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## Is the distribution pattern of the stone crayfish in the Carpathians related to karstic refugia from Pleistocene glaciations?

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**Abstract.** The stone crayfish, *Austropotamobius torrentium*, is one of the oldest freshwater crayfish species in Europe. Most Carpathian populations are in Romania, with a distribution clustered in 2 compact metapopulations in the western part of the country. Our goal was to understand if this pattern is the result of a coincidence or a complex set of restrictive circumstances. Romania is an ideal place to analyze crayfish distributions because they have not been disturbed by species translocations or massive loss of populations. We recorded the presence/absence of crayfish and measured 15 habitat variables at 428 randomly chosen headwater sampling sites, and analyzed the crayfish distribution pattern with a boosted regression tree model. Our results show that most of the Romanian territory is ecologically suited to support stone crayfish under current conditions. The most important influences on probability of presence were water velocity, conductivity, altitude, river size, and dissolved O<sub>2</sub>. When included as a supplementary predictor in the model, the distance from karst became the strongest variable accounting for the probability of presence and explaining the current distribution of the stone crayfish. We propose the hypothesis that at least one cycle of Pleistocene glaciation shaped the current distribution pattern by causing massive extinction in the Carpathians, except in karstic underground water bodies that offered ecological refuges. After the glaciations, stone crayfish expanding from these refuges competed with colonizing noble crayfish *Astacus astacus*, restricting stone crayfish to insular areas.

**Key words:** *Austropotamobius torrentium*, boosted regression trees, glacial refuge, noble crayfish, population history, species-distribution modeling.

The current distribution of a crayfish species can provide useful insights into its biogeographic history. Over time, many factors have influenced the geographical distributions of living crayfish species, especially the climate fluctuations of the Pleistocene (2.6 million to 11,500 y ago), which dramatically shifted or reduced the distribution of many taxa (Hewitt 1996, Grandjean et al. 2006). In recent decades, native crayfish populations have experienced pressures (e.g., loss of habitat, competition from nonindigenous species, and the crayfish plague)

that have caused declines in wild stocks (Füreder et al. 2003, Holdich et al. 2009, Kozák et al. 2011). Crayfish populations are susceptible to extirpation (Holdich and Reeve 1991, Matthews and Reynolds 1992), and their natural ability for recovery is very low (Bohl 1987). Therefore, understanding the causes of species' distribution is crucially important to adequate conservation.

Five indigenous freshwater crayfish species are presently living in Europe (Holdich 2002): the stone crayfish *Austropotamobius torrentium* (Schrank 1803), the white-clawed crayfish *Austropotamobius pallipes* lato (Lereboullet 1858), the noble crayfish *Astacus astacus* (Linnaeus 1758), the narrow-clawed crayfish *Astacus leptodactylus* Eschscholtz 1823, and the thick-clawed crayfish *Astacus pachypus* Rathke 1837. The

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stone crayfish is one of the oldest crayfish species in Europe. It originated from an ancestral *Austropotamobius*, which split into the *pallipes* (white-clawed crayfish) and the *torrentium* (stone crayfish) lineages in the Miocene (Albrecht 1982). Speciation was associated with the uplift of the Alps and Dinarides that separated the Paratethys and Mediterranean seas (Trontelj et al. 2005). Early colonization of the Carpathian Danube basin is estimated to have taken place in the late Pliocene or early Pleistocene from the western Balkans (Trontelj et al. 2005, Klobučar et al. 2013). The noble crayfish probably colonized most of the watersheds in the Danube basin after the last glaciation (Albrecht 1983, Schulz and Grandjean 2005, Schrimpf et al. 2011), and it is plausible that historically, this species constituted an important competitor for the stone crayfish in this region.

