Spatial and phylogenetic structure of DNA-species of Alpine stonefly community assemblages across seven habitats

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Abstract

1. Stream ecosystems are spatially heterogeneous environments due to the habitat diversity that define different microhabitat patches within a single area. Despite the influence of habitat heterogeneity on the biodiversity of insect community, little is known about how habitat heterogeneity governs species coexistence and community assembly. Here, we address the question if habitat heterogeneity may drive changes in community composition of the stonefly (Plecoptera, Insecta) community in different sampling locations, by assessing the relative role of the habitats that explain beta biodiversity patterns (spatial structure) and evolutionary processes (phylogenetic signal) in structuring communities.

2. We sampled across seven habitats types among 20 sampling sites in Alpine rivers, and we used mitochondrial DNA, *cox1*, and nuclear DNA, ITS, genetic markers on 21 stoneflies morpho-species to estimate putative DNA-species by General Mixed Yule Coalescent model (GMYC). With the use of putative DNA-species, we first analyzed the patterns of variation of DNA-species richness, composition, and diversity of stonefly community assessing their habitat correlates. Then, we assessed through a phylogenetic clustered pattern if DNA-species with similar physiological requirements co-occur due to environmental filtering.

3. Based on 52 putative DNA-species, we found that corridors contributed to DNA-species richness where the meandering corridor section displayed the highest contribution. While, habitats contributed to DNA-species diversity, where glide, riffle, and pool influenced the spatial structure of the stonefly community possible owed to the high species turnover observed.

4. Among the habitats, pool showed a significant phylogenetic clustering, suggesting evolutionary adaptation and strong habitat filtering. This pattern of community phylogenetic structure could have resulted from the long-term stability of the habitat and physiological requirements of the species that cohabitate.

5. Our study shows the importance of different habitats on the spatial and phylogenetic structure of stonefly community assemblies and sheds light on the habitat-specific diversity that may help improve conservation practices.

KEYWORDS

DNA-species, habitats, spatial structure, phylogenetic structure, Plecoptera

1. INTRODUCTION

Understanding species diversity patterns and the process that governs their coexistence in a community is a fundamental question in ecology and biodiversity studies. The development of an effective and suitable conservation strategy for biodiversity maintenance is unraveling the process of community assembly variation (Socolar, Gilroy, Kunin, & Edwards, 2016) along a temporal and spatial gradient (Anderson et al., 2011). Previous studies demonstrated that community assemblies were the outcome of a relationship between species diversity and habitat availability (e.g., Wiens et al., 2010); however, there is still no clear consensus on the type of habitats that influences this relationship.

Stream ecosystems provide one of the most heterogeneous landscapes because of the dynamic interaction between spatial elements (as topography) and ecological process (such as hydrology) (Benda et al., 2004; Tockner & Stanford, 2002). This interaction creates a variety of habitat throughout the longitudinal (upstream-downstream) dimension of the river, classified as lotic (running) and lentic (standing) (Calow & Petts, 1996; Dobson & Frid, 1998; Hauer & Lamberti, 1996). At a biogeographic scale, habitat heterogeneity in a river channel defines environmental patches that affect aquatic taxa composition and distribution (Brasil, Da Silva, Batista, Olivera, & Ramos, 2017; Dias-Silva, Cabetter, Juen, & De Marco JR, 2013); while at a local scale, creates environmental filtering to sort species with similar requirements (Saito, Cianciaruso, Siqueira, Fonseca-Gessner, & Povoine, 2016; Webb, Ackerly, McPeek, & Donoghue, 2002). Among aquatic taxa, aquatic insects are the most abundant, diverse, and broadly distributed taxa in aquatic ecosystems (Lancaster & Downes, 2013). Although the strong positive relationship between species diversity of aquatic insect community and spatial habitat heterogeneity has been observed (e.g. Arscott, Tockner, & Ward, 2005; Astorga, Death, Death, Paavola, Chakraborty, & Muotka, 2014; Batista, Buss, Dorville, & Nessimian, 2001; Benda et al., 2004; Karaus, Larsen, Guillong, & Tockner, 2013), little is known about the direct relationship between assembly process on both biogeographical and local scale. Thus, to be able to understand the biodiversity of a locality is crucial to understand the interaction between habitats and species diversity.

However, counting the biodiversity of a locality is not a simple task. One of the main problems in studies of biodiversity is species delimitation. Due to limitations in taxonomic expertise, and the small-sized organisms or taxa with incomplete taxonomic classifications, some species delimitation may result into unclassified identification (Bickford et al., 2007), which makes it challenging to estimate the biodiversity of a locality. Genetic methods developed over the last decade have helped with species identifications and the clarification of species boundaries of a wide range of taxa, including aquatic insects (e.g., Serrana, Miyake, Gamboa, & Watanabe 2019). The analysis of species-level entities delimitation by recognizing putative species based on variation in DNA sequences (DNA taxonomy; Vogler & Monaghan, 2006) using Generalized Mixed Yule Coalescent model (GMYC; Pons et al., 2006) is one of the most common methods employed for recognizing putative DNA-species. Briefly, GMYC identify the

transition rate between inter and intra-species branching events on a time-calibrated ultrametric tree (distances from the root of every branch is equal), using molecular markers, as mitochondrial DNA (e.g. Mynott, Webb, & Suter, 2011; Vogler & Monaghan, 2006), or based on the congruency between mitochondrial and nuclear DNA (e.g. Rutschmann et al., 2016).

Stream ecologist had found a variety of community assembly patterns using putative DNA-species diversity of aquatic insect along the river (Astorga et al., 2014; Baselga et al., 2013; Finn, Bonada, Múrria, & Hughes, 2011). However, majority of the observations has been based on DNA-species diversity changes along longitudinal dimension of the river (Finn et al., 2011; Finn, Zamora-Muñoz, Múrria, Sáinz-Bariáin, & Alba-Tercedor, 2013; Finn & LeRoy Poff, 2011; Gill, Harrington, Kondratieff, Zamudio, Poff, & Funk, 2013; Hughes, Schmidt, & Finn, 2009; Jackson, Battle, White, Pilgrim, Stein, Miller, & Sweeney, 2013), where the influence of other river dimensions, such as habitats remains limited to a few species such as beetles (Ribera & Vogler, 2008) or caddisfly (Marten, Brandle, & Brandl, 2006). To explore the effect of DNA-species diversity on the community assemblies within and among different habitats, we examined the stonefly community in an Alpine region. Stoneflies (Plecoptera) are aquatic insects considered as an important component of the river channel because of their high sensibility to environmental changes (Stewart & Stark, 2008). Stoneflies have a complex response to habitat change (Bojková, Rádková, Soldán, & Zahrádková, 2014) with high impact on ecological (Lancaster & Downes, 2013) and evolutionary responses (Gamboa & Watanabe, 2019) than other insects. Hence, investigating the spatial patterns of stonefly community composition in the river channel in relation to habitat heterogeneity will shed light on the understanding for the community assembly in aquatic ecosystems.

The understanding of local community assembly has not only focused on spatial structure, and composition change approaches. Phylogenetic approaches have been proposed to provide additional insights on environmental filtering as one of the main drivers of community assembly (Violle et al., 2011; Webb et al., 2002). The basis of this approach is to compare observed traits and phylogenetic structures in communities with those expected under null models (phylogenetic signal). When species share similar physiological requirements, environmental filtering should cause related species to co-occur (phylogenetic clustering) more than expected by chance. Contrary, when species compete for the same limiting resources, competitive exclusion should cause the opposite pattern (phylogenetic overdispersion) (Cavender-Bares & Wilczek, 2003). Although phylogenetic clustering and overdispersion has been observed in aquatic insect community assemblies along spatial scales (Saito, Soininen, Fonseca-Gessner, & Siquiera, 2015a; Saito, Siquiera, & Fonseca-Gessner, 2015b; Saito et al., 2016) or by specific trait-based approaches, as respiration strategy (Buchwalter, Jenkins, & Curtis, 2002), and anthropogenic pollutants (e.g., Martin, Cain, Luoma, & Buchwalter, 2007), the assessed relationship between phylogenetic structure in different habitats have not been explored.

