RESEARCH ARTICLE

Headwater refuges: Flow protects *Austropotamobius* crayfish from *Faxonius limosus* invasion

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Abstract

This study explores the geospatial relationship between the invasive crayfish species *Faxonius limosus* and the native *Austropotamobius bihariensis* and *A. torrentium* crayfish populations in Eastern Europe, identifying the environmental factors which influence the invasion. We used species distribution modelling based on several climatic, geophysical and water quality variables and crayfish distributional data to predict sectors suitable for each species within the river network. Thus, we identified the sectors potentially connecting invasive and native population clusters and quantified the degree of proximity between competing species. These sectors were then extensively surveyed with trapping and hand searching, doubled by eDNA methods, in order to assess whether any crayfish or the crayfish plague pathogen *Aphanomyces astaci* are present. The predictive models exhibited excellent performance and successfully distinguished between the analysed crayfish species. The expansion of *F. limosus* in streams was found to be limited by flash-flood potential, resulting in a range that is constrained to lowland rivers. Field surveys found neither crayfish nor pathogen presence in the connective sectors. Another interesting finding derived from the screening efforts, which are among the most extensive carried out across native, apparently healthy crayfish populations, was the existence of a latent infection with an *A. astaci* strain identified as A-haplogroup. Our results provide realistic insights for the long-term conservation of native *Austropotamobius* species, which appear to be naturally

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protected from *F. limosus* expansion. Conservation efforts can thus focus on other relevant aspects, such as ark-sites establishment for preventing the spread of more dangerous invasive crayfish species and of virulent crayfish plague pathogen strains, even in locations without direct contact between crayfish hosts.

Introduction

Invasions of non-native species are a global phenomenon affecting almost every region and taxonomic group (Catford et al. 2012; Essl et al. 2020). Although the knowledge of invasions continues to improve, efforts to stop or slow them have had limited success (Clavero and García-Berthou 2005; Crooks 2005; Wilson et al. 2009). To preserve remaining native populations, a practical and low cost approach is to find "ark-sites", areas where invasions are unlikely to reach in the existing conditions or with minor interventions (Holdich et al. 2009; Nightingale et al. 2017). Our goal in this study is to assess the vulnerability of the native *Austropotamobius bihariensis* Pârvulescu, 2019 and *A. torrentium* (von Paula Schrank, 1803) crayfish populations in the natural conditions offered by the mountain habitats in Romania, in light of the ongoing invasion of *Faxonius limosus* (Rafinesque, 1817).

The impact of crayfish invasions on native species populations can manifest as declines in density (Gherardi et al. 2011), changes in community structure (Kuhlmann 2016; Galib et al. 2021), or in trophic position (Pacioglu et al. 2020; Chucholl and Chucholl 2021), and even habitat alteration (Gherardi 2007; Albertson and Daniels 2016). Invasive crayfish can exhibit plasticity and can employ various tactics to increase their chances of success. Some of these are: making use of superior phenotypic traits (Chuang and Peterson 2016; Messager and Olden 2019), changing reproductive strategies (Pârvulescu et al. 2015; Francesconi et al. 2021a), acting as pathogen reservoirs and transmitting disease (Strauss et al. 2012), or even using sheer boldness (Hudina et al. 2015; Pârvulescu et al. 2021; Roessink et al. 2022).

While significant international efforts have gone towards regulating probable invasion entry points through legislation, the prevention and control methods available in natural habitats are still quite limited. Measures such as building mechanical barriers (e.g., dams) to block upstream movement can be effective in some specific conditions, but these are not stopping the invasions completely (Krieg and Zenker 2020; Krieg et al. 2020, 2021). Commercial activities such as food and pet trade or leisure activities (Andriantsoa et al. 2020; Bláha et al. 2022) and even animal-mediated spread of non-native taxa (Águas et al. 2014; Anastácio et al. 2014) can facilitate the invasion of some crayfish species. On top of that, the presence of the *Aphanomyces astaci* pathogen carried by most of the invasive crayfish species (Mrugała et al. 2015) makes prevention an urgent matter. However, it should be pointed out that some native crayfish

populations have shown signs of long-term survival and coexistence with invasives. For example, the *Pontastacus leptodactylus* Eschscholtz, 1823 seems to have good resistance to the crayfish plague pathogen (Panteleit et al. 2018; Ungureanu et al. 2020) and has reportedly lived alongside invasive crayfish for a long time, showing signs of recovery (Pacioglu et al. 2020). Despite analyses based on large datasets, a clear temporal trend has not been established for crayfish invasions in Europe (Soto et al. 2023).

Faxonius limosus originates from North America and was introduced to Europe for commercial reasons in the late 19th Century (Holdich 2002). It has since spread widely in Western Europe, reaching Romania in 2008 (Parvulescu et al. 2009). This species is harmful to the native crayfish populations (Gherardi et al. 2011; Capinha et al. 2013; Aluma et al. 2023), as it competes with them for food and habitat (Lele and Pârvulescu 2017; Veselý et al. 2021), and also brings the fatal crayfish plague disease pathogen (Pârvulescu et al. 2012), which is listed among the 100 worst alien species in the world (Luque et al. 2014). The pathogen is almost permanently present alongside invading populations or even far ahead of the invaded areas (Schrimpf et al. 2012; Panteleit et al. 2018; Ungureanu et al. 2020).