Most stone crayfish populations in the Carpathians are in Romania (Holdich 2002). Romania is entirely within the Danube watershed (Ujvari 1972), thereby offering homogeneous conditions for crayfish dispersal throughout history. The distributions of native crayfish species may differ from historical distributions because of crayfish harvesting and astaciculture in central and western European countries (Albrecht 1983, Grandjean et al. 2000, Trontelj et al. 2005, Machino and Holdich 2006, Güner and Harlioğlu 2011, Schrimpf et al. 2011). However, crayfish farming did not develop in Romania (Băcescu 1967, Perdikaris et al. 2012), and the country is ideal for analyzing natural drivers of patterns of crayfish distributions.

Recent declines in native European crayfish species have been related to the crayfish plague caused by *Aphanomyces astaci*, a fungus-like pathogen (Lowe et al. 2004). Massive population losses were recorded in countries where introductions of North American crayfish species (*Orconectes limosus*, *Pacifastacus leniusculus*, and *Procambarus clarkii*) were extensive, and these species have now become invasive (Diéguez-Uribeondo 2006, Holdich et al. 2009). Only one report was made of mass mortality in the Romanian Danube basin. This die-off affected the noble crayfish population in the lower Olt River in 1934–1935 (Băcescu 1967), but the association with crayfish plague has never been proven. *Orconectes limosus* and *Aphanomyces astaci* have been found in the main course of the Danube River (Pârvulescu et al. 2012, Schrimpf et al. 2012), but no other invasive species have been reported in Romania so far.

The 1<sup>st</sup> comprehensive survey of the distribution of stone crayfish in Romania revealed 2 insular metapopulations in the southwestern and northwestern parts of the country (Pârvulescu and Zaharia 2013). Molecular investigations showed that these metapop-

ulations have a high degree of genetic differentiation, which suggests a long period of isolation (Popa et al. 2011). Stone crayfish usually inhabit small and medium-sized rivers, brooks, and streams with pristine waters and moderate current speeds (Pöckl and Streissl 2005, Vlach et al. 2009, Pârvulescu et al. 2011). Generally, the species is found in cold waters and is active at water temperatures  $>5^{\circ}\text{C}$  (Bohl 1987). Occasional reports have been made of stone crayfish living in caves (Koutrakis et al. 2005). For example, a healthy and viable population was found 7000 m from the entrance to Aggitis Cave in an isolated gallery that has been blocked for years (Koutrakis et al. 2005). The affinity of this crayfish for karstic areas has been recognized, (e.g., Machino 1997, Maguire and Gottstein-Matoèec 2004, Simiæ et al. 2008), but no evidence exists of a causal relationship between the distribution of karst and the distribution of the crayfish.

We used a biogeographic approach to examine the distribution of the stone crayfish in relation to karst. This research extends the knowledge of the species' history, opens new perspectives for phylogeographical approaches, and may provide useful information on the ecological requirements for crayfish conservation in Europe and elsewhere.

## Methods

The uneven distribution pattern of the stone crayfish in Romania (Pârvulescu and Zaharia 2013) motivated us to examine whether freshwater habitats in the central and eastern parts of the country are suitable for this species. We studied 428 randomly selected river sectors in the mountain and hill areas of Romania in summer 2008–2011. We recorded species occurrence and measured environmental variables for inclusion in a geospatial database that was analyzed to predict species distribution. A significant part of the region under study is populated by the noble crayfish (Fig. 1). Therefore, we compared the ecological preferences of the 2 species.

### *Species data and environmental variables*

We assessed crayfish presence/absence at each site by hand sampling an ~200-m section of the river bed. We asked local residents about the presence of crayfish, but we used only our direct observations in the data set.