Here, we address the question if habitat heterogeneity may drive changes in community composition of stonefly community in different sampling locations, by assessing the

relative role of seven habitats that could explain beta biodiversity patterns (spatial structure) and evolutionary processes (phylogenetic signal) in structuring stonefly communities. We hypothesize that species distribution and community assembly are related to certain habitats and habitat filtering act on phylogenetically closely related species with similar physiological requirements. In contrast, high levels of species diversity are expected to limit structure similarity of the assembly and phylogenetic overdispersion response. Using two molecular markers (*cox1* and ITS), we aim (1) to quantify the habitats contribution to overall DNA-species diversity, (2) to determinate the influence of habitats on spatial community assemblies along sampling sites, (3) to understand which turnover or nestedness process among habitats contribute to DNA-species diversity, and (4) to quantify if phylogenetic clustering is influenced by the habitats.

2. METHODS

2.1 Study sites and sample collection

We selected seven habitats types (Fig. 1), waterfall (vertical descent of watercourse), riffle (high-turbulent water flow), glide (low-turbulent water flow), side-channel (small-sized water pass flowing on a sandy bar), wando (as an analogy of side-channel in the head and tail on a sandy bar without connection between them; Ishida, Abekura, & Takemon, 2005), pond (isolated still water habitat on sandy bar) and pool (>1mt deep-high river section) on 20 sampling sites in two alpine rivers (Tagliamento and Fella rivers) (Fig. S1). These sampling sites were located among four geomorphologically distinct corridor sections (4 to 1556 m asl in altitude): headwater constrained, meandering (stipe river movement), bar-braided floodplain sections (convergent river bankfull width), and lowland spring-fed streams. Many spring-fed streams that frequently occur in the fluvial floodplain are a characteristic of this river (Doering, Uehlinger, & Tockner, 2013). Fifteen sampling sites were selected from the Tagliamento River (T01 to T15), and five were selected from its major tributary, the Fella River (F01 to F05) (Fig. S1). The habitat diversity per site was measured by using the Simpson index.

We collected qualitative samples of stoneflies nymph using D-frame nets (250 µm mesh), on five replicate and spending at least two person-hours per habitat type in a site. All stoneflies samples were preserved in 99.5% ethanol in the field, and the ethanol was replaced twice with fresh 99.5% ethanol upon returning to the laboratory. Morpho-species identification was conducted under a stereoscopic microscope (80 X) following Consiglio (1980) and Fochetti & Tierno de Figueroa (2008) taxonomical keys. Field samplings were repeated twice in summer (10 July to 10 September) 2009 and once in early spring (24 March to 15 April) 2010. We used 75 merged samples (summer and spring) in the following analysis.

2.2 DNA extraction and sequencing analysis

Three hundred seventy-five individuals were collected and used for genetic analysis. Genomic DNA was extracted using DNeasy tissue kits (Qiagen GmbH, Hilden, Germany), following the manufacturer's instruction. A 658-bp fragment of mitochondrial *cox1* was amplified using LCO-1490 and HCO-2198 primers (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994) with 48 °C annealing temperature and 30 PCR cycles. Internal transcribed spacer (ITS) maker of nuclear DNA was also analyzed for a subset sample of 116 individuals that represent all morphological taxonomic groups in this study. A 930-bp fragment of ITS marker was amplified using universal primers of 28S and 18S (McLain, Wesson, Oliver, & Collins, 1995) with 58 °C of annealing temperature and 40 PCR cycles. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced in both directions using the same primers as above. *Cox1* sequences were analyzed by ABI 3500xL automated sequencer (Applied Biosystems), and ITS sequences were analyzed by Eurofins – Operon, Tokyo. All sequence data reported here have been deposited to GenBank.

Forward and reverse sequences were assembled and edited using CodonCode Aligner v 3.5 (Codon Code Corporation, Dedham, USA). All sequences were aligned using ClustalW (align.genome.jp; Larkin et al., 2007). ITS data were first aligned to remove un-align-able regions (indel regions) using the Indel Module of the Seqfire application (http://www.seqfire.org, Ajawatanawong, Atkinson, Watson-Haigh, MacKenzie, & Baldauf, 2012) with the default settings. The initial alignment had approximately 1016 bp long but reduced to 790 bp after the removal of indel regions. These sequences were used for a second alignment with an outgroup (see phylogenetic analysis section). We reduced redundancy by collapsing identical sequences using CleanCollapse software (http://sray.med.som.jhmi.edu/SCRoftware/CleanCollapse/). All sequences of mtDNA and nDNA markers were compared to the NCBI nucleotide database using blastn queries (http://blast.ncbi.nlm.nih.gov) to corroborate species identification (DNA barcoding, similarity > 98%) and discard possible sequence errors.

Genetic diversity was calculated by the number of polymorphic sites, nucleotide diversity (Kimura 2-parameter model), and the number of haplotypes using DnaSp v5.10 (Librado & Rozas, 2009).

2.3 DNA-based species delimitation

Putative DNA-species were delineated using the General Mixed Yule Coalescent model (GMYC; Fujisawa & Barraclough, 2013). An ultrametric gene tree of *cox1* gene was constructed using BEAST v. 1.8.3 (Drummond, Suchard, Xie, & Rambaut, 2012) with a relaxed clock model, a coalescent prior, and 50 million generation parameters. The result was summarized with Tree Annotator (BEAST package) and visualized using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). The GMYC analysis was run using the splits package (Ezard, Fujisawa, & Barraclough, 2014) in R v. 3.3 (R Core Team). We used a single version of the GMYC model. The maximum likelihood of the GMYC model was tested using the

likelihood ratio test against a one-species null model (i.e., the entire tree is considered as a single coalescent). The putative DNA-species diversity per sampling sites and per habitats was calculated with Shannon-Weaver and Simpson diversity index in vegan package in R v. 3.3 (R Core Team, Oksanen, et al., 2012).

2.4 Spatial structure of stonefly community assemblies

In order to quantify the habitat contribution to overall DNA-species diversity, we compared the relative contribution of the four corridor sections and seven habitats using the additive partition of species diversity analysis (Lande, 1996). This analysis allows alpha and beta values to be simultaneously calculated in order to assess which (corridor or habitats) contribute to overall diversity (Gering, Crist, & Veech, 2003). This method analyzes an abundance matrix hierarchically organized as: among habitats (alpha diversity, α) and among corridor sections (beta diversity, β). Species richness and Shannon diversity index were partitioned into components to observe if the value of overall species richness/diversity was ≥ than the average richness/diversity within a community (Lande, 1996). We compared statistical significance for each component by a randomization procedure. This procedure tested a null hypothesis that the observed alpha and beta diversity components not differ from those by a random distribution (expected). Therefore, a total of 10,000 randomizations were generated and were used to calculate the null distribution of alpha and beta. The significance was calculated as the proportion of null values that were greater or lesser than the actual estimates. The test was performed using the adipart function of the vegan package in R v. 3.3 (R Core Team, Oksanen et al., 2012). Additionally, in order to identify if differences in community assemblies where associated either with species turnover (i.e., replacement of species by other species in different habitats) or species nestedness (i.e., species loss or gain between habitats) among the habitats, we partitioned the beta diversity into these components (turnover and nestedness) using a Sorenson dissimilarity matrix performed by betadisper function of the vegan package in R v. 3.3 (R Core Team, Oksanen et al., 2012). We used a null model with 10,000 randomizations to test if the results of the components were greater than expected by chance. The positive values indicated higher than expected contribution.

