for all freshwater macroinvertebrates and plain thin lines to the same without Diptera. Dashed thick lines of the pGLS regressions correspond to the model for insects and dashed thin lines to the same without Diptera (See Table S7 for further details on models). Clades names were shortened to the first three letters: PLA, Planarioidea; ODO, Odonata; EPH, Ephemeroptera; VEN, Veneroidea; PLE, Plecoptera; UNI, Unionoidea; TRI, Trichoptera; HYG, Hygrophila; CUL, Culicomorpha; NEP, Nepomorpha; ADE, Adepaga; GER, Gerromorpha; CER, Cerithioidea; BYR, Byrrhoidea; TIP, Tipulomorpha; AST, Astacoidea; EMP, Empidoidea; HYD, Hydrophiloidea.

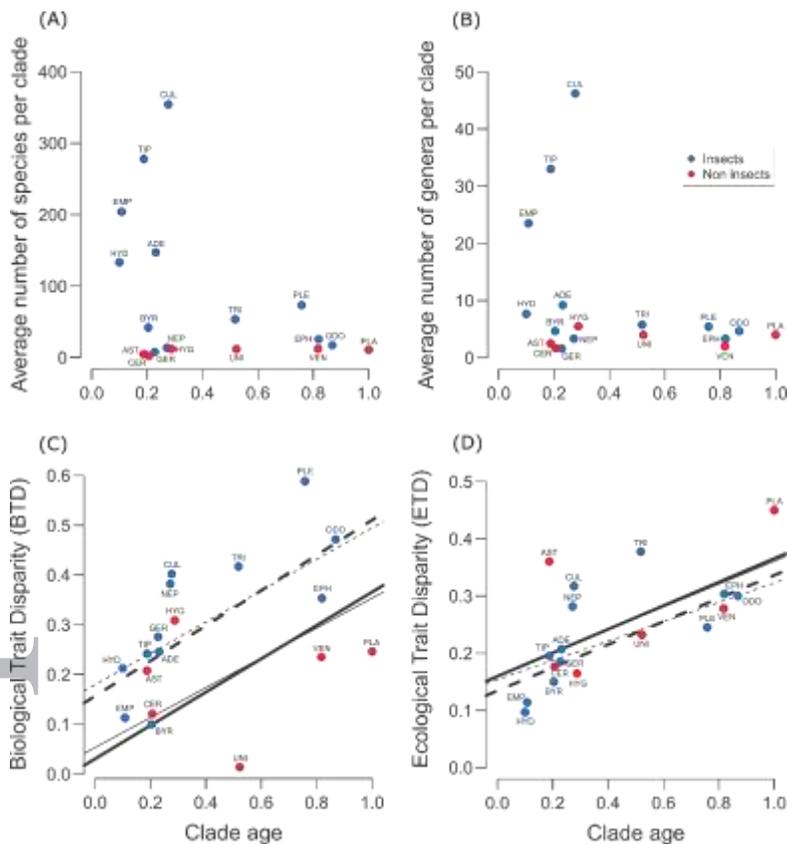


Fig. 4 Correspondence analysis performed on the variance of extant biological trait disparity (A) and ecological trait disparity (B) among the 18 freshwater clades; scores for each trait are plotted at the weighted average COA scores of their clades. Joint ordination of biological (C) and ecological (D) traits and genera; each dot is a genus and the hulls rounds show the grouping by relative age of the corresponding clades; traits involved in the similarities are ordered in decreasing variance in biological and ecological traits (Table S11). In green clades with clade age >0.7 , in orange clades with clade age between 0.5-0.6, and in blue clades with clade age <0.3 . Biological trait names were shortened to: ASTA, Aquatic stages; RESP, Respiration; DISP, Dispersion; FHAB, Feeding habits; LOCO, Locomotion and substrate relation; FOOD, Food; REPR, Reproduction; RESI, Resistance forms; RCYC, Potential number of reproduction cycles per year; MSIZ, Maximal size; LDUR, Life duration cycle. Ecological trait names were shortened to: SALI, Salinity; TROP, Trophic level; LONG, Longitudinal distribution; SAPR, Saprobity; CURR, Current velocity; SUBS, Substrate; ALTI, Altitude; TEMP, Temperature.

