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β-Diversity Scaling Patterns Across Different Bioregionalisations for a Megadiverse Neotropical Fish Family

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Keywords: beta diversity | conservation | freshwater ecosystems | killifish | Rivulidae | scale-dependent | spatial analysis | wetlands

ABSTRACT

Aim: Bioregionalisation frameworks represent unique assemblages of species resulting from geographic isolation and environmental heterogeneity. Understanding how different bioregionalisations capture community compositional variation is crucial, as underlying patterns and processes are scale-dependent. This study aimed to (1) explore the underlying ecological processes through the decomposition of beta diversity (turnover and nestedness); (2) identify which bioregionalisation framework offers the optimal spatial granularity for distinguishing between communities; and (3) evaluate the effective number of compositionally distinct areas.

Location: Neotropical domain.

Taxon: Rivulidae—annual species.

Methods: Presence–absence data of fish species were analysed using pairwise β-diversity and hierarchical clustering methods (UPGMA) and compared with 14 comprehensive bioregionalisation frameworks, including terrestrial ecoregions (TEOW), freshwater ecoregions (FEOW), neotropical provinces and watersheds (HydroBasins).

Results: The study revealed that (1) turnover is the dominant component of β-diversity, surpassing nestedness across all bioregionalisation frameworks; (2) turnover increases non-linearly as regionalisation area decreases, with a threshold identified beyond which further area reduction does not significantly increase turnover; and (3) the optimal spatial granularity for bioregionalisation is achieved at smaller watershed scales (146–414 km^2), where turnover is maximised and the optimal number of bioregions (> 180) is identified.

Main Conclusions: Turnover patterns are linked to factors such as high endemism, low dispersal capacity and the significant isolation of temporary wetlands. The scale dependence of β-diversity is influenced not only by the area of bioregionalisations but also by the underlying design of these units, such as those based on hydrogeomorphological features (HydroBasins) or taxon distribution patterns (FEOW and TEOW). Finer spatial scales are more effective for assessing biodiversity patterns for endemic taxa and in habitats with low connectivity. These findings can enhance the understanding of how bioregionalisation frameworks reflect species compositional variation, with important implications for interpreting ecological patterns and developing scale-dependent conservation strategies.

1 | Introduction

The definition of spatial scale is the cornerstone of disciplines such as ecology, biogeography and conservation biology because most underlying patterns and processes are scale-dependent (Levin 1992; Chave 2013). Studying these dependencies is crucial for advancements in these disciplines, as they determine how nature is observed and understood and, ultimately, how conservation strategies are formulated. Based on Whittaker (1960) seminal contribution, diversity has been distinguishing between alpha diversity (α—local diversity), beta diversity (β—variation in community composition among sites) and gamma diversity (γ—regional diversity) to gain a deeper understanding of the processes that structure communities at different spatial scales. Baselga (2010) expand this view by partitioning β-diversity into two components that reveal antithetical processes: turnover and nestedness. Turnover denotes the replacement of species between sites, while the nestedness component arises from changes in species richness—sites with fewer species are strict subsets of more species-rich sites. These two opposing patterns are associated with (though not limited to) distinct processes, such as dispersal, connectivity, extinction–colonisation dynamics, biotic interactions and environmental gradients (Soininen, McDonald, and Hillebrand 2007; Ulrich, Almeida-Neto, and Gotelli 2009; Soininen, Heino, and Wang 2018). Moreover, environmental heterogeneity can exert a strong influence on β-diversity and its components (Heino, Melo, and Bini 2015). In heterogeneous habitats, a broader range of ecological niches is generally available, favouring greater species diversity (Tews et al. 2004; Stein, Gerstner, and Kreft 2014). In such environments, turnover tends to be higher because each site can harbour a distinct set of specialised species, increasing the rate of species replacement in the landscape (Soininen, Heino, and Wang 2018). Conversely, more homogeneous habitats tend to host more similar and less diverse communities, thereby favouring nestedness patterns (Ulrich, Almeida-Neto, and Gotelli 2009; Soininen, Heino, and Wang 2018).

β-diversity can be measured using various metrics, each with its advantages and limitations, driven by specific proposals and hypotheses (Anderson et al. 2011). Studies have revealed that β-diversity and its components (turnover and nestedness) are strongly scale-dependent, including both the grain size and the spatial extent of a study (Barton et al. 2013; Sreekar et al. 2018; Rolls et al. 2023; Ohyama, Bogota-Gregory, and Jenkins 2024). Spatial scale comprises 'grain' (resolution or size of the sampling unit, e.g., grid cell, buffer, watershed) and 'extent' (size of the region encompassing all sampling units in a study, e.g., hydroregion, country, continent, domain) (Wiens 1989; Heino, Melo, and Bini 2015). This dependency raises a fundamental question: does the scale-dependent nature of β-diversity pose a problem, or is it the key to identifying the spatial scale that best distinguishes community differences?

The answer to this question is not yet consensual and depends on the context and the metric used. The scale dependency is negative when the effect of scale is not controlled or when the scale is arbitrarily established, leading to methodological artefacts that can bias the interpretation of underlying patterns and processes (Whittaker, Willis, and Field 2001). Contrastingly, scale dependence can be positive when β-diversity is evaluated at multiple scales, indicating the optimal spatial granularity where the separation between ecological communities is most distinct (Ohyama, Bogota-Gregory, and Jenkins 2024). Indeed, modern biogeography has increasingly relied on this principle of β-diversity to identify different ecological communities as well as to more precisely delineate biogeographical regions (Kreft and Jetz 2010; Oliveira et al. 2024). A non-consensual issue is that multiscale approaches using different β-diversity metrics but under the same scaling framework (fixed extent and varying grain) produce varying β-diversity peaks, with peaks occurring at regional scales (Ohyama, Bogota-Gregory, and Jenkins 2024) or local scales (Antão et al. 2019). Additionally, β-diversity may show similar peaks at regional scales when using distinct metrics and scaling approaches (Ohyama, Bogota-Gregory, and Jenkins 2024—fixed extent and varying grain vs. Barton et al. 2013—varying extent and grain). Therefore, this complexity highlights the need to continue exploring multiscale approaches to determine how different metrics and scales influence the interpretation of β-diversity and, consequently, the precise delimitation of ecological communities and biogeographical regions.