Our focus is to find if there is a favourable spatial and ecological context for the invasion of *F. limosus* to progress into the habitats of the two *Austropotamobius* native species naturally living in the upper regions of mountain ranges in Romania, and thus, to assess how protected the native populations are from this invasion. To this aim, we used several climatic, geophysical and water quality variables known to be relevant in describing crayfish distribution, as well as species presence/absence data, to predict the most suitable river sectors occupied by the targeted species, and checked for proximity and potential spatial overlap between the invasive and natives.

Methods

The study was designed in two stages. The first stage involved species distribution modelling (SDM) aimed to learn ecological preferences of the assessed species. This allowed us to predict which sectors would be suitable for each species within the river network determined by the areal of *A. bihariensis* and *A. torrentium* in Romania. The second stage identified the sectors potentially connecting invasive and native population clusters and quantified the degree of proximity between competing species to identify areas of concern. These areas were then visited in the field, using trapping and hand searching, doubled by modern molecular techniques, to detect crayfish and the crayfish plague pathogen, *A. astaci*.

Crayfish distribution data

Special attention was given to the location data of crayfish presence and absence (Fig. 1A). Although citizen science is increasingly popular, its reliability is still debated (Zizka et al. 2020), which led us to rely solely on scientific publications. Our dataset
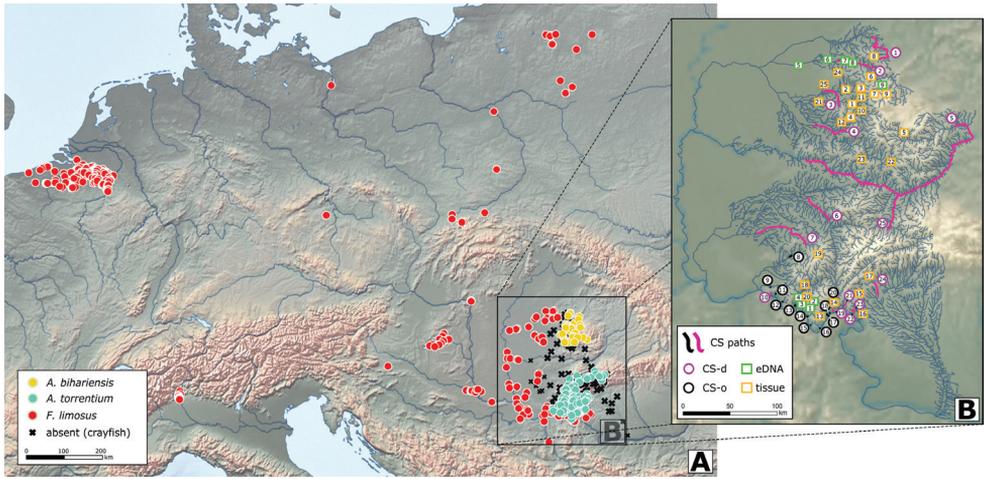
Figure 1. A sites with crayfish presence/absence data used for training SDM **B** paths connecting closest river sectors with positive predictions for native and invasive species (codes according to Table 4), and crayfish plague tissue and eDNA sampling locations (codes according to Suppl. material 3 for eDNA and Table 5 for tissue samples).

comprised existing data from previous studies on native *Austropotamobius* crayfish in Romania (Pârvulescu and Zaharia 2013; Pârvulescu 2019; Pârvulescu et al. 2020) and literature data from the first detection of *F. limosus* invasion in this region (Pârvulescu et al. 2009) to the latest publication (Pacioglu et al. 2020). As Romania is still in the early stage of the invasion of *F. limosus*, we expanded the dataset with available data in Europe from published literature. Additional new data was collected in the field during 2019–2022 to get the most up to date situation in the investigated area. We captured crayfish by using hand searching for native species, and baited traps (Pirate type, with double entrance) for the invasive species. Capture effort was set as one daylight visit per site for hand searching, visiting a river sector of at least 150 m, whereas trapping was applied for at least two nights with batches of 3 traps per site. Data on crayfish absences were obtained exclusively from field investigations (Fig. 1).

Geospatial database

Selecting the most important variables that will be included in the model and on which certain decisions will be made is essential and not always trivial (Mac Nally 2002; van de Pol et al. 2016; Fourcade et al. 2018). We considered variables whose relevance in predicting crayfish distributions has already been demonstrated in the literature (Chucholl and Schrimpf 2016; Krause et al. 2019; Pârvulescu et al. 2020; Dornik et al. 2021; Soto et al. 2023), focusing on elevation, habitat quality, substrate, and climatic variables.

Data was processed using ArcGIS Pro software (ESRI, Redlands) and Saga 8.5.0. (https://saga-gis.sourceforge.io/en/index.html). Elevation data, at 3 arc-second

(90 m × 90 m) spatial resolution digital elevation model (DEM), was downloaded from USGS Earth Explorer data portal (https://earthexplorer.usgs.gov/). The DEMs were first combined into a raster mosaic for the whole area of interest. ArcGIS Pro Hydrology Tools were used to create the stream network by modelling the flow of water across the raster surface.