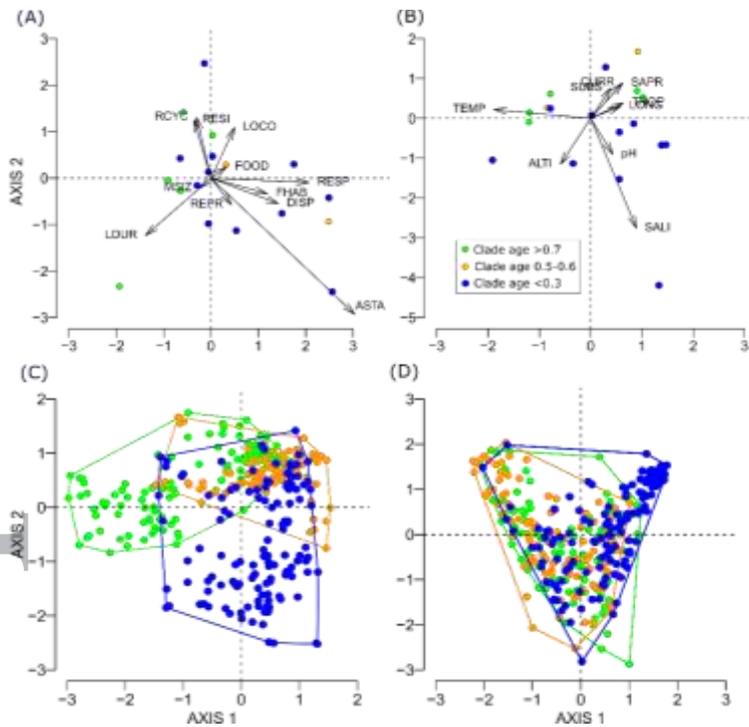


Fig. 5 Example of the three hypothetical relationship between clade age (relative time) and biological trait disparity within-clade (BTD) of (A) life cycle duration (positive), (B) feeding habits (un-related), and (C) aquatic stages (negative); and ecological trait disparity within-clade (ETD) of (D) substrate preferences (positive), (E) pH (un-related), and (F) salinity tolerance (negative, but non-significant). In red non-insects, and in blue insects. Plain lines and ** correspond to models for p -value <0.05 . Dashed lines and * correspond to models for $0.05 < p$ -value <0.1 . Dashed lines without * correspond to models for p -value >0.1 . Short names of clades as in Fig. 3 (see Figs. S3 and S4 for plots of all traits).

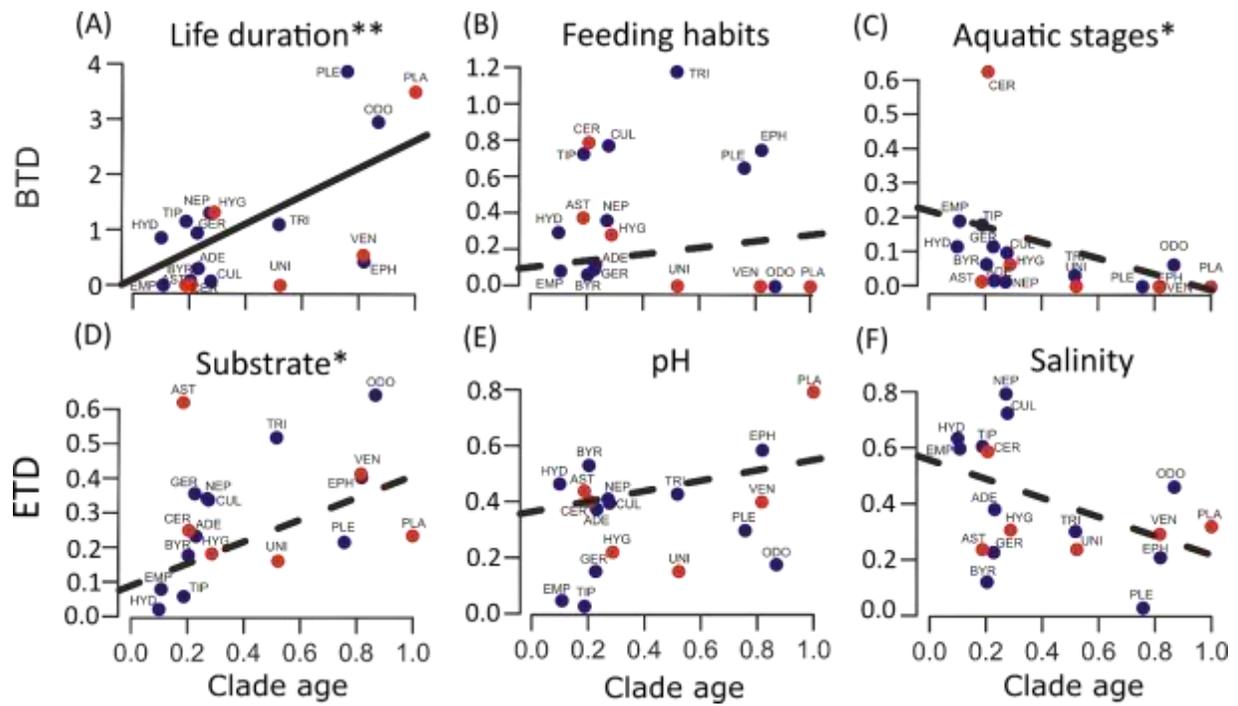


Table Legend

Table 1. Taxonomic rank, relative clade age and family composition for the 18 freshwater clades used in the statistical analyses. Clades are divided in three groups: clade age >0.7, clade age between 0.5-0.6, and clade age <0.3.