There is significant variability in how the scaling of β-diversity has been addressed in the literature, both in terms of area size, number of grains (or levels) and the shape (square, circular, hexagonal and natural boundaries) of the scales. The studies include (but are not limited to): comparisons of different boundary delineations (political boundaries, natural geomorphic breaks and watershed boundaries; Stoczynski et al. 2023); square grids with different grain sizes (Calderón-Patrón et al. 2013); levels of a scale gradient through the systematic partitioning of buffer subdivisions (Antão et al. 2019); central hexagons as the average of their β paired with their adjacent neighbouring hexagons (Ohyama, Bogota-Gregory, and Jenkins 2024). Although this arbitrary segmentation (e.g., grids and hexagons) provides a fast, easy and standardised method for spatial analyses (Birch, Oom, and Beecham 2007), it imposes artificial boundaries that may obscure important ecological processes operating along environmental gradients or within heterogeneous landscapes (Oliveira, Brescovit, and Santos 2015).

Currently, a variety of global bioregionalisation frameworks are available, based on different criteria for delineating regions, reflecting distinct evolutionary history, environmental heterogeneity and geographic isolation in shaping biodiversity patterns. For example, the Terrestrial Ecoregions of the World (TEOW; Olson et al. 2001) divide terrestrial areas into ecoregions on the basis of distinct sets of fauna and flora species shaped by biogeographic barriers, such as mountain ranges and rivers. The

Freshwater Ecoregions of the World (FEOW; Abell et al. 2008) follow a similar logic but focus on freshwater systems, reflecting the influence of drainage areas and aquatic connectivity. The neotropical provinces (Morrone et al. 2022) consider combining climatic, geological and biotic criteria, particularly in the tropics, where geographic isolation plays a central role in species diversification. Finally, frameworks such as HydroBasins (Lehner and Grill 2013) organise aquatic and terrestrial systems hierarchically, using nested sub-basins that reflect hydrological connectivity and environmental gradients. Little is known about how these different bioregionalisation frameworks capture the scaling of β-diversity. Moreover, most previous studies are multitaxa, involving species with different life history traits, dispersal patterns and endemism (Qian 2009; Calderón-Patrón et al. 2013; Soininen, Heino, and Wang 2018; Antão et al. 2019; Ohyama, Bogota-Gregory, and Jenkins 2024), which may introduce confounding factors in the assessment of scaling.

Neotropical freshwater fishes of the family Rivulidae (484 valid species; 9th most speciose fish family in the world; Fricke, Eschmeyer, and Fong 2024) may serve as an important case study for understanding β-diversity scaling patterns for two main reasons. First, because most species exhibit restricted distribution patterns (high endemism) and share similar life history traits, such low dispersal ability, small body size, rapid growth, pronounced sexual dimorphism and a life cycle synchronised with the rainy season (Costa 2010; Loureiro et al. 2018; Guedes, Luz, et al. 2023; Alonso et al. 2023; Loureiro et al. 2024); reducing confounding factors related to phylogeny, seasonality and life history traits in multitaxa analyses of β-diversity. Rivulidae species are categorised into two life cycles: annual or perennial, according to the presence (or absence) of eggs with embryonic diapause (Furness et al. 2018). Eggs with developmental arrest (i.e., embryonic diapause) are present in annual species, a characteristic that allows these species to survive extreme water fluctuations in temporary wetlands and endure for months without water in dry terrestrial habitats (Guedes, Gomes, et al. 2023; Guedes, Luz, et al. 2023). The evolution of an annual life cycle in vertebrates is peculiar and aligns these fishes with the survival strategies (seeds, eggs and cysts) developed by plants in desert environments or by insects in temperate zones exposed to freezing winters (Furness 2016). Second, because Rivulidae faces a significant extinction threat (Volcan and Lanés 2018). In Brazil, one of the most megadiverse countries on the planet, Rivulidae is the most numerically threatened vertebrate family, with this single family alone harbouring more threatened species (130 spp.) than all other marine fish families combined (97 spp.) (ICMBio—Instituto Chico Mendes de Conservação da Biodiversidade 2022). Consequently, defining the scales that best represent the biodiversity patterns of Rivulidae is urgent and essential for formulating conservation strategies for these species.

Therefore, the main goal of this study was to determine how different bioregionalisation frameworks capture the scaling of β-diversity for annual fish species (Rivulidae) in the Neotropical region. The specific objectives are: (O1) To assess the underlying ecological processes through the decomposition of β-diversity (turnover and nestedness). (O2) To identify which bioregionalisation framework includes the optimal spatial granularity where the separation between communities is most distinct. (O3) To determine the effective number of compositionally distinct areas through a hierarchical clustering approach. To achieve this, presence–absence data for annual species were compiled from temporary wetlands across the entire Neotropical region, from Mexico (18°N) to Argentina (37°S), and compared with 14 widely used bioregionalisation frameworks in the literature (e.g., TEOW, FEOW and HydroBasins). Two hypotheses were tested. H1—Turnover (species replacement between sites) is the dominant component of β-diversity, surpassing nestedness in all bioregionalisation frameworks. H2—β-diversity increases as the area of bioregionalisation decreases. However, the scaling of β-diversity is not linear; there exists a threshold beyond which further reductions in the area of bioregionalisation no longer significantly impact β-diversity. H1 is supported by meta-analyses that reveal the dominance of species turnover across different ecosystems and taxonomic groups (Soininen, Heino, and Wang 2018). This phenomenon may be particularly relevant for annual fish due to the pattern of isolated populations, low active dispersal capacity and the low connectivity of temporary wetlands. H2 was formulated on the basis of the principle that, in multiscale analyses based on classic β-diversity metrics (e.g., pairwise and multiple-site) where grain varies and extent is fixed, higher β-diversity values are expected at smaller spatial scales (Barton et al. 2013). The non-linearity in β-diversity scaling is supported by a consistent power law observed among different taxa by Antão et al. (2019), which may be related to how different scales capture habitat heterogeneity, species dispersal capacity, and the intra- and interspecific aggregation patterns of communities. In summary, this study aims to advance the understanding of how different bioregionalisation frameworks capture the compositional variation of communities. By exploring the decomposition of β-diversity and determining the optimal spatial granularity, this research can not only clarify the underlying ecological processes but also provide a refined scale for formulating conservation strategies for these highly threatened fish species in the Neotropical region.