Regarding environmental factors used as predictors, we used a set of 12 variables that described the ecological, climatic and edaphic conditions. Altitude (ALT) was sampled at each point location from the DEM. Annual mean temperature (BIO1) and another six climatic variables, considered relevant for the species in question, were downloaded from the WorldClim data portal (https://www.worldclim.org/). This database provides free historical climate data for 1970–2000 (Fick and Hijmans 2017). The used variables were the maximum temperature of the warmest month (BIO5), the mean temperature of the driest quarter (BIO9), the annual precipitation (BIO12), the precipitation of the wettest quarter (BIO16), the precipitation of the driest quarter (BIO17) and the precipitation of the warmest quarter (BIO18). Soil type (TYS) was extracted from a raster layer with data on the soil classes based on the international standard for soil classification system World Reference Base (Hengl et al. 2017). We obtained this raster from the SoilGrids portal (Poggio et al. 2021). The thickness of soil up to the bedrock (THS) was extracted from the world-level estimated model provided by (Pelletier et al. 2016). The 30 arc-second climatic and soil data were sampled for each point location of our study dataset.

Remote Water Quality (RWQ) is an ecological index that measures the anthropogenic pollution potential in the upstream areas (Şandric et al. 2019). For its computation, the 44 third-level inventory classes of CORINE Land Cover 2018, (https://land.copernicus.eu/pan-european/corine-land-cover/clc2018) were regrouped into 4 new classes based on their human-based potential impact (Burkhard et al. 2012). RWQ represents the average of class scores weighted by their specific watershed area. We ran an iterated chain of specific steps using Model Builder. This process delineates unique watersheds and computes the corresponding RWQ index at each point location at 3 arc-second (90 m × 90 m) resolution.

Flash-flood potential (FFP) estimates stream disturbance potential according to the local and upstream drainage velocity (Pârvulescu et al. 2016). This index is calculated starting with the river network and two land-surface variables: slope gradient (accounting for the potential water velocity at a given site) and catchment slope (estimating the average gradient of a surface that drains towards a given site, thus accounting for the potential of flash floods as a consequence of heavy rain events). Higher values of FFP indicate a high risk of water velocity increasing rapidly and temporarily. The slope was derived in ArcGIS Pro using the Spatial Analyst Slope tool, in a 3 × 3 window neighbourhood, using the 3 arc-second (90 m × 90 m) previously processed DEM mosaic. The spatial resolution of output data was preserved at 90 m × 90 m. The catchment slope was calculated using Saga hydrological tools. FFP was computed in each river network cell location as the product of slope gradient and catchment slope, using ArcGIS Pro raster mathematical tools.

Species distribution modeling and prediction

In order to understand the interaction between the two *Austropotamobius* species and *F. limosus*, both the locations for presence, as well as absence of crayfish, are essential. The data for each of the three species were analysed separately using machine learning modeling techniques. The dataset for *A. bihariensis* contained 106 records (31 presences and 75 absences), the one for *A. torrentium* had 213 records (93 presences and 120 absences), while the one for *F. limosus* had the largest sample size with 711 records (482 presences and 229 absences). The species presence was predicted using Random Forest (RF) method on the 12 input (predictor) variables, using the "sklearn" package in Python. To obtain the best fitting random forest architecture we performed hyperparameter tuning for each dataset.

The prediction task was done in several stages. The first step was to reveal which of the predictor variables have relevance on predicting the crayfish locations, i.e. feature importance (FI), based on the mean decrease in impurity. In the second step we trained machine learning models using the most important variables detected above. For each of the three crayfish species we used Scikit-Learn's GridSearchCV that evaluated various hyperparameter candidates from the grid of parameter values. The hyperparameters considered were the number of trees to be used in the model, the maximum features in each tree, the maximum number of splits each tree can take and how many divisions of nodes should be done. The best solutions are presented in Suppl. material 1. Once the models were fitted, they were used to predict crayfish presence/absence on the full river network for each of the three species. In each case, the prediction cut-off was set to a default value of 0.5. Predicted suitable ranges (SR) were calculated using partial dependence plots to understand the variable intervals on which each species fit the best. Observing high compliance between SRs and observed ranges (OR) provides us with noteworthy information that SRs can be trusted and used for further interpretation (Cutler et al. 2007). Additionally, to understand the species' relationship with each variable retained in the model, we calculated the percentage of overlap of the SR from the total range (TR) of the variable (further denoted %Ov). The distributions of relevant ecological variables were compared between the investigated crayfish species using two samples Welch's t-test.

Finally, the hydrographic network dataset used in the study was loaded into GIS software to identify positive predictions for each native and invasive species with the aim to observe and understand each species spatiality. We compared the positively predicted river network between the two *Austropotamobius* species, and between each and the invasive *F. limosus* separately, determining the degree of overlap.

Concerning situations for contact zones between native and invasive crayfish species

To identify potential areas of concern for the colonization of native *Austropotamobius* crayfish populations by the invasive species *F. limosus*, we identified continuous network sectors with positive prediction for one of the native species that contained at least one confirmed presence point for that species. Subsequently, following the river

course, each such sector was associated with the closest river sector with positive prediction for the invasive species, thus defining "paths".

Based on these paths, we measured the level of separation (LoS). It serves as a proxy for the "ecological cost" representing the sum of challenges that migrating individuals would encounter at each point within the habitat (Zeller et al. 2012). The LoS was determined by summing the differences between the threshold (0.5) and the predicted probability of *F. limosus* occurrence for each network cell along the path. We then computed the LoS per kilometer of river path for better comparability. Two cases are possible: (i) LoS > 0 (implying that a physical distance existed along the path between two sectors), and (ii) LoS ≤ 0 (when the two sectors overlapped).