We employed a distance-based redundancy analysis (db-RDA; Legendre & Anderson, 1999) to quantify the influence of habitats on spatial community assemblies along sampling sites. We first conducted Principal Coordinates Analysis (PCoA) using community dissimilarity matrix calculated as Bray-Curtis index. The resulting eigenvalues of PCoA were used for the following db-RDA. Statistical significance of db-RDA models was tested using ANOVA. In addition, a permutation test was conducted to determine which habitat contribute to the spatial structure of the stonefly community. The calculation of dissimilarity matrix, db-RDA, and permutation test was conducted using vegdist, capscale and ordistep functions, respectively in vegan package in R v. 3.3 (R Core Team, Oksanen et al., 2012).

We employed multivariate dispersion as a measure of beta diversity based on a community dissimilarity matrix using DNA-species to further investigate the influence of a particular habitat on the beta diversity. We calculated beta diversity as the distance to group centroid (homogeneity of a community in a given habitat) based on a Bray-Curtis dissimilarity matrix. The community matrix consisted of the DNA-species richness per sampling site combining Tagliamento River (T01 to T15), and Fella River (F01 to F05). We employed an analysis of homogeneity of multivariate dispersions using a linear model in betadisper function in vegan package in R v. 3.3 (R Core Team, Oksanen et al., 2012) with a permutation test of significance (1000 permutations).

2.5 Phylogenetic structure of stonefly community

The phylogenetic relationship among species was estimated using a Maximum-Likelihood (ML) trees for *cox1* and ITS genes separately using PhyML 3.1 (Guindon & Gascuel, 2003) with the default settings under a GTR+I+G model as obtained by jModeltest 3.0 (Posada, 2008). The trees were bootstrapped using 10,000 replications. We used sequences of Orthoptera as outgroup in the *cox1* (HQ962558) and ITS (KT440350) trees based on the phylogenetic relationship with stoneflies (Misof et al., 2014).

A congruency index (MAST) (de Vienne, Giraud, & Martin, 2008) was conducted to test the congruency of tree topologies between *cox1* and ITS using the web version (http://max2.ese.u-psud.fr/icong/index.help.html). MAST index reveled congruency of tree topologies between both markers (Icong = 1.9, $p<0.05$). Therefore, a DNA supermatrix of the two markers (*cox1* + ITS) was generated by concatenating the output files of PhyML into one using FASconCAT-G perl script (https://github.com/PatrickKueck/FASconCAT-G). This supermatrix was used to observe the community phylogenetic signal of stoneflies among habitats using three types of analysis. First, we quantified the multiple-site phylogenetic similarities (i.e., similarity measures comparing more than two sites (here, as habitats)). We computed multiple-site phylogenetic turnover, nestedness and phylo-beta diversity index (Sorensen similarity index) as a total to communities per habitat for each sampling sites, in order to represent the proportion of shared branch lengths between pairs of sampling sites (Bryant, Lamanna, Morlon, Kerkhoff, Enquist, & Green, 2008) using phylo.beta.multi function in betapart package in R v. 3.3 (R Core Team, Baselga & Ornme, 2012). Second, we estimated the phylogenetic diversity by observing the niche conservatism (i.e., closely related species are ecologically similar and thus share similar habitats, Wiens et al., 2010) in the phylogenetic tree using Blomberg's K-statistic test and phylogenetic independent contrast (PIC) test. High values of Blomberg's K indicate a strong similarity in habitats among closely related species (positive phylogenetic autocorrelation), while values close to zero indicates no similarities in habitats among closely related species (random phylogenetic autocorrelation) (Blomberg, Garland, & Ives, 2003). This test was implemented per habitat using phylosig function in phytools package in R v. 3.3 (R Core Team, Revell, 2012). The PIC was implemented in picante package in R v.

3.3 (R Core Team, Kembel et al., 2010). We ran 10,000 iterations to obtain the null distribution. If the p-values of observed vs. random variance of PICs were lower than 0.05, we interpreted them as evidence of phylogenetic clustering for a given habitat. Third, we calculated the Net Relatedness Index (NRI) and the Nearest Taxon Index (NTI) to measure the degree of phylogenetic clustering at the community level (Webb et al., 2002) within each of the seven habitats (waterfall, riffle, glide, wando, side channel, pond and pool). The NRI measures the phylogenetic dispersion of an assemblage-community (community associated with the same habitat type) by comparing the observed mean pairwise phylogenetic distance between species in an assemblage-community to the null model. The NTI measures the phylogenetic dispersion of an assemblage-community by comparing the observed mean nearest phylogenetic neighbor distance between species in an assemblage-community to the null model. Positive values of NRI and NTI indicate that assemblage-community with the same habitat preferences are phylogenetic clustered (more closely related than expected), while negative values for NRI and NTI indicate that assemblage-community with the same habitat preferences show phylogenetic overdispersion (more distantly related than expected). The NRI and NTI were implemented using ses.mpd and ses.mntd functions with 1000 replications, respectively in picante package in R v. 3.3 (R Core Team, Kembel et al., 2010). If the four tests (Blomberg's K, PIC, NRI, and NTI) displayed statistically significant ($p<0.05$) positive results, it provides clear evidence of phylogenetic clustering.

3. RESULTS

Twenty-one morpho-species were collected from the 20 sampling sites. From each sampling site, 5 - 40 individuals/sampling site (mean 19) and 1 - 8 morpho-species/sampling sites (mean 4) were collected (Table 1). The 21 morpho-species were composed of 12 genera from two superfamilies of Nemouridae (Euholognatha) and Perloidea (Systellognatha) (Table S1).

We found 184 and 87 haplotypes among the 375 *cox1* (658-bp length) and 116 ITS (790-bp length) sequences, respectively. The highest haplotypes richness per sampling sites was found in T12 and the lowest number at T14 and T15. Stonefly community was dominated by *Leuctra major* (50 % of the total haplotypes, Table S1). Intraspecific nucleotide diversity for mitochondrial marker ranged 0 – 13 % among putative DNA-species, being the highest value for *L. major* (Table S1).

The log-likelihood of the GMYC model for *cox1* at the optimal threshold (2514.495) was significantly better than the null model of a single coalescent (logL = 2400.932) in the likelihood ratio test ($p < 0.001$). Most clades have GMYC-support values higher than 0.9, implying that the probability of the clades being delimited as separate GMYC-species among the alternative models of delimitation (within a 95% confidence set) is higher than 0.9. The single-threshold model delimited 52 putative species (Table S1) (confidence interval: 38 – 59) composed of 42 clusters (confidence interval: 32 – 49), indicating that ten (10%) out of the 52 inferred putative

species were singletons (i.e., only one sequence). Fourteen morpho-species showed multiple putative DNA-species (hereafter DNA-species), which suggest the presence of cryptic species (i.e., hidden species classified as nominal species because of indistinguishable morphological characters, Bickford et al., 2007). The congruence of ITS phylogenetic groups provided confirmation of DNA-based groups detected by GMYC. DNA-species co-occurred in different sampling sites and habitats throughout the corridor; however, a high percentage of cryptic species were found at the headwater section (78% of the total cryptic DNA-species).

3.1 Spatial structure of stonefly community assemblies

Out of 52 DNA-species, the meandering corridor section displayed the highest number of DNA-species (52 DNA-species) following by bar-braided (45 DNA-species) (Table 1). T08 showed the highest number of DNA-species (15 species) and the lowest at T14 and F05 (Table 1). Riffle displayed the highest number of DNA-species (31 DNA-species) following by glide (25 DNA-species) (Table 2, S2). Meandering corridor section showed the highest number of DNA-species (97 DNA-species) diversity per habitats (0.89 and 0.03, Shannon-Weaver and Simpson diversity index respectively, Table S2).