2 | Methods

2.1 | Valid Species and Delimitation of Annualism

Eschmeyer's Catalog of Fishes was used as the taxonomic reference for determining valid species and genera within the Rivulidae family (Fricke, Eschmeyer, and Fong 2024). The species were categorised on the basis of the presence (or absence) of embryonic diapause and an annual life cycle, according to four categories (1–4) derived from the three possible stages of diapause (I–III) in killifish, as recognised by Furness et al. (2018): (1) species without diapause and with typical teleost development; (2) and (3) species with delayed hatching and potential specific phases of diapause, representing intermediates in diapause evolution; and (4) species with typical embryonic diapause of an annual life cycle, which exhibit the dispersed cell phase (diapause I), diapause II and delayed hatching/diapause III. Our study focused exclusively on taxa that fit the fourth (4) pattern described above (see Appendix S1). Additionally, annual killifishes that complete two or more generations in 1 year are also referred to as 'seasonal' or 'multivoltine' (Žák et al. 2021). Classifying a rivulids species as annual, seasonal or multivoltine does not seem to be a pertinent topic for our goals because: (1) the same species/population might display an annual cycle in 1 year and a seasonal/multivoltine cycle in another, depending on hydroclimatic conditions (García et al. 2019); (2) species with fragmented populations across a wide geographical range (e.g., *Pterolebias longipinnis* and *Neofundulus paraguayensis*) may potentially exhibit an annual life cycle in one area and a seasonal life cycle in another due to variations in the duration and pulses of rainy season. Therefore, our data include all species designated elsewhere as annual, seasonal and multivoltine.

2.2 | Occurrence Data

Occurrence records of annual killifish were exhaustively compiled between January 2021 and September 2023 from various sources, including articles, books and public digital repositories that aggregate information from collections/museums, such as the Global Biodiversity Information Facility (GBIF, gbif.org), Brazilian Biodiversity Information System (SIBBR, sibbr.gov.br), SpeciesLink (splink.org.br) and Biodiversity Extinction Risk Assessment System (SALVE, salve.icmbio.gov.br). To include occurrences in our database, we applied the following filters: (i) only records identified at the species level (sp., cf. or aff. excluded) and (ii) geographic coordinates consistent with distribution information available in the literature. Species distributions were validated by plotting individual species maps and comparing them with those published in the primary taxonomic literature. For species with unavailable/unknown geographic coordinates, we estimated approximate locations on the basis of georeferenced maps or descriptions of type localities. All occurrences used in this study were compiled during the respective rainy seasons in the areas where the species occur. Our final database includes 1635 occurrence records, encompassing all 261 annual species (41 genera) currently recognised in the Neotropical (Tables S1.1 and S1.2 in Appendix S1).

2.3 | Bioregionalisation Frameworks

Species occurrence data were overlaid with 14 different bioregionalisation frameworks: terrestrial ecoregions (TEOW; Olson et al. 2001), freshwater ecoregions (FEOW, Abell et al. 2008), neotropical provinces (Morrone et al. 2022) and 11 different levels of hierarchically nested sub-basins (HydroBasins; Lehner and Grill 2013). This results in presence or absence matrices of species for the 14 different bioregionalisations (Figure 1), with mean area and number of grains/levels ranging from 3,251,927 to 146 km^2, and 6–767 levels, respectively (Table 1). These bioregionalisations were chosen for different reasons. Watersheds represent natural boundaries that can significantly affect the distribution and endemism of fish. Freshwater ecoregions were specifically delineated on the basis of the distribution of fish and other aquatic organisms (Abell et al. 2008). Terrestrial ecoregions provide a complementary context for understanding the interaction between terrestrial and aquatic habitats, as annual fishes occupy temporary wetlands, which are essentially terrestrial environments during the dry period. Finally, the neotropical provinces offer a broad and integrative scale that encompasses large ecological provinces with distinct climatic and biogeographic characteristics (Morrone et al. 2022). The overlay of occurrence data and vector layers with the boundaries of the regionalisations were performed in QGIS software version 3.32.3 Lima (QGIS Development Team 2024).