Situations belonging to the first category were further analysed by field investigations aimed at detecting whether any crayfish or *A. astaci* were present in the area. In-field crayfish searches used manual methods or traps as described above. In some instances, we also collected water filtrate (as in Shogren et al. 2017) by filtering three replicates (minimum of 5 L of water each) per site, one replicate from the center of the river channel and two close to the riverbanks. These water samples were taken to give a better resolution assessment through eDNA analysis (Seymour et al. 2021). In total, 27 filters from 9 sites, including Oradea as control (see Fig. 1B), were collected in the summer season to enhance detectability success (Baudry et al. 2023), and stored in ethanol. A high salt DNA extraction method (modified after Aljanabi and Martinez 1997) was applied for DNA isolation from the filters. For the qPCR analysis for each sample 6 µl Environmental PCR Master Mix, 1.6 µl nuclease-free water and 0.4 µl of each forward and reverse primer and the probe were mixed. Then in total 11 µl of this mixture were added to a PCR tube. In each tube, 1.2 µl of DNA from each sample was added. For the detection of *F. limosus* DNA the primers and probe from Rusch et al. (2020) were used, for the stone crayfish DNA the primers and probe from Chucholl et al. (2021) were used and for *A. astaci* DNA the above-mentioned primers and probe were applied.

Screening of native crayfish populations

Since none of the in-field investigations of paths with LoS > 0 found any crayfish, we extended the search upstream, in known native population sites. The goal was to verify whether *A. astaci* had spread there, even without *F. limosus*. Native crayfish were captured and sampled (see Fig. 1B) by collecting a piece of the uropod, to harm the crayfish as little as possible. Exuviae or dead animals were collected as well where available. Samples were stored in 96% ethanol. In total, 353 samples from 25 crayfish populations (12 of *A. bihariensis*, 6 of *A. torrentium* and 6 of *Astacus astacus*, and one mixed population of *A. torrentium* and *A. astacus*) were used for the crayfish plague analysis. In the laboratory we applied a CTAB DNA extraction protocol modified after Vrålstad et al. (2009). The whole uropod was used; additionally, in the case of exuviae or dead animals, other parts of the carapax were used as well (Vrålstad et al. 2009). For the real-time PCR a new set of primers and probe was used according to Francesconi et al. (2021b) and the annealing times and temperatures were also adapted.

In order to identify the *A. astaci* haplotype, samples with a high ct-value were selected to be sequenced using two primer pairs amplifying the mitochondrial ribosomal rnnS (AphSSUF and AphSSUR) and rnnL (AphLSUF and AphLSUR) according to Makkonen et al. (2018).

Availability of data

The data underpinning the analyses reported in this paper are deposited in the Elsevier's Mendeley Data repository at https://doi.org/10.17632/5vg35hc58m.2.

Results

Species distributions modeling and prediction

The SDMs performed well in predicting the occurence of native species (Fig. 2), even with the small sample size in the particular case of the endemic *A. bihariensis*. The relevance of predictors for each species can be found in Tables 1–3 providing a visualisation of the predictor influence for each species. The important predictors for *A. bihariensis* included ALT, FFP, RWQ and several climatic variables (BIO1, BIO5, BIO16, BIO12, BIO9) providing an accuracy of 85.2% (F1 = 0.745). For *A. torrentium*, the important predictors were RWQ, BIO17, ALT, BIO12, FFP, BIO1, BIO5, BIO16 and BIO9, with the RF classifier providing an accuracy of 85.4% (F1 = 0.851). In the case of *F. limosus*, the method performed the best, with a high accuracy 99.5% (F1 = 0.99) using the predictors FFP, ALT, BIO9, BIO16, THS, and BIO1. Notably, variables related to soil had a low influence on the accurate location detection for both *Austropotamobius* species, whereas water quality (RWQ) was found to be one of the most important predictors for both species. For the invasive species *F. limosus*, the variables RWQ, BIO5, BIO12, and BIO17 had very limited influence on prediction.

We found an overlap of 27.2% of the predicted river network of *A. torrentium* over *A. bihariensis* (see Fig. 2A), and 17.5% of *A. bihariensis* over *A. torrentium* (see Fig. 2B). Furthermore, the overlap of the prediction of invasive species over either of the two native species was minimal, with 0% of the predicted network for *A. bihariensis* and 0.88% for *A. torrentium*.