The relative contribution of the habitats and corridors to DNA-species diversity was observed (Fig. 2). The additive partition showed that the major contributor to DNA-species richness was corridor sections (57%, $p < 0.001$). While DNA-species diversity (Shannon index) analysis showed that habitats were the major contributors (88%, $p < 0.001$). DNA-species among habitats resulted in significantly high species turnover (0.60) than species nestedness (0.16) to overall beta diversity (0.79) ($p = 0.0016$).

The effect of habitat on DNA-species spatial variation among 20 sampling sites was observed using db-RDA. We found that DNA-species spatial variation was affected by three habitats ($p<0.05$, Fig. 3). Riffle correlated with DNA-species diversity of headwater and bar-brained floodplain section, while glide with bar-brained floodplain section and pool with the downstream section. Riffle and glide resulted with the highest diversity dissimilarity among the seven habitats (Linear model $F = 1.43$, $p = 0$, Fig. 4). This means that the community in these two habitats were less similar among each other that the community in the rest of the habitats.

3.2 Phylogenetic structure of stonefly community

The ML tree of mtDNA (*cox1*) showed two clades (Fig. S2), which correspond to the superfamilies of Nemouridae (Euholognatha) and Perloidea (Systellognatha). The Euholognatha branch group contains four families: Taeniopterygidae, Nemouridae, Capniidae, and Leuctridae, while the Systellognatha branch group contains three families: Choloperlidae, Perlidae, and Perlodidae. mtDNA agreed on 21 morpho-species delimitations; however, 11 species were composed of multiple terminal branches. Nuclear ITS phylogenetic tree showed congruence on tree topology with *cox1* tree. Except for haplotypes of *Nemoura cinerea* and

Nemoura mortini that were located in the same branch as *Capnia nigra* and *Capnia vidua* in ITS tree (Figure S1).

The stonefly community phylogenetic signal among habitats was observed. The multiple-site phylogenetic similarities showed that species turnover resulted significantly higher (0.927) than species nestedness (0.055) to overall beta diversity (0.98) ($p < 0.005$) in agreement to the values of species turnover for DNA-species composition among habitats (see above section). The pool habitat showed a significant phylogenetic clustering for all four tests (Blomberg's K test, PIC, NRI, and NTI, p-values < 0.05; Table 2), suggesting similarity among closely related species within this habitat. Additionally, a negative value for NRI and NTI indices was statistically observed (p-values<0.05, Table 2) by wando and side channel, and riffle, respectively, indicating that the assemblage-community show phylogenetic overdispersion.

4. DISCUSSION

The community assemblies within the stream are strongly related to the local environmental heterogeneity, as habitats availability (e.g., Astorga et al., 2014). We observed that different habitat types influenced Alpine stoneflies DNA-species diversity, their spatial structure, and their evolution.

The seven habitats were the major contributor to DNA-species diversity (diversity partition analysis, Shannon index). Habitat heterogeneity has been identified as a key driver of beta diversity of aquatic invertebrates (Astorga et al., 2014; Finn & LeRoy Poff, 2011; Finn, Theobald, Black, & Poff, 2006; Finn et al., 2011; Marten et al., 2006; Ribera & Vogler, 2008), and apart from the increase in niche availability, heterogeneous landscape may influence beta diversity through dispersal limitation (Finn & LeRoy Poff, 2011; Astorga et al., 2014). Stoneflies exhibit limited airborne dispersal with stream corridors (Consiglio, 1980; Fochetti & Tierno de Figueroa, 2008), and they tend to avoid migration among habitats even in response to a predator (Tiziano, Fenoglio, Lopez-Rodriguez, Tierno de Figueroa, Grenna, & Cucco, 2010). Supporting evidence of dispersal limitation is species turnover. A high species turnover has been associated with poor dispersal abilities (Thompson & Townsend, 2006). We found a high replacement of the species by other species among habitats (species turnover = 0.60), suggesting that a non-random migration is likely to occur among habitats caused by environmental filtering or spatial restriction, which likely promote high diversity, as previously observed in the freshwater fish, *Fecunduls heteroclitus* (Wagner, Baris, Dayan, Du, Oleksiak, & Crawford, 2017). However, a high species turnover could also be associated with sampling bias. Non-common species among habitats could be a consequence of a non-equal sampling effort or habitat availability. Here, habitat availability was found to be similar throughout the sampling sites (average = 0.75 Simpson diversity, range = 0.55-0.84, Table 1), and despite similar sampling effort implemented per habitat (2 hours), some species were not detected. An increase of sampling effort per habitats should be implemented in future studies in order to estimate sampling bias.

Among seven habitats, three (i.e., riffle, glide, and pool), were the major contributor of stonefly community structure (db-RDA analysis) and two habitats (riffle and glide) providers of high diversity (multivariate dispersion analysis) among sampling sites. Riffle (high-turbulent flow) and glide (low-turbulent flow) play an essential role for habitat suitability for many species of aquatic insects (Benda et al., 2004; Lancaster & Downes, 2013), especially stoneflies (Batista et al., 2001; Lancaster & Downes, 2013). The density-dependent local competition and high organisms drifting make then dynamic habitats harboring high biodiversity (Arscott et al., 2005; Batista et al., 2001; Hughes et al., 2009). On the contrary, pool (deep-high river section with slow/inexistence flow) reported a week relation with aquatic insect community diversity (Herrera-Vasquez, 2008), mainly because of the habitat-specialized taxa usually found (Pastuchova, Lehotsky, & Greeskova, 2008).

Surprisingly, we found that the pool habitat influenced on the phylogenetic clustering of stonefly community according to four employed indices (Blomberg's K, PIC, NRI, NTI). Long-term adaptation (e.g., Lososova et al., 2015), dispersal limitations (Saito et al., 2015a, 2015b), colonization history (Aizen, Gleiser, Sabatino, Gilarranz, Bascompte, & Verdu, 2015), and low species competition for resources (Webb et al., 2002) has been associated with phylogenetic clustering. However, a common factor detected by previous studies, it was that the phylogenetic clustering was usually detected at refugia associated with long-term stable habitats. Pool has been reported as a habitat with low impact of physical perturbations (Buffington, Lisle, Woodsmith, & Hilton, 2002), that may persist despite high flow (Calow & Petts, 1996), discharge (Rolls, Leigh, & Sheldon, 2012) and drought (Lake, 2003). The pool habitat could influence the evolution of stonefly community by phylogenetic clustering species associated with low-term steady habitat characteristics. However, also it has been observed that a greater competitive asymmetry (i.e., unequal division of resources) among distant phylogenetic relatives and facilitation among close phylogenetic relatives could cause phylogenetic clustering (Mayfield & Levine, 2010; Sargent & Ackerly, 2008). Future studies should address this concern by comparison of resources (i.e., food) among habitats, in order to obtain a clear picture of the evolutionary process on co-occurring species.

For riffle, wando and side channel habitats, a phylogenetic overdispersion pattern was detected instead of clustering. This pattern is commonly observed in aquatic insects (Saito et al., 2016; Violle et al., 2011). Efficient colonization and high dispersal capability patterns were previously reported to explain the phylogenetic overdispersion structure of several organisms, such as birds (Sobral & Cianciaruso, 2016), mammals (Cardillo, Gittleman, & Purvis, 2008) and insects (Violle et al., 2011). Efficient colonization and high dispersal ability were documented rarely for some stoneflies species (e.g., *Leutra ferruginea*, Macneale, Peckarsky, & Likens, 2005) but it has been documented that their dispersal is mainly dominated by in-stream drift (Stewart & Stark, 2008); however, their phylogenetic signal is unknown. Phylogenetic overdispersion usually was detected in a highly disturbed area that tends to preserve high phylogenetic diversity (Xiu et al., 2017). High-turbulent water flow habitats (riffle) and high physical perturbation habitats as those associated with sandy bar (wando and side-channel) at

the Tagliamento river tend to a high degree of aquatic habitat turnover (62%, Tockner et al., 2003) mainly because of two annual flooding (Ward et al., 1999). Therefore, the phylogenetic overdispersion of stonefly community at riffle, wando, and side-channel could be promoted by the high degree of perturbation that these habitats undergo.