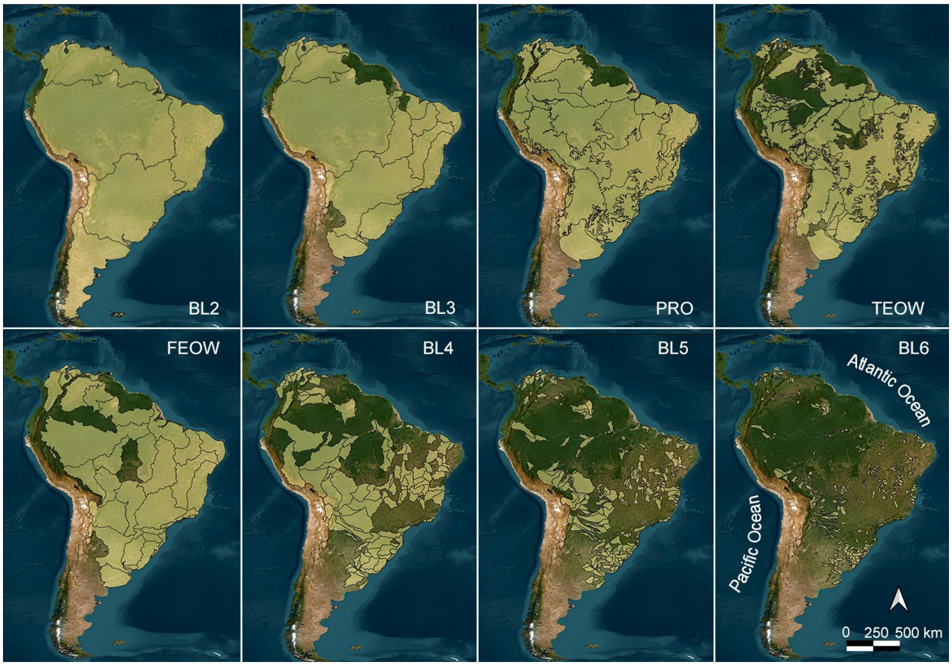


FIGURE 1 | Map illustrating the occurrence of annual killifishes (yellow polygons) according to different regionalisations within the neotropical domain. The maps are organised in descending order on the basis of the mean area (km^2) of polygons (levels) with species occurrences. BL, HydroBasins 2–6; FEOW, Freshwater ecoregions; PRO, neotropical provinces; TEOW, Terrestrial ecoregions. Occurrence records in Mexico and basins Levels BL7-12 were omitted to enhance figure readability.

TABLE 1 | Summary of the characteristics of the 14 analysed regionalisations: Average area (km^2) and number of grains/levels (subdivisions); mean ± standard deviation of β-diversity (βsor) and the components of turnover (βsim) and nestedness (βsne). Clusters—optimal number of clusters according to the UPGMA hierarchical clustering algorithm. The regionalisations are listed in descending order on the basis of the mean area (km^2) of polygons (levels) with species occurrences (see Figure 1).

Regionalisation	Area (km^2)	Levels	Clusters	βsor	βsim	βsne
BL2	3,251,927	6	5	0.991 ± 0.03	0.948 ± 0.17	0.042 ± 0.16
BL3	885,202	17	13	0.98 ± 0.09	0.945 ± 0.19	0.035 ± 0.16
PRO	621,064	25	20	0.973 ± 0.09	0.944 ± 0.14	0.028 ± 0.08
TEOW	369,159	52	31	0.961 ± 0.13	0.920 ± 0.23	0.04 ± 0.15
FEOW	240,433	33	24	0.979 ± 0.08	0.962 ± 0.13	0.016 ± 0.07
BL4	210,486	56	34	0.977 ± 0.1	0.957 ± 0.17	0.02 ± 0.11
BL5	64,979	114	55	0.973 ± 0.13	0.953 ± 0.20	0.02 ± 0.11
BL6	14,740	263	114	0.983 ± 0.11	0.975 ± 0.14	0.007 ± 0.06
BL7	4165	394	146	0.985 ± 0.11	0.979 ± 0.13	0.005 ± 0.05
BL8	1207	516	163	0.986 ± 0.1	0.982 ± 0.12	0.004 ± 0.04
BL9	414	678	181	0.987 ± 0.1	0.983 ± 0.12	0.004 ± 0.04
BL10	179	754	188	0.987 ± 0.1	0.983 ± 0.12	0.003 ± 0.04
BL11	148	766	188	0.987 ± 0.1	0.983 ± 0.12	0.003 ± 0.04
BL12	146	767	188	0.987 ± 0.1	0.983 ± 0.12	0.003 ± 0.04

Abbreviations: BL, HydroBasins 2–12; FEOW, freshwater ecoregions; PRO, neotropical provinces; TEOW, terrestrial ecoregions.

2.4 | Data Analysis

To assess the underlying ecological processes through the decomposition of β-diversity (O1 and H1), the additive partitioning model by Baselga (2010) was adopted, based on pairwise dissimilarity, with the Sørensen index reflecting total β-diversity (βsor), considering all aspects of variation in composition. The Simpson dissimilarity index (βsim) measures species turnover, disregarding species richness gradients and the nestedness component (βsne) is measured as the fraction resulting from Sørensen dissimilarity nestedness. Pairwise distances were calculated for each of the 14 bioregionalisations separately using the *'beta.pair'* function from the *betapart* package (Baselga and Orme 2012).

To investigate the patterns of turnover (βsim) scaling in relation to the mean area of bioregionalisations (H2), four distinct models were fitted: linear, quadratic, power and exponential. This approach follows Kreft and Jetz (2010), who argue that richness-independent turnover is more informative for biogeographical regionalisations and recommend using metrics that are minimally affected by variation in species richness. Although a non-linear relationship was expected, the choice for using different models was based on previous studies showing that the relationship between area and beta diversity could be better explained by power (Antão et al. 2019) or quadratic (Ohyama, Bogota-Gregory, and Jenkins 2024) models. The area (in km^2) was normalised by dividing each value by the maximum area, in order to standardise comparisons. The selection of the best model was based on the coefficient of determination (R^2), which measures the proportion of variance in βsim explained by each model, allowing the identification of the relationship that best describes the data.

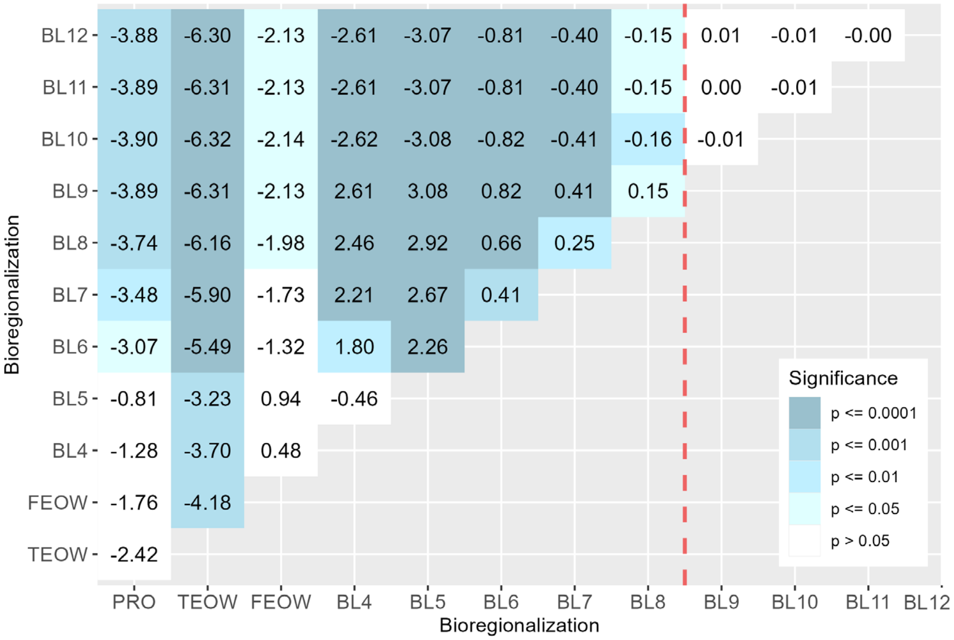
To verify the existence of a threshold beyond which further reductions in the area of bioregionalisation no longer significantly impact β-diversity (O2), pairwise distance values of βsim (1.35 million pairwise values) were compared among the bioregionalisations using Welch's F-test and the Games–Howell multiple comparisons ($p < 0.05$). These tests were chosen because they are robust to heteroscedasticity and do not require equal group sizes (Games and Howell 1976), as is the case in this study. Two regionalisations with few levels (BL2 = 6; BL3 = 17) were excluded from this analysis to ensure the robustness of the results and to avoid biases related to β-diversity when sample sizes are very small (Bennett and Gilbert 2016). This analysis was conducted using the *'games_howell_test'* function from the *rstatix* package (Kassambara 2023).