Although the SR and OR were highly consistent, the species %Ov (Tables 1–3) revealed how each species could explore the ecological niche within a geographic area as indicated by the TR for each variable. *Austropotamobius* species only tolerated the lower values of the RWQ, with 20.44 %Ov (0.3–1.2) for *A. bihariensis* and 27.26 %Ov for *A. torrentium* (0–1.2) out of the TR found in the study area. Also notably, there were differences in altitude between the natives and invasive species, with *A. bihariensis* exploring only 28.31 %Ov (between 259 to 796 m asl) of the range in the region, while *A. torrentium* was found slightly more permissive in its lower altitude margin, with an SR ranging from 120 to 798 m asl (35.77 %Ov), whereas *F. limosus* ranged between 0–370 m asl with 19.52 %Ov. Another difference between the species was their toler-
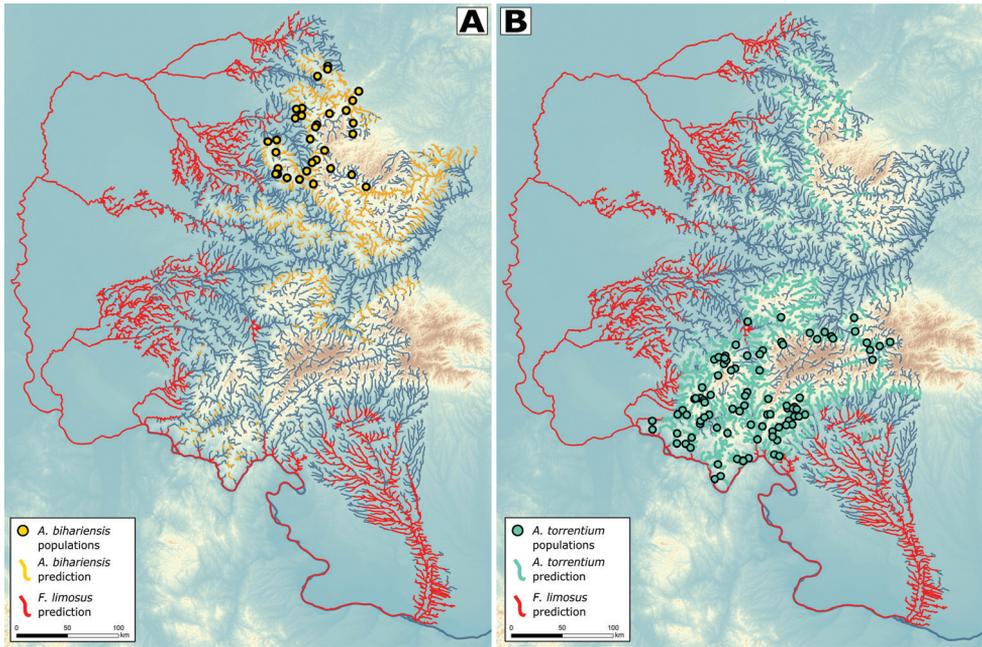
Figure 2. Prediction results over the investigated network for **A** *A. bihariensis* vs *F. limosus* and **B** *A. torrentium* vs *F. limosus*.

Table 1. Analysis of variables retained in *A. bihariensis* species distribution model: FI – feature importance; OR – observed range; SR – suitable range; TR – total range; %Ov – percentage of overlap of the SR from the TR. For more information on habitat variable codes, we refer readers to the "Geospatial database" section in Methods.

Variable	FI	OR	SR	TR	%Ov
BIO1	0.164	5.09–9.5	5–9.8	0.2–12.3	39.68%
ALT	0.120	259.4–935.6	259.4–796	0–1921	28.31%
BIO5	0.114	20.5–25.5	19.3–25.6	13.8–30.2	38.41%
BIO16	0.105	229–311	230–280	166–386	22.72%
BIO12	0.096	637–826	520–730	491–1047	37.76%
BIO9	0.094	-2.8–1.4	-2.8–1.5	-7.5–17.7	16.98%
FFP	0.089	0.18–1.7	0.2–1.8	0–12.27	13.03%
RWQ	0.086	0–1.01	0.3–1.2	0–4.40	20.44%

ance for temperature, as *A. torrentium* had a much lower %Ov for variables describing temperature (BIO1, BIO5, BIO9) in comparison with *A. bihariensis*. Important differences were observed between the natives and invasive species regarding temperature variables (BIO1, BIO9), which were considerably higher for the invasive species (see Tables 1–3). Similar differences were observed for climatic variables describing precipitation BIO12 with much higher requirements for *A. bihariensis* and BIO17 for *A. torrentium*. BIO16 was found relevant for both native species and had a high %Ov. A noticeable difference between native *Austropotamobius* and invasive *F. limosus* was

Table 2. Analysis of variables retained in *A. torrentium* species distribution model: FI – feature importance; OR – observed range; SR – suitable range; TR – total range; %Ov – percentage of overlap of the SR from the TR. For more information on habitat variable codes, we refer readers to the "Geospatial database" section in Methods.

Variable	FI	OR	SR	TR	%Ov
RWQ	0.138	0–1.03	0–1.2	0–4.40	27.26%
BIO17	0.132	106–142	114–145	73–175	30.39%
ALT	0.126	119.4–868.7	120–798	0–1921	35.77%
BIO12	0.122	642–828	650–775	491–1047	22.48%
FFP	0.104	0–4.3	0.2–3	0–12.27	22.81%
BIO1	0.072	6.2–11.2	6.9–10.8	0.2–12.3	32.24%
BIO5	0.065	20.9–28.3	22.3–27.8	13.8–30.2	33.53%
BIO16	0.063	207–295	170–296	166–386	57.27%
BIO9	0.062	-1.7–2.7	-1.1–2.5	-7.5–17.7	14.21%

Table 3. Analysis of variables retained in *F. limosus* species distribution model: FI – feature importance; OR – observed range; SR – suitable range; TR – total range; %Ov – percentage of overlap of the SR from the TR. For more information on habitat variable codes, we refer readers to the "Geospatial database" section in Methods.