Overall, the spatial and the phylogenetic structure of stonefly community assemblies in Tagliamento and Fella rivers were promoted by the habitat availability and stability. Among the four corridor sections, meandering section harbored the major number of DNA-species (52 DNA-species) with high diversity among habitats (0.89 and 0.03, Shannon-Weaver and Simpson diversity index, respectively). Meandering is a highly complex morphodynamic corridor section of the river that supports a high benthic invertebrate diversity because of habitats suitability and resilience in fluvial ecosystems (Garcia, Schnauder, & Push, 2012). However, the headwater corridor section displayed a high number of cryptic species (78% out of 100% total cryptic species). Headwater highly contribute to the proportion of cryptic species in freshwater ecosystems (e.g., Finn et al., 2011; Hughes et al., 2009; Jackson et al., 2013; Murria, Bonada, Arnedo, Prat, & Vogler, 2013) due to less canalization, loss of the connectivity with the stream network and low human disturbances (Arscott et al., 2005; Karaus et al., 2013; Tockner et al., 2003). Cryptic species are often observed in stoneflies (e.g., Viteck, Vincon, Graf, & Pauls, 2017), may be due to introgression (Boumans, Hogner, Brittain, & Johnsen, 2017) or hybridization (Young, Smith, Pilgrim, Fairchlid, & Schwartz, 2019) which remain unresolved species. Tagliamento and Fella River headwaters have narrow valleys and gorges with low sinuosity and confinement (Tockner et al., 2003); therefore, their headwater section could promote to an increase of local speciation and intraspecific genetic differences among stoneflies species. Both meandering and headwater corridor sections showed a strong role in maintaining the biodiversity of stoneflies in the Alpine rivers; therefore, their maintenance should be a priority on conservation practices.

In conclusion, we demonstrated that different habitats played an essential role in spatial and phylogenetic structure of stonefly community assemblies in Alpine regions. Community diversity was linked to high-turbulent water flow, but their evolutionary adaptation was linked with steady habitats that allow long-term stability. Our results highlight the importance of molecular studies on the assessment of counting biodiversity in a locality and the essential role of habitats in a region, which is especially important for community studies with a conservation concern (Esteban & Finlay, 2010).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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TABLE 1 Summary table of morpho-species, DNA-species, haplotype number, and DNA-species sampled per habitats per sampling sites (DNA-species per habitats, refer to Table S2) found at longitudinal corridor section of Tagliamento (decoded by T) and tributary Fella (decoded by F) River

Corridor Section	Sampling sites	Altitude (m)	Habitat simpson diversity	Morpho-species	DNA-species	Haplotype	DNA-species per habitats
Headwater	T01	1174	0.77	2	3	3	3
	T02	1064	0.77	3	4	6	7
	T04	1556	0.76	3	5	5	10
	T06	1346	0.78	8	10	10	21
	F01	1042	0.80	4	9	9	26
	F03	978	0.80	3	4	15	12
Meandering	T05	486	0.76	6	10	12	16
	T08	625	0.81	5	15	12	27
	T13	183	0.80	5	10	13	25
	T15	5	0.81	2	5	2	6
	F02	769	0.80	4	10	13	17
	F04	488	0.76	2	2	2	2
Bar-braided (floodplain)	T03	661	0.81	6	10	13	21
	T07	524	0.78	3	6	10	9
	T09	393	0.76	3	11	11	16
	T10	287	0.78	5	8	13	18
	T12	131	0.84	5	9	16	20
	F05	298	0.82	1	1	1	2
Spring-fed	T11	170	0.55	4	11	15	19
	T14	4	0.55	1	1	3	3

TABLE 2 Phylogenetic signal (clustering or overdispersion) of stoneflies communities at Tagliamento and Fella River in seven habitats. Bold asterisk values indicate statistical significance. N = total number of DNA-species per habitat, n taxa= number of DNA-species per sampling site per habitat, PIC = phylogenetic independent contrasts, NRI = net relatedness index, NTI = nearest taxon index

	N	**n taxa**	**Blomberg's K**	**p-value**	**PIC**	**p-value**	**NRI**	**p-value**	**NTI**	**p-value**
Waterfall	21	47	2.30	0.21	2.41	0.082	-1.29	0.115	-0.79	0.22
Riffle	31	99	2.30	0.37	41.73	0.034	1.52	0.959	-1.85	**0.027***
Glide	25	62	2.71	0.23	26.47	0.006	0.11	0.496	-0.87	0.188
Pool	10	23	4.03	**0.04***	76.8	**0.002***	2.53	**0.022***	1.79	**0.036***
Wando	6	14	1.73	0.55	10.89	0.583	-6.44	**0.001***	-1.26	0.094
Side Chanel	4	6	1.25	0.85	64.83	0.856	-2.06	**0.039***	-1.06	0.154
Pond	17	29	2.09	0.27	16.59	0.186	0.15	0.505	-0.82	0.209