To determine the effective number of compositionally distinct areas, a hierarchical clustering approach was employed (O3). Given the lack of a priori justification for selecting a specific method for each of the 14 βsim matrices, eight widely used hierarchical clustering algorithms (e.g., UPGMA, Ward and UPGMC) were compared on the basis of the degree of data distortion, using Sokal and Rohlf's (1962) cophenetic correlation coefficient (function *select_linkage*). UPGMA exhibited the highest correlation values across all regionalisations, resulting in regions with maximum internal similarity and maximum differentiation from other areas (Tables S1.3 in Appendix S1). Therefore, UPGMA was used to determine the optimal number of clusters that best describe each observed βsim matrix using the 'elbow' (or 'knee') method, corresponding to the point of maximum curvature (Salvador and Chan 2004; function *optimal_phyloregion*, argument '*k*' = total number of levels in each bioregionalisation or algorithm limit: 514). All analyses described in this paragraph were performed using the *Phyloregion* package (Daru, Karunarathne, and Schliep 2020) following the framework proposed by Kreft and Jetz (2010). All analyses described in this article were conducted in the R environment (R Core Team 2024), and the data and scripts used in the analyses are available in Appendix S2.

3 | Results

The β-diversity (βsor) of annual fishes was high across all regionalisations, with an overall mean >0.96 (Table 1). H1: Partitioning revealed turnover as the dominant component (overall mean βsim = 0.92), surpassing nestedness (negligible contributions, βsne = 0.01) in all 14 bioregionalisations (Table 1). Turnover and nestedness showed opposite trends; as the area of the bioregionalisation decreased, turnover experienced a slight increase, whereas nestedness declined (Table 1).

Turnover (βsim) was exceptionally high across all bioregionalisations (Table 1). Despite the mean βsim varying little among the different bioregionalisations, the statistical analyses showed highly significant differences (Fwelch = 43.39, $p < 0.0001$). Games–Howell multiple comparisons indicated significant differences in βsim among most regionalisations ($p < 0.05$; Figure 2). The freshwater ecoregions (FEOW), with a mean area of 240,433 km^2, captured a similar species turnover ($p > 0.05$; Figure 2) to regionalisations with substantially smaller areas (e.g., HydroBasins; BL7–4165 km^2). From the bioregionalisation sub-basin level 9 (HydroBasins; BL9; 414 km^2), the reduction in regionalisation area to lower levels (BL10-12; lower limit 146 km^2) did not result in significant gains in βsim ($p > 0.05$; Figure 2). The power model, with an R^2 value of 0.74, provided



	PRO	TEOW	FEOW	BL4	BL5	BL6	BL7	BL8	BL9	BL10	BL11	BL12
BL12	-3.88	-6.30	-2.13	-2.61	-3.07	-0.81	-0.40	-0.15	0.01	-0.01	-0.00	
BL11	-3.89	-6.31	-2.13	-2.61	-3.07	-0.81	-0.40	-0.15	0.00	-0.01		
BL10	-3.90	-6.32	-2.14	-2.62	-3.08	-0.82	-0.41	-0.16	-0.01			
BL9	-3.89	-6.31	-2.13	2.61	3.08	0.82	0.41	0.15				
BL8	-3.74	-6.16	-1.98	2.46	2.92	0.66	0.25					
BL7	-3.48	-5.90	-1.73	2.21	2.67	0.41						
BL6	-3.07	-5.49	-1.32	1.80	2.26							
BL5	-0.81	-3.23	0.94	-0.46								
BL4	-1.28	-3.70	0.48									
FEOW	-1.76	-4.18										
TEOW	-2.42											

FIGURE 2 | Results of the Games–Howell Multiple Comparisons test comparing turnover (βsim) between different bioregionalisations. Values within the graph indicate the estimated mean difference between the compared groups (Estimate = [Mean of Group 1 − Mean of Group 2] × 10^2). Positive and negative estimate values indicate the direction of the difference between group means, with Group 1 on the *x*-axis and Group 2 on the *y*-axis. The colour gradient reflects the statistical significance of the paired comparisons. The red dashed line on the right marks the threshold where βsim shows no significant differences between bioregionalisations with different average areas (identified by the Games–Howell Multiple Comparisons test). The *x*-axis of the graph is organised in descending order of the average area of the bioregionalisations.

TABLE 2 | Results of four different models (linear, quadratic, power and exponential) fitted to investigate the relationship between turnover (βsim) and the mean area (normalised) of bioregionalisations. R^2—coefficient of determination; The parameters (*a*, *b* and *c*) represent the coefficients for each model equation.