Variable	FI	OR	SR	TR	%Ov
FFP	0.372	0–1.229	0–0.94	0–12.27	7.66%
ALT	0.216	0–358	0–370	0–1921	19.52%
BIO9	0.096	-2.9–6.6	-0.8–6.6	-7.5–17.7	99.53%
BIO16	0.088	168–383	168–383	166–386	97.72%
THS	0.081	1–50	0–50	0–50	100%
BIO1	0.067	5.8–12.2	6.5–12.3	0.2–12.3	47.95%

particularly evident for FFP, with the invasive species exploring significantly less of the TR with %Ov of 7.66% (0–0.94), than *A. bihariensis* (0.2–1.8, 13.03 %Ov) and *A. torrentium* (0.2–3, 22.81 %Ov). The differences between the three species regarding the environmental parameters can also be seen by analysing the comparisons between their values in sites where each respective species was observed (Suppl. material 2).

Concerning situations for contact zones of native and invasive crayfish species

We have identified 25 paths connecting native and invasive species (Fig. 1B). Out of these, 11 were cases of LoS ≤ 0 (overlap), which are generally short, with lengths ranging from 90 to 2250 m (Table 4), being found only for the *A. torrentium* populations. As for LoS > 0 (separation by distance), we found 14 cases, with lengths ranging from 26820 to 269730 m (average 73902 m) for *A. bihariensis*, and 720 to 160560 m (average 30120 m) for *A. torrentium*. The extremes are four paths with LoS greater than 100, and five paths with a highly concerning LoS below 10 (Table 4). A better view on the adequacy of native crayfish habitats to act as ark-sites is given by LoS/Km (see Table 4), showing the difficulties faced by *F. limosus* to traverse the river sectors separating it from the natives.

Table 4. The paths and the level of separation (LoS) between sectors with positive predictions for native crayfish (*A. bihariensis* or *A. torrentium*) and the invasive *F. limosus*. LoS ≤ 0 represents overlap.

ID	Native species	Length (m)	LoS	LoS/Km
1	*A. bihariensis*	45990	135.22	2.94
2		39060	97.99	2.51
3		26820	42.72	1.59
4		41130	54.17	1.32
5		269730	716.18	3
6	*A. torrentium*	32400	71.21	2.19
7		62190	125.44	2.01
8		2160	≤ 0	-
9		180	≤ 0	-
10		990	0.65	0.66
11		540	≤ 0	-
12		540	≤ 0	-
13		990	≤ 0	-
14		810	≤ 0	-
15		90	≤ 0	-
16		180	≤ 0	-
17		2250	≤ 0	-
18		360	≤ 0	-
19		990	1.08	1.10
20		450	≤ 0	-
21		2430	3.42	1.41
22		2070	4.15	2.01
23		720	0.69	0.96
24		13950	35.89	2.57
25		160560	418.46	2.61

Screening of native crayfish populations

From a total of 27 water samples (Suppl. material 3), three were positive for *F. limosus* DNA. All three filters with water filtered from Oradea showed a positive signal in the qPCR analysis, confirming the field observations. We could not detect DNA from the two *Austropotamobius* species nor from *A. astaci* in any other filter.

The analysis of crayfish tissue samples revealed an infection rate of 64% (16 infected out of 25 investigated populations) in apparently healthy native crayfish. In total, 34 samples out of 353 (9.6%) analysed tissue samples were positive for an *A. astaci* infection (Table 5). Concerning the different species, 20 out of 191 analysed tissue samples from *A. bihariensis* (10.4%), 8 out of 83 samples from *A. torrentium* (9.6%) and 6 out of 79 samples from *A. astacus* (7.6%) were positive. Most ct-values were relatively low (35.32 to 45.96). Three samples returned results from sequencing attempts (see Table 5), one from *A. bihariensis* (site Rănușa), one from *A. torrentium* (site Plopa), and one from *A. astacus* (site Brebu), all those being detected with a ct-value suitable for sequence analysis and revealed identical to reference sequences As (named after *A. astacus*, the species on this genetic variant has been identified the first time) and Up (named after "Úpořský brook" where this genetic variant was detected the first time, on *A. torrentium*) of the A-haplogroup (Kozubíková et al. 2008; Grandjean et al. 2014).

Table 5. Results of the crayfish plague analysis of samples collected during the screening of native populations. Asterisk (*) indicates sites with genotyping results.