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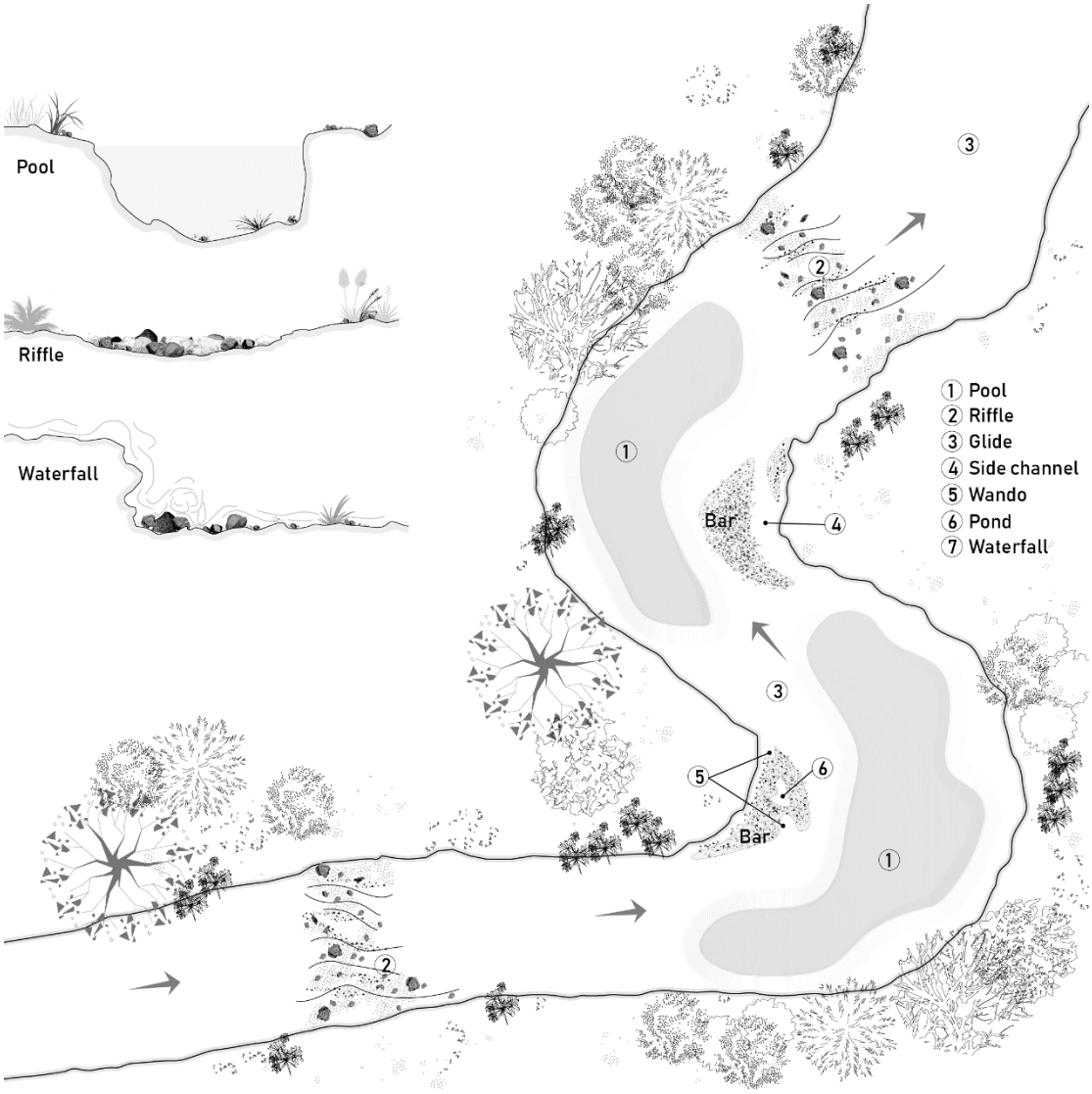
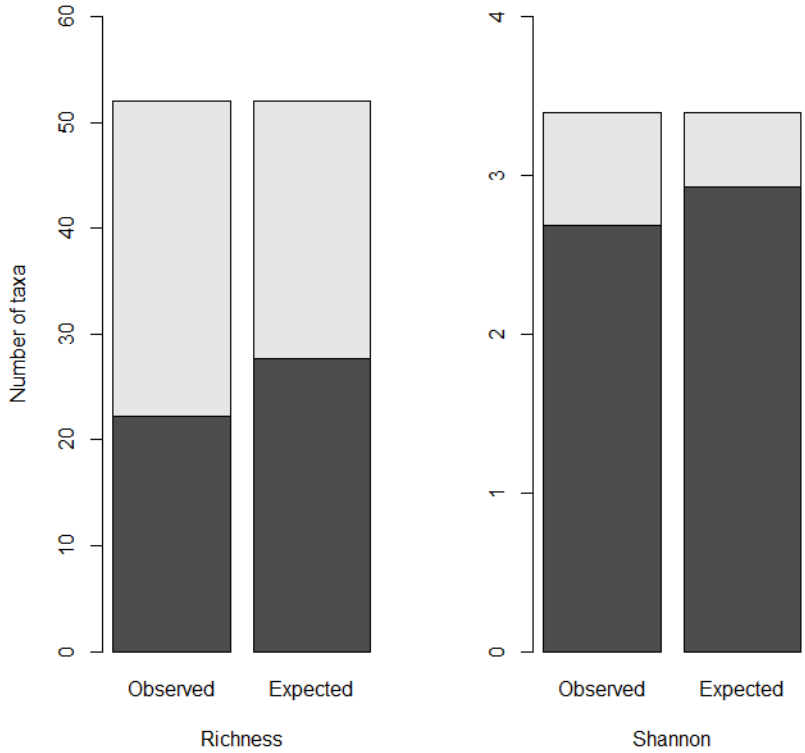
FIGURE 1 Habitats types sampled for each sampling site (Fig. S1) along the corridor of Tagliamento and Fella rivers. The arrows indicate the flow direction

FIGURE 2 Additive diversity partition of DNA-species richness and DNA-species diversity (Shannon-Wiener index) into observed and expected hierarchical components of corridors (light grey) and habitats (dark grey). Both partition analyses were statistical significance (<0.001) against null estimates based on 10,000 randomizations. Note the different scales in both graphics

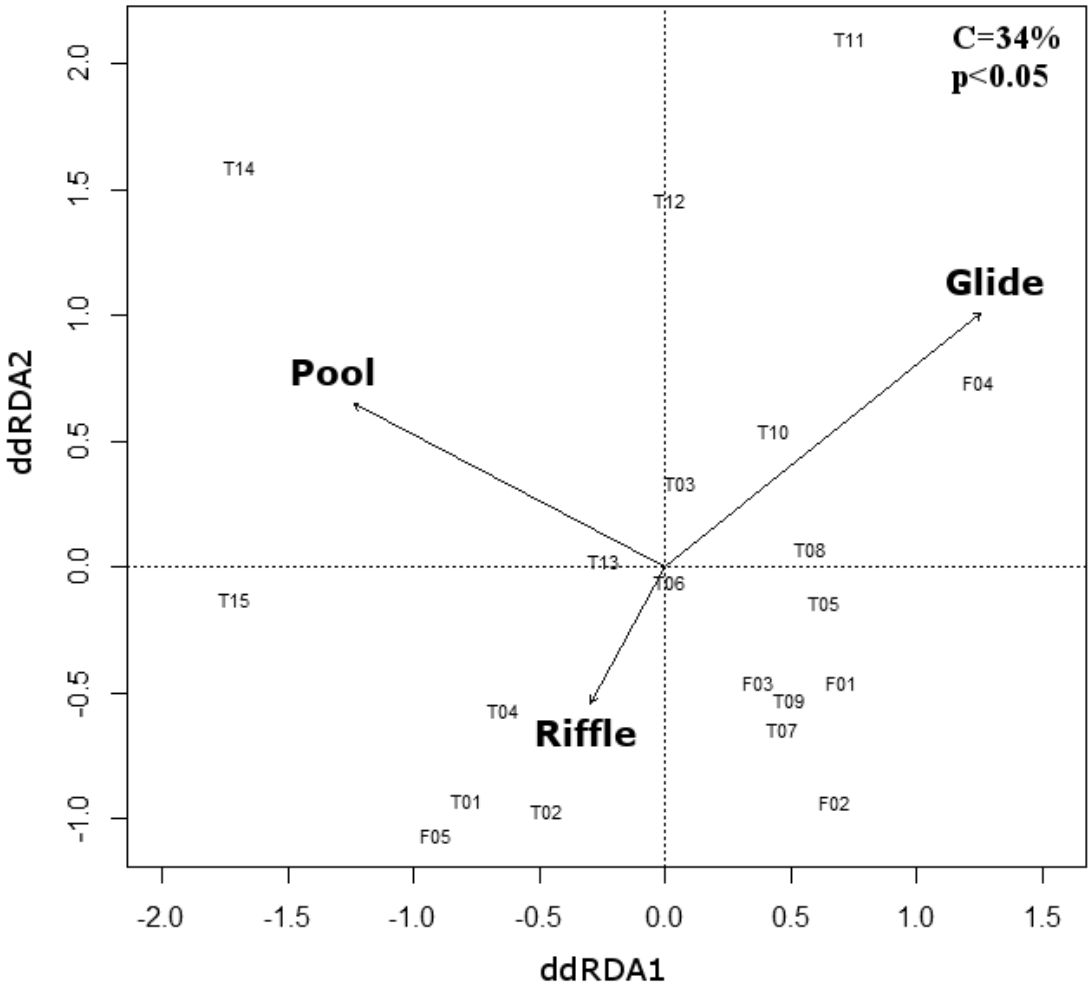
FIGURE 3 Distance based-redundancy analysis of DNA-species of stoneflies communities among 20 sampling sites displaying the influence of habitats. T = Tagliamento river, F = Fella river. Three habitats (pool, glide, and riffle) explained the highest proportion of total variation

FIGURE 4 Beta diversity of stonefly community in seven habitats base on the distance of values of beta diversity of each habitat in relation to their centroid (homogeneity of a community in a given habitat, see methods). Bold line represents median, and the bottom and top of the box represent lower and upper quartiles