Model	Equation	R^2	*a*	*b*	*c*
Linear	$\beta\text{sim} = a \times \text{area\_norm} + b$	0.196	−0.034	0.969	
Quadratic	$\beta\text{sim} = a \times \text{area\_norm}\hat{}2 + b \times \text{area\_norm} + c$	0.622	0.202	−0.229	0.977
Power	$\beta\text{sim} = a \times \text{area\_norm}\hat{}b$	0.744	0.940	−0.005	
Exponential	$\beta\text{sim} = a \times \exp(b \times \text{area\_norm})$	0.199	0.969	−0.036	

the best fit (H2), followed by the quadratic model ($R^2=0.62$; Table 2). The linear and exponential models showed weaker explanatory power ($R^2=0.19$; Table 2). A slightly negative parameter b in the power model (−0.005) indicated that the relationship between area and turnover (βsim) slightly decreased as area increased (Table 2).

The number of optimal clusters (elbow method; UPGMA clustering algorithm) ranged from 5 to 188 (Table 1), showing a coupling pattern with the number of levels of the bioregionalisations between BL2 and BL4 (HydroBasins), and decoupling from BL5 onwards (Figure 3). As the number of levels in the regionalisation increases, the initial number of optimal clusters grows rapidly, but the growth rate diminishes and eventually stabilises as more levels with smaller areas are added to the bioregionalisations (Figure 3). For the watershed at level 9 (BL9), 181 compositionally distinct areas (bioregions) were identified (maximum explained variance of 0.95) for Neotropical annual killifishes (Figure S3.1 in Appendix S3). Each bioregion comprises between 1 and 10 species (Table S3.1 in Appendix S3), with approximately 72% (187 species) being endemic to a single bioregion.

4 | Discussion

4.1 | Turnover Was the Dominant Component of β-Diversity

A clear pattern observed in this study is that annual fishes of the family Rivulidae exhibit a predominant pattern of species turnover, surpassing nestedness regardless of the scale of bioregionalisation, thereby supporting H1. The dominance of turnover suggests that each bioregionalisation represents a unique assemblage of annual fish species due to geographic isolation and environmental heterogeneity, rather than following a nested pattern where less diverse communities are subsets of more diverse ones. Although the predominance of turnover in Rivulidae was widely expected, it had never been previously tested. This study, for the first time, confirms this assumption by compiling species distribution data across the entire Neotropical region using a multiscale approach.

The high turnover pattern observed for annual fishes may be associated with multiple interactive factors, such as high endemism, low dispersal capacity and the significant isolation of the temporary wetlands that these species occupy (Costa 2010; Loureiro et al. 2018, 2024; Guedes et al. 2020; Alonso et al. 2023). In general, the components of β-diversity largely reflect the differential dispersal capacities and endemism among taxa (Laffan and Crisp 2003; Barton et al. 2013; Qian 2009; Soininen, Heino, and Wang 2018; Antão et al. 2019; Cortés-Guzmán et al. 2024). For instance, birds exhibit greater dispersal and lower turnover than mammals, which in turn show greater dispersal and lower turnover than reptiles and amphibians (Qian 2009). In the case of aquatic vertebrates, such as fishes, turnover increases with the decline in habitat connectivity and for non-migratory species (Griffiths 2017). Specifically for annual fishes, dispersal between temporary wetlands is limited because of the natural fragmentation of these ecosystems, occurring both spatially, with small isolated wetlands within a specific bioregionalisation, and temporally, with hydrological cycles of flooding and

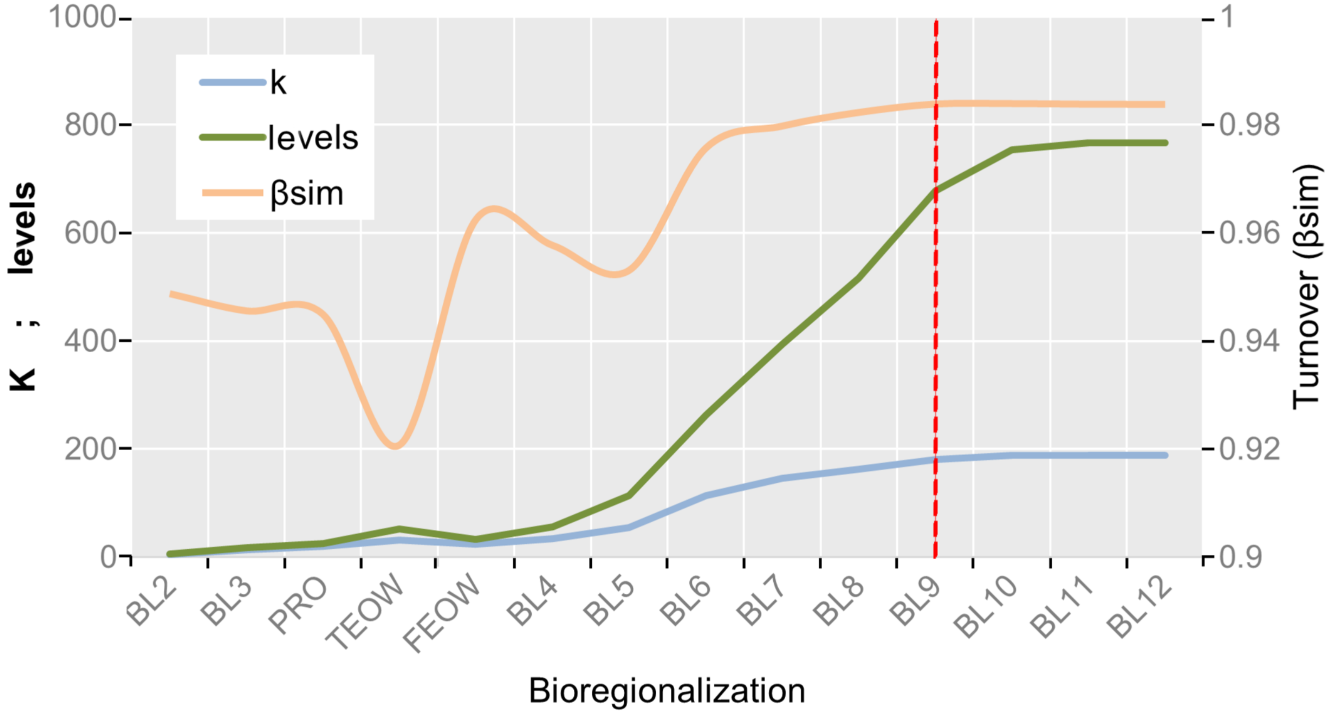


FIGURE 3 | Plot showing the relationship between the optimal number of clusters (k; blue line; primary y-axis), the number of levels (green line; primary y-axis) and the turnover component of beta diversity (βsim; orange line; secondary y-axis) for the 14 different bioregionalisations (x-axis). The x-axis is organised in descending order of the average area of the bioregionalisations. The red dashed line indicates the threshold to the right, where βsim shows no significant differences between bioregionalisations with varying average areas.