Species	ID	Site	GPS coordinates	Sample	Total	Positive	Negative	%
A. bihariensis	1	Boga	46.6107°N, 22.6610°E	uropods, exuvia	27	3	24	11.1
	2	Crăiasa	46.5443°N, 22.5964°E	uropods	11	0	11	0
	3	Racu	46.6631°N, 22.5255°E	uropods	13	3	10	23.1
	4	Tâlniciorii	46.4182°N, 22.4672°E	uropods, claw	13	1	12	7.7
	5	Valea Bistrii	46.4059°N, 23.0541°E	uropods	12	2	10	16.7
	6	Valea Anişelului	46.7883°N, 22.8872°E	uropods	8	0	8	0
	7	Preluca	46.7257°N, 22.8813°E	uropods	19	1	18	5.3
	8	Valea Mare	47.1242°N, 22.6216°E	uropods	28	1	27	3.6
	9	Valea Iadului	46.7447°N, 22.5597°E	uropods	20	0	20	0
	10	Cuţilor	46.8311°N, 22.3977°E	uropods	14	1	13	7.1
	11	Ciur Ponor	46.8188°N, 22.3800°E	uropods, legs	7	0	7	0
	12	Rănuşa*	46.4391°N, 22.2672°E	uropods, dead	19	8	11	42.1
		total for *A. bihariensis*			**191**	**20**	**171**	**10.4**
A. torrentium	13	Sirinea	44.6387°N, 22.0863°E	uropods, dead	16	2	14	12.5
	14	Valea Satului	44.6294°N, 22.2461°E	uropods	15	1	14	6.7
	15	Jidoştiţa	44.7268°N, 22.5619°E	uropods, dead	16	0	16	0
	16	Coşuştea	44.9665°N, 22.6573°E	uropods	16	1	15	6.3
	17	Aninoasa	46.9557°N, 22.3457°E	legs	5	0	5	0
	18	Plopa*	45.0286°N, 21.8369°E	uropods	10	3	7	30
	19	Brebu	45.2288°N, 22.1436°E	uropods, exuvia	5	1	4	20
	20	Valea Poienii	44.6387°N, 22.0863°E	uropods	8	2	6	25
		total for *A. torrentium*			**91**	**10**	**61**	**10.9**
A. astacus	21	Crişul Negru	46.6112°N, 22.4035°E	uropods	4	1	3	25
	22	Băcaia	46.0163°N, 23.1741°E	legs	16	0	16	0
	23	Ţebea	46.1461°N, 22.7022°E	legs	9	0	9	0
	24	Peştireului	46.9888°N, 22.4582°E	legs	20	0	20	0
	25	Valea Mare	46.6416°N, 22.2447°E	uropods	2	1	1	50
	19	Brebu*	45.2288°N, 22.1436°E	uropods, exuvia	20	2	18	10
		total for *A. astacus*			**71**	**4**	**67**	**5.6**
		totals			**353**	**34**	**319**	**9.6**

Discussions

The ecological particularities of the species

From a habitat quality perspective, the *Austropotamobius* species are generally known to be sensitive (Füreder and Reynolds 2003; Demers et al. 2006; Trouilhéet al. 2007; Piyapong et al. 2020). This aspect is consistent with the results of this study, which showed the species` tolerance only for the lower range of the RWQ found in the study area, indicating low anthropogenic impact. Regarding altitude preferences, *Austropotamobius* crayfish inhabit mountain and submountain areas (Streissl and Hödl 2002; Pârvulescu and Zaharia 2013). Our study revealed that *A. bihariensis* has a preference for upstream areas at a higher altitude compared to *A. torrentium*, making the latter more exposed to contact with the invasive *F. limosus*. *A. bihariensis* has less tolerance for high temperatures and low precipitation than *A. torrentium*. This result is to be expected since it is known that, in general, karstic habitats (where this species is living) are ex-

posed to dryness (Bonacci 1993; Fiorillo and Guadagno 2010) but are more balanced with respect to temperature (O'Driscoll and DeWalle 2006; Cantú Medina et al. 2021).

The overall overlap between the prediction of invasive species and either of the native species was found to be marginal. This is most importantly due to the fact that for *F. limosus*, the FFP, which is the most relevant variable predicting the species presence, has a much lower suitable range than the other two species. Stream flow regulates many aspects of an aquatic ecosystem, increasing oxygen supply and impacting substrates, detritus, and benthic communities (Pacioglu et al. 2019a). A possible explanation for why *F. limosus* does not choose stream habitats could be from its trophic perspective (Vojkovská et al. 2014; Šidagyte et al. 2017; Pacioglu et al. 2019b; Mathers et al. 2020), considering that streams offer less diverse food which is also likely more difficult to find compared to larger rivers (Romanuk et al. 2006; Hette-Tronquart et al. 2016), possibly in relation with water velocity (Finlay et al. 1999; Light 2003; Kerby et al. 2005).

The other important variable influencing *F. limosus* distribution, altitude, is also related to stream flow velocity (Pârvulescu et al. 2016). Although the presence of the species has also been reported at higher altitudes (see Bonk and Bobrek 2020), moderate FFP can be patchy in this area (Pârvulescu et al. 2016), and therefore not optimal for invasion expansion. Those locations may actually be the result of human or other vector-mediated relocation. This is supported by other prediction approaches which show a marked decrease of presence probability for altitude over 500 m (Piyapong et al. 2020). As the native areal of the *F. limosus* is also largely oriented towards lowland habitats (Bloomer and Taylor 2020; Lieb et al. 2011), we conclude that the variables selected by our model are representative of the species' ecology.

The adequacy of habitats for conservation

In order to control invasive species, one must discover and understand habitat conditions that sustain or harm them. The findings of this study highlight the significant advantage *A. bihariensis* and *A. torrentium* have against the imminent invasion of *F. limosus*. It appears that the aquatic habitat conditions at the limit between lowland and submontane areas act as a decisive factor against the establishment of *F. limosus* populations (also noted by Petrusek et al. 2006; Bonk and Bobrek 2020). Although some studies suggest the potential spread of *F. limosus* into subalpine areas (Garzoli et al. 2020; Boggero et al. 2023), their findings fall within our predicted range for the species (below 370 m asl). The likelihood of high FFP values is low at such altitudes, as it is based on slope gradient and catchment slope.