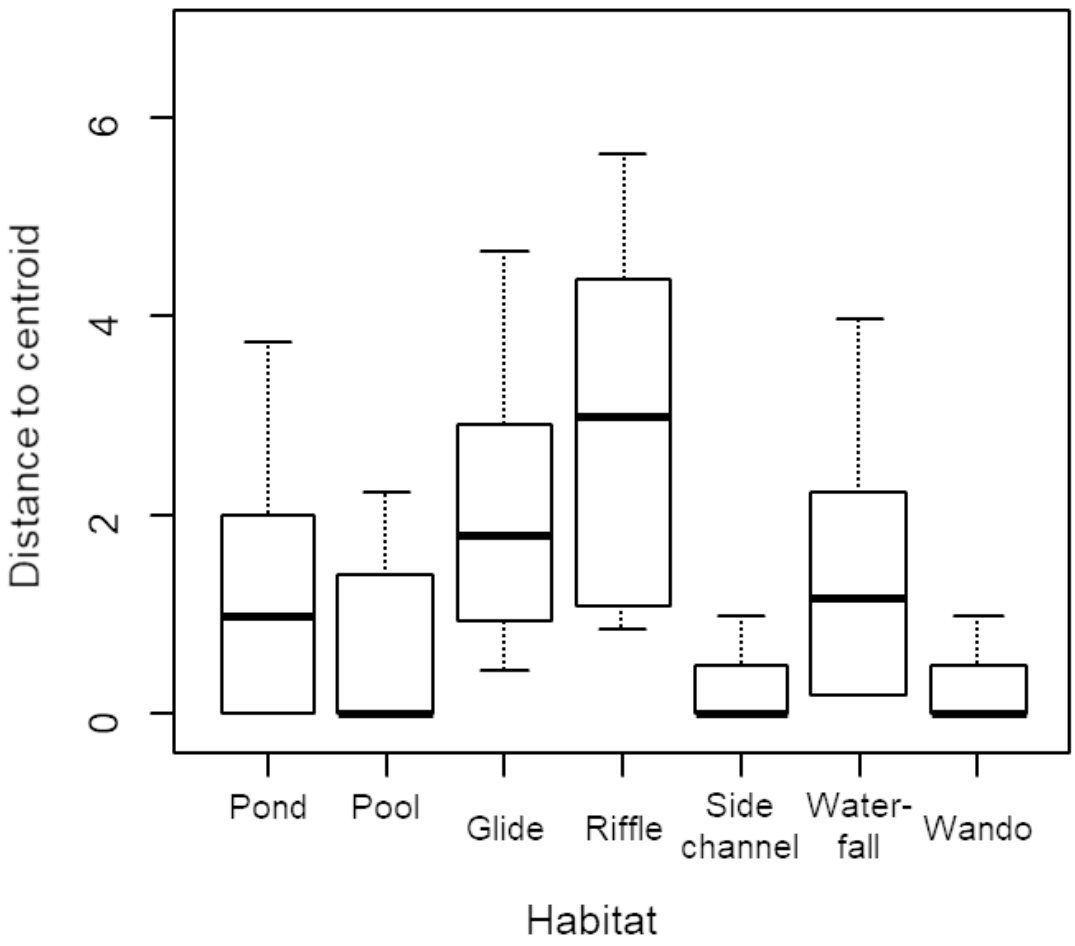

Pool
Riffle
Waterfall
① Pool
② Riffle
③ Glide
④ Side channel
⑤ Wando
⑥ Pond
⑦ Waterfall
Bar
Bar


Number of taxa
0
10
20
30
40
50
60
Observed
Expected
Richness
0
1
2
3
4
Observed
Expected
Shannon

778


C=34%
p<0.05
Glide
Pool
Riffle
T11
T14
T12
F04
T10
T03
T08
T13
T06
T15
T05
F03
F01
T09
T04
T07
T01
T02
F02
F05
ddRDA2
ddRDA1
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5

779


Distance to centroid
0
2
4
6
Pond
Pool
Glide
Riffle
Side channel
Water-fall
Wando
Habitat

780

Supplementary

TABLE S1 DNA-species list for all sampling sites

Species	Total n	DNA-species	Haplotypes	Present sampling sites	Present habitats	Genetic Diversity (%)	Altitude range (m)
Systellognatha							
Perlodidae							
Isoperla cf. *andreinii*	2	1	1	1	1	0	1346
Isoperla grammatica	15	2	9	5	4	2	486-1346
Besdolus sp.	28	3	14	7	5	3.5	183-1556
Perlodes microcephalus	16	2	10	6	2	2	287-769
Perlodes intrincatus	4	1	3	3	2	0	287-625
Perlidae							
Dinocras cephalotes	13	2	8	4	2	2.6	4-183
Perla marginata	24	2	15	6	5	6.5	131-661
Perla grandis	4	2	3	2	1	3	183-625
Chloroperlidae							
Chloroperla susemicheli	13	5	9	2	5	9.5	287-661
Euholognatha							
Capniidae							
Capnia nigra	17	3	9	2	2	7	131-170
Capnia vidua	8	1	2	1	2	0	170
Nemouridae							
Nemoura mortini	8	3	5	4	4	8.2	486-1346
Nemoura cinerea	10	1	2	1	2	0	1346
Amphinemoura sp.	2	1	1	1	1	0	524
Protonemoura cf. *nimborum*	3	2	3	3	4	5.2	393-1174
Protonemoura nimborella	7	1	1	1	1	0	1556
Protonemoura nitida	6	2	4	1	2	1.3	1346
Leuctricidae							
Leuctra fusca	25	7	12	6	3	3.7	131-287
Leuctra braueri	10	2	6	2	3	4.2	978-1346
Leuctra major	91	8	64	18	7	13.1	5-1556
Taenioterygidae							
Brachyptera risi	6	1	3	1	2	0	131

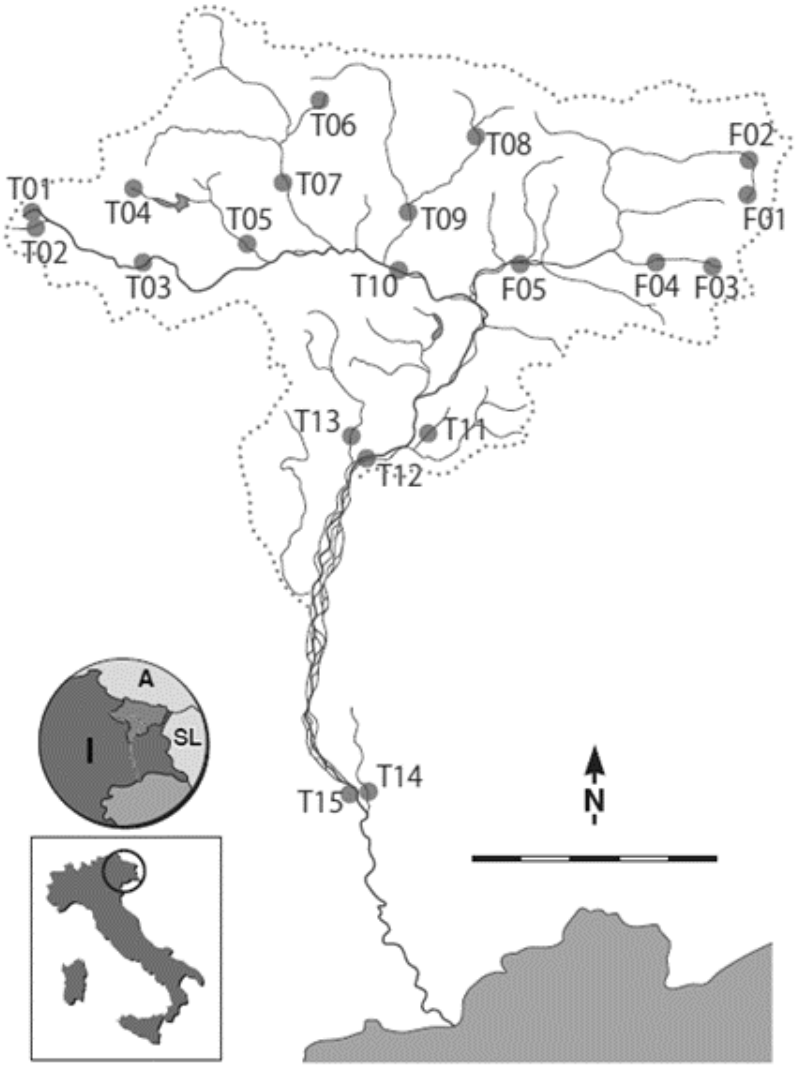
TABLE S2 DNA-species richness among habitats per sampling sites.