drying varying over time across different bioregionalisations. Dispersal may occur through rare and complex passive mechanisms, such as endozoochory and large floods (Incagnone et al. 2015; Silva et al. 2019). As a result, taxa with higher endemism and lower dispersal capacity that occupy fragmented environments tend to have higher turnover than those groups with lower endemism and better dispersal capacity (Barton et al. 2013; Qian 2009; Antão et al. 2019), which may have promoted the conspicuous species turnover observed among the different biogeographic units in this study.

4.2 | β-Diversity Scaling Patterns Across Different Bioregionalisation Proposals

The results indicate an increase in turnover (βsim) as the regionalisation area decreases, supporting H2. This pattern observed in Neotropical annual fish species is consistent with findings reported for various taxonomic groups, including vertebrates, invertebrates and plants (Calderón-Patrón et al. 2013; Antão et al. 2019). Generally, the increase in turnover as area size decreases is a consistent property of β-diversity, especially when a 'varying grain, fixed extent' approach is employed using classic β-diversity metrics (e.g., pairwise and multi-beta) (Barton et al. 2013; Antão et al. 2019). The effect of area on β-diversity may be associated with several factors. First, as the area size decreases, fewer species are shared, and environmental differences become more pronounced (Antão et al. 2019). Second, annual fish exhibit low dispersal capacity and are primarily endemic to small, specific biogeographic units. This high pattern of endemism in small, isolated habitats dilutes the effects of species with broader distribution ranges (e.g., *Pterolebias longipinnis* and *Neofundulus paraguayensis*), which typically have less impact on beta dissimilarity because of their ability to colonise multiple areas. Consequently, the low dispersal and high degree of endemism exacerbate species composition variation among small areas, increasing turnover.

The observation that the scaling of turnover with the area of bioregionalisation does not follow a linear pattern but is instead better described by a power-law function aligns with other findings in studies on β-diversity scaling (Zhang et al. 2015; Antão et al. 2019; Xing and He 2021). Another significant aspect identified in this study is the existence of a threshold where further reductions in the area of bioregionalisation did not result in significant increases in species turnover. This pattern may be associated with the power-law in β-diversity (Antão et al. 2019), which suggests that the relationship between area and β-diversity follows a non-linear pattern, where species turnover predictably increases as the area decreases, but not indefinitely. In this specific case, the BL9 threshold seemingly represents a point where the power-law function reaches a form of saturation or curve stabilisation. In the context of this study, the observed scaling patterns should not be attributed solely to the area of the bioregionalisations but also to the differences in how these bioregionalisations are designed. Regionalisations based on hydrogeomorphological features (e.g., HydroBasins; Lehner and Grill 2013) or on the distribution patterns of different taxa (e.g., TEOW—Olson et al. 2001, FEOW—Abell et al. 2008, Provinces—Morrone et al. 2022) likely had a significant impact on the scaling of β-diversity. This effect is particularly evident in freshwater ecoregions (FEOW), which are designed based on a variety of aquatic organisms (including fishes) and captured species turnover similar to that of watershed-based regionalisations with an area 57.7 times smaller (FEOW—240,433 km^2 vs. BL7–4165 km^2). These variations in the principles and design of bioregionalisations can, therefore, produce complex and non-linear patterns of β-diversity scaling, reflecting the interaction between environmental and biological factors operating at different spatial scales. These findings emphasise the importance of considering not only the area but also the underlying design factors of bioregionalisations, which have a crucial impact on how β-diversity scales.

4.3 | What Is the Optimal Spatial Scale?

Ohyama, Bogota-Gregory, and Jenkins (2024) argue that peaks in β-diversity indicate the ideal spatial granularity, where the separation between ecological communities is most pronounced. For the purposes of bioregionalisation, this spatial granularity serves as an essential foundation for determining the optimal number of bioregions, which are defined by clustering assemblages of grains (in the case of grids) or levels (for bioregionalisations) with similar taxa (Kreft and Jetz 2010). In this context, regionalisations based on smaller watershed areas (from BL9 to BL12) have proven to be more suitable in terms of spatial granularity, as they maximise turnover (~0.983) and result in a similar optimal number of bioregions ($k = 181–188$). It is worth noting that although the number of bioregions is comparable, the average areas of the levels within these bioregionalisations vary significantly, ranging from 146 to 414 km^2. As a result, the clustering of watersheds with similar ecological communities produced bioregions with average areas ranging from 598 km^2 (BL9) to 1551 km^2 (BL12). However, these areas should not be confused with the actual distribution of communities. For example, annual fish species do not occur broadly and diffusely within these watersheds but are instead found in small, fragmented temporary wetlands (Guedes, Luz, et al. 2023; Alonso et al. 2023; Loureiro et al. 2024). The distribution patterns of annual species reflect different paleogeographic events that altered the barriers and connectivity of drainage systems in the Neotropical region, such as past allopatric fragmentation caused by various vicariance events, formation of tectogenic basins, river capture and rearrangements, marine transgressions and regressions, continental drift and past climatic changes (García et al. 2012; Costa 2013; Costa, Amorim, and Mattos 2017, 2018; Loureiro et al. 2024). Therefore, our findings reinforce that β-diversity is scale-dependent and that finer spatial scales are more effective for assessing biodiversity patterns for endemic taxa and in habitats with low connectivity.