Invasions may impede native species populations connectivity (Groza et al. 2021), hindering migration between existing populations which may otherwise be apparently safe within protected areas (Pârvulescu et al. 2020). We can easily observe that *A. bihariensis* is relatively safe in this respect, as the separation distances between the predicted invasion boundary and the native species suitable network are quite substantial, indicating that they might be also secure in the near future. However, it is essential not to overlook the possibility of other invasive species' expansion, which could be more aggressive in this respect (e.g.,

Pacifastacus leniusculus, whose presence has been recorded (Weiperth et al. 2020) in neighboring Hungary). As for *A. torrentium*, it is in a more alarming situation, as only 45% of populations are at some distance from the potentially invaded zone, and these distances are smaller compared to *A. bihariensis* (see Table 4). Over half of the populations are already in potential contact, even though field investigations did not find mixed populations with *F. limosus*. This situation has not changed in the past ten years (see Pârvulescu et al. 2012; Pârvulescu et al. 2015; Pacioglu et al. 2020). Further efforts should go towards establishing, monitoring and managing "ark-sites" for the long-term conservation of this native species.

We did not detect *F. limosus* DNA in the qPCR analysis from paths. Still, we cannot exclude the possibility of false negative results given by a low number of crayfish at the marginal sectors of the invasion front. Moreover, the specific environmental conditions in the upstream sectors could be suspected to hinder eDNA detectability (Curtis et al. 2021; Snyder et al. 2023).

Overall, we need to remain cautious, especially considering the potential expansion of crayfish plague pathogen *A. astaci* virulent strains. To the best of our knowledge, this screening represents the most extensive investigation of *A. astaci* prevalence in native crayfish populations. We found an infection rate of 64% in apparently healthy native crayfish populations, with no observed mass mortality events. The rate may be underestimated because of the low amount of tissue used for the qPCR analysis. However, killing healthy, protected crayfish for more reliable results was not an ethical option.

The haplotype found in the three locations with native crayfish species (including *A. astacus* found in a mixed population with *A. torrentium*, see Table 5) was identical in the sequenced fragment of the ribosomal rnnS and rnnL region to sequences of the A-haplogroup (containing the genotype As and Up), a haplogroup with strains ranging from non-virulent to highly virulent (Becking et al. 2015; Boštjančić et al. 2022). While the genotype As has been identified as latent infection in several European crayfish populations (e.g., Viljamaa-Dirks et al. 2013; Jussila et al. 2021), the genotype Up is known from mass mortality in Czechia (Kozubíková et al. 2008; Grandjean et al. 2014). Since *A. astaci* has been present in the Danube and its delta since the first infection wave (Alderman et al. 1987) and is now coexisting with native crayfish (Schrimpf et al. 2012), it is possible that this has also caused the latent infection we found in native populations in this study.

Although the current conditions appear to be stable, this may change in the future. Since *P. leniusculus* is also present in the Danube, but still far from the analysed area at this study date (Weiperth et al. 2020), mortality caused by a more infectious *A. astaci* haplotype is a forthcoming threat, as different vectors other than crayfish may also be responsible for the pathogen's spread (Oidtmann et al. 2002; Águas et al. 2014; Svoboda et al. 2017).

Conclusion

The predictive models enabled the identification and quantification of the degree of proximity between competing species (two native *Austropotamobius* and the invasive *F. limosus*). The expansion of *F. limosus* in streams was found to be limited by flash-flood potential

(a variable measuring stream disturbance potential according to the local and upstream drainage velocity) in a range that is characteristic to lowland rivers. The study revealed *A. bihariensis* is safe against invasion, having large sectors separating it from the invasion front, sectors in which neither *F. limosus* nor the pathogen *A. astaci* was detected. The situation is worrying for *A. torrentium*, as it has many populations at high risk of contact with the invader. A latent infection with *A. astaci* (A-haplogroup) in apparently healthy populations of both species was detected with a low virulent strain, without mass mortality events. Consequently, the conservation efforts in the areal of *A. bihariensis* must focus on preventing the spread of other more virulent crayfish plague pathogen strains, whereas a careful monitoring and management of the ongoing situation of *A. torrentium* is required.

Acknowledgments

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Supplementary material 1

Hyperparameter tuning using Grid Search
Authors: Alina Satmari, Kristian Miok, Mihaela C. Ion, Claudia Zaharia, Anne Schrimpf, Lucian Pârvulescu
Data type: docx
Link: https://doi.org/10.3897/neobiota.89.110085.suppl1

Supplementary material 2

Results of two samples Welch t-tests for comparisons between species with respect to the geospatial variables in occurrence sites
Authors: Alina Satmari, Kristian Miok, Mihaela C. Ion, Claudia Zaharia, Anne Schrimpf, Lucian Pârvulescu
Data type: docx
Link: https://doi.org/10.3897/neobiota.89.110085.suppl2

Supplementary material 3

Overview of the results from the eDNA analysis for the detection of
A. bihariensis/A. torrentium*, *F. limosus* and *A. astaci
Authors: Alina Satmari, Kristian Miok, Mihaela C. Ion, Claudia Zaharia, Anne Schrimpf, Lucian Pârvulescu
Data type: docx
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