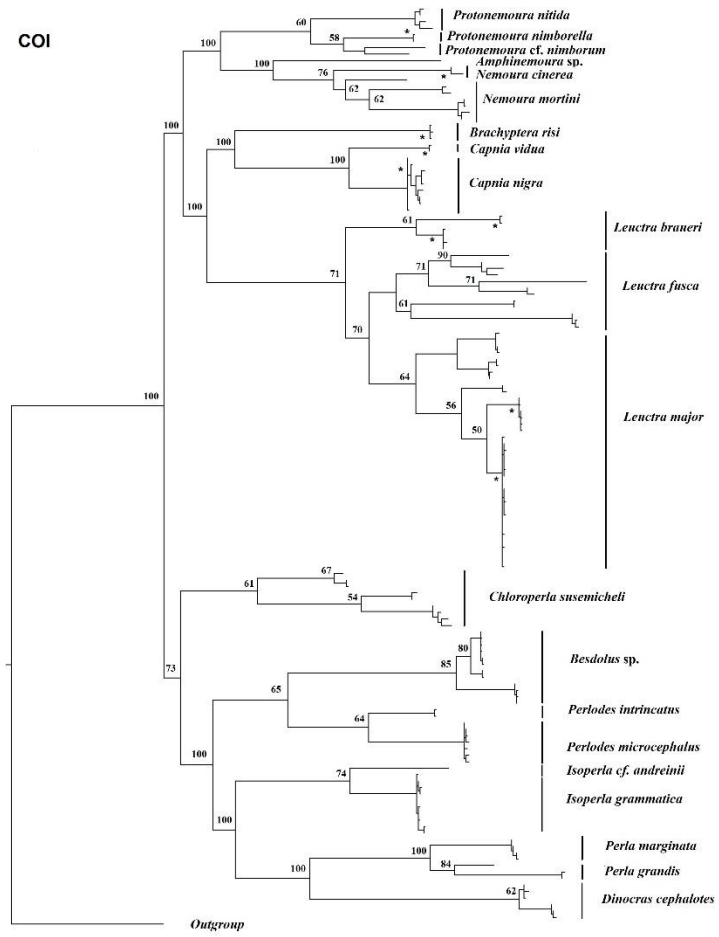
Corridor Section	Sampling sites	Waterfall	Riffle	Glide	Pool	Wando	Side channel	Pond	Shannon diversity	Simpson diversity
Headwater	T01	0	0	3	0	0	0	0	0.048602	7.68E-05
	T02	4	2	1	0	0	0	0	0.092222	0.000538
	T04	3	3	1	0	0	0	3	0.119007	0.001152
	T06	4	10	4	0	0	1	2	0.19427	0.005376
	F01	13	7	1	0	2	0	3	0.220693	0.008321
	F03	0	5	7	0	0	0	0	0.134995	0.00169
Meandering	T05	3	4	2	3	0	0	4	0.163554	0.003072
	T08	1	17	1	0	6	0	2	0.225542	0.008986
	T13	5	6	8	0	2	1	3	0.215707	0.00768
	T15	0	0	5	1	0	0	0	0.082351	0.000384
	F02	1	11	1	0	3	0	1	0.170096	0.003482
	F04	0	1	0	1	0	0	0	0.035297	2.56E-05
Bar-braided (floodplain)	T03	6	8	1	2	0	1	3	0.19427	0.005376
	T07	0	2	4	1	1	0	1	0.110493	0.000922
	T09	2	7	7	0	0	0	0	0.163554	0.003072
	T10	2	4	4	2	0	0	6	0.176427	0.003917
	T12	1	6	8	3	0	1	1	0.188504	0.004864
	F05	0	0	0	0	0	2	0	0.035297	2.56E-05
Spring-fed	T11	2	5	4	8	0	0	0	0.18256	0.004378
	T14	0	1	0	2	0	0	0	0.048602	7.68E-05
	Total	47	99	62	23	14	6	29	2.802043	0.936585

List of supplementary figures

FIGURE S1 Map of sampling sites of Tagliamento and Fella rivers. The northeast location of the river in Italy and sampling sites coding by T1-15 (T=Tagliamento) and F1-05 (F=Fella) are shown

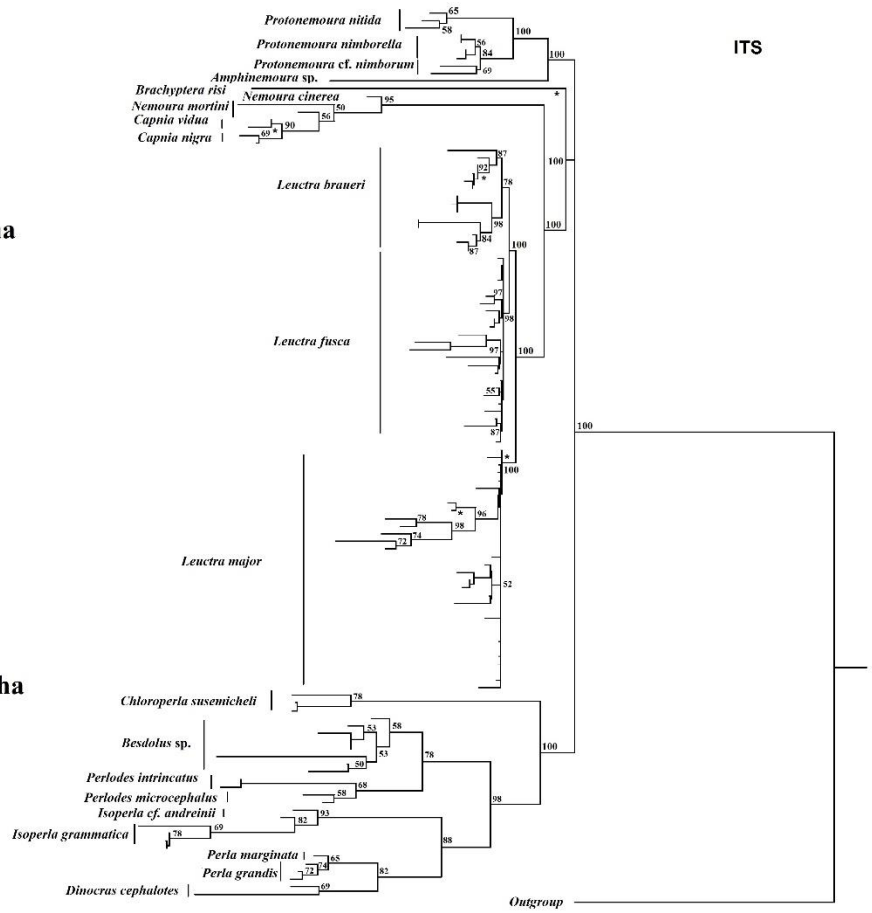
FIGURE S2 Maximum likelihood phylogenetic tree of sequences of stoneflies species located at Tagliamento River. Right is cox1 (mtDNA) and left is ITS (28S, nDNA). Numbers above branches indicate likelihood bootstrap support values. Asterisks show adult position. The subdivision of two groups for main river clade: Euholognatha and Systellognatha is showed


T01
T02
T03
T04
T05
T06
T07
T08
T09
T10
T11
T12
T13
T14
T15
F01
F02
F03
F04
F05
A
I
SL
N



Euholognatha

Systellognatha



799