4.4 | Implications and Limitations

The predominance of species turnover in β-diversity among annual fishes of the Rivulidae family underscores the importance of considering spatial heterogeneity and geographic isolation in conservation strategies. This study reveals a high number of bioregions (> 180), each representing a unique assemblage of annual fish species, emphasising the need for decentralised conservation efforts that account for the distinct ecological communities present in different watersheds. These conservation efforts are particularly urgent given that, of the 261 species analysed in this study, 159 (~61%) are threatened with extinction in countries such as Brazil (ICMBio—Instituto Chico Mendes de Conservação da Biodiversidade 2022), Bolivia (MMAyA—Ministerio de Medio Ambiente y Agua 2009), Venezuela (Rodríguez, García-Rawlins, and Rojas-Suárez 2015), Colombia (Mojica et al. 2012), Uruguay (Soutullo, Clavijo, and Martínez-Lanfranco 2013) and Mexico (Lyons 2019). The primary threat faced by annual fishes is habitat loss, with temporary wetlands being destroyed due to uncontrolled urban, agricultural and industrial expansion (Volcan and Lanés 2018). Additionally, small water bodies are commonly underrepresented in conservation policies (Hill et al. 2018, 2021). Protecting these fragmented and isolated habitats is, therefore, crucial, particularly for species with high endemism and low dispersal capacity, as their survival heavily depends on the conservation of specific environmental conditions within their limited range. Guedes, Luz, et al. (2023) indicate that the establishment of protected areas (PAs), even when small in size, has been beneficial for the preservation of temporary wetlands where annual species occur. According to their findings, PAs tend to have greater coverage of natural landscapes and show a trend towards ecological restoration. In contrast, areas without conservation policies have faced higher rates of deforestation and conversion into anthropogenic landscapes. The high turnover pattern of β-diversity and the trend towards environmental restoration in small protected areas support the idea that several small protected areas may be more effective for the conservation of annual fishes than a single large reserve (*SLOSS* debate—Single Large or Several Small; Socolar et al. 2016, Fahrig et al. 2022). Moreover, the identification of β-diversity thresholds can have direct implications for the planning of conservation units, as such critical points may indicate the minimum area required to maintain species turnover at biologically relevant levels. Thus, by detecting when area reduction reaches a stage of turnover saturation, it becomes possible to optimise the design of protected areas to effectively preserve regional β-diversity.

The lack of detailed information on the distribution and habitats of annual fishes continues to be a significant obstacle for threat assessment and the formulation of effective conservation strategies. Brazil, for example, uses the IUCN Red List Categories and Criteria (International Union for Conservation of Nature; www.iucnredlist.org/) to develop and update the *Red Book of Endangered Brazilian Fauna* (ICMBio—Instituto Chico Mendes de Conservação da Biodiversidade 2022). In some cases, particularly for aquatic species, HydroBasins are used to calculate the Extent of Occurrence (EOO) and apply criterion ‘B’ (i.e., Restricted geographic distribution with fragmentation, decline or fluctuation). The results presented in this study may contribute to a more accurate application of these categories and criteria by demonstrating that finer spatial scales (HydroBasins: BL9-BL12) may be more effective in assessing biodiversity patterns. Additionally, the metadata provided in this study may help fill existing knowledge gaps and serve as a foundation for future ecological, historical and conservation studies.

Although this study provides valuable insights, there are inherent limitations that must be acknowledged. First, the reliance on species distribution data, which inevitably depends on an incomplete sampling effort for the Neotropical region, is a significant limitation, as new species continue to be regularly described (Ramos et al. 2023; Alonso et al. 2024) and actual distribution ranges may change as new occurrences are reported (Costa et al. 2024). Second, this study was based on traditional taxonomic classification rather than phylogeny-based classification. Incorporating phylogenetic information into β-diversity measurement has the potential to offer new insights into bioregionalisation schemes (Azevedo et al. 2021). Third, although this study evaluates a wide range of area sizes (from 3,251,927 to 146 km^2), it primarily focuses on regional scales. The inclusion of bioregionalisations at local scales, which are currently unavailable, would be important to determine whether the β-diversity threshold holds at even smaller spatial scales. A complementary approach could involve applying grains (grid cells) of different sizes (Calderón-Patrón et al. 2013), comparing the results with the bioregionalisations presented here. Fourth, despite the low numerical variability in turnover among different bioregionalisations, statistical analyses revealed highly significant differences. Statistically, these significant differences, despite small variations, are related to the large sample size (involving 1.35 million pairwise βsim values). With such a large dataset, the standard error decreases as the sample size increases, and statistical tests gain substantial power to detect even minor differences between group means (Jan and Shieh 2013). Fifth, other characteristics of bioregionalisations (such as perimeter, design and connectivity) also affect β-diversity outcomes (Zhang et al. 2015). Therefore, new studies that decompose the effect of each characteristic on β-diversity scaling are necessary. Sixth, β-diversity is a dynamic field, with ongoing findings and debates. The scaling and patterns of β-diversity observed in this or any other study are invariably affected by the metric used (e.g., pairwise, multi and null models), the approach (variation in grain and/or extent), hierarchical or non-hierarchical structure and data type—abundance or presence/absence (Barton et al. 2013; Bennett and Gilbert 2016; Antão et al. 2019; Xing and He 2021; Ohyama, Bogota-Gregory, and Jenkins 2024). Finally, this study was not designed to exhaust all possibilities related to β-diversity scaling across different bioregionalisation proposals, and therefore, future studies may further elucidate the patterns described here.

Author Contributions

Gustavo Henrique Soares Guedes: conceptualisation (lead), formal analysis (lead), investigation (lead) methodology (lead), supervision (equal), validation (lead), writing – original draft (equal), writing – review and editing (equal). **Jayme Magalhães Santangelo:** visualisation (equal); review and editing (equal). **Aliny Patrícia Flauzino Pires:** visualisation (equal), writing – review and editing (equal). **Francisco Gerson Araújo:** formal analysis (supporting); supervision (equal), writing – review and editing (equal).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the Appendix S1–S3 of this article. Data and computer code used for data compilation/manipulation and analysis are openly available in the Dryad database (https://doi.org/10.5061/dryad.zpc866tjz).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.