CLADE (taxonomic rank)	CODE	RELATIVE CLADE AGE	CLASS	ORDERS	FAMILY (ordered by phylogenetic relation)
PLANARIOIDEA (Superfamily)	T-PLA	1	TURBELLARIA	TRICLADIDA	Planariidae, Dendrocoelidae
ODONATA (Order)	I-ODO	0.868	INSECTA	ODONATA	Lestidae, Calopterygidae, Platycnemididae, Coenagrionidae, Corduliidae, Gomphidae, Aeschnidae, Libellulidae, Cordulegastridae
EPHEMEROPTERA (Order)	I-EPH	0.819	INSECTA	EPHEMEROPTERA	Oligoneuriidae, Caenidae, Baetidae, Amelletidae, Siphonuridae, Heptageniidae, Isonychiidae, Leptophlebiidae, Ephemerellidae, Polymitarcidae, Ephemeridae, Potamanthidae, Prosopistomatidae
VENEROIDA (Order)	B-VEN	0.816	BIVALVIA	VENEROIDEA	Dreissenidae, Sphaeriidae, Corbiculidae
PLECOPTERA (Order)	I-PLE	0.757	INSECTA	PLECOPTERA	Nemouridae, Capniidae, Leuctridae, Perlidae, Chloroperlidae, Perlodidae, Taeniopterygidae
UNIONOIDA (Order)	B-UNI	0.522	BIVALVIA	UNIONOIDA	Unionidae, Margaritiferidae
TRICHOPTERA (Order)	I-TRI	0.517	INSECTA	TRICHOPTERA	Rhyacophilidae, Hydropsychidae, Psychomyiidae, Ecnomidae, Polycentropodidae, Philopotamidae, Glossosomatidae, Philopotamidae, Ptilocolepidae, Leptoceridae, Hydroptilidae, Calamoceratidae, Molannidae, Helicopsychidae, Sericostomatidae, Beraeidae, Odontoceridae, Brachycentridae, Limnephilidae, Goeridae, Phryganeidae, Uenoidae, Lepidostomatidae
HYGROPHILA (Taxonomic clade)	G-HYG	0.287	GASTEROPODA	PULMONATA	Ancylidae, Planorbidae, Acroloxidae, Physidae, Lymnaeidae
CULICOMORPHA (Infraorder)	I-CUL	0.276	INSECTA	DIPTERA	Culicidae, Ceratopogonidae, Chironomidae, Thaumaleidae, Simuliidae
NEPOMORPHA (Infraorder)	I-NEP	0.271	INSECTA	HETEROPTERA	Corixidae, Nepidae, Notonectidae, Pleidae, Aphelocheiridae, Naucoridae
ADEPHAGA (Suborder)	I-ADE	0.231	INSECTA	COLEOPTERA	Halipilidae, Gyrinidae, Dytiscidae, Hygrobiidae, Noteridae
GERRMORPHA (Infraorder)	I-GER	0.227	INSECTA	HETEROPTERA	Hebridae, Mesoveliidae, Hydrometridae, Gerridae, Veliidae
CERITHIOIDEA (Superfamily)	G-CER	0.206	GASTEROPODA	NEOTAENIOGLOSSA	Thiaridae, Melanopsidae, Pleuroceridae
BYRRHOIDEA (Superfamily)	I-BYR	0.204	INSECTA	COLEOPTERA	Dryopidae, Elmidae, Psephenidae
TIPULOMORPHA (Infraorder)	I-TIP	0.187	INSECTA	DIPTERA	Tipulidae, Limoniidae
ASTACOIDEA (Superfamily)	M-AST	0.187	MALACOSTRACA	DECAPODA	Cambaridae, Astacidae
EMPIDOIDEA (Superfamily)	I-EMP	0.107	INSECTA	DIPTERA	Empididae, Dolichopodidae
HYDROPHILOIDEA (Superfamily)	I-HYD	0.1	INSECTA	COLEOPTERA	Hydrophilidae, Hydrochidae, Helophoridae