At each of the sampled sites, we measured altitude, mean river width and depth, and water velocity. We estimated water velocity as the mean of 10 to 20 measurements made per site with a flow meter (JDC Electronic SA, Waadt, Switzerland). We measured pH, dissolved  $\text{O}_2$ , conductivity, water hardness,

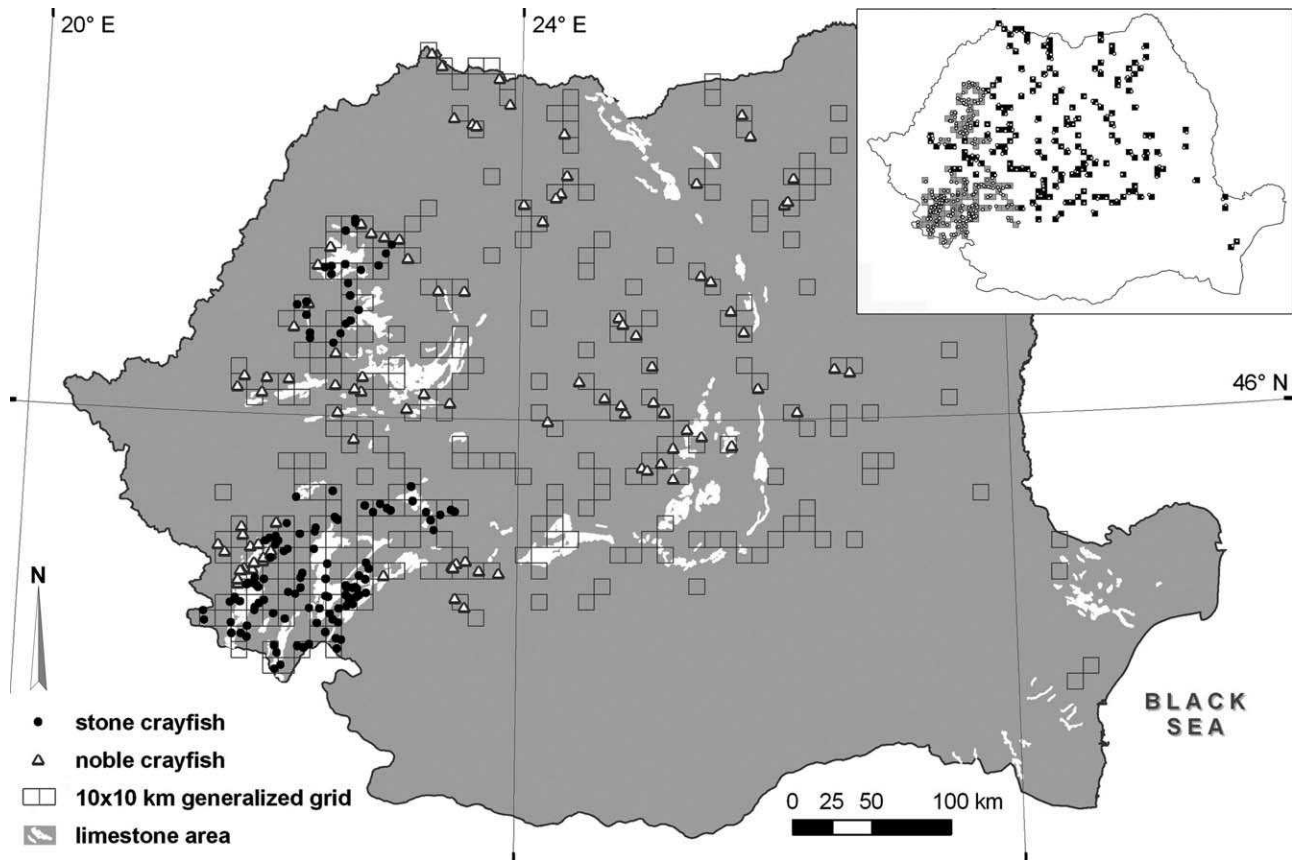


FIG. 1. Map showing the distribution of stone crayfish and noble crayfish in relation to limestone areas in Romania. Grid squares represent 10 km  $\times$  10 km areas including field sampling sites. Inset: sites included in data subsets used to create the statistical model (subset A in gray) and to test the probability of occurrence (subset B in black).

dissolved Ca and Mg ion concentration, dissolved inorganic N forms ( $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ ), and soluble reactive P (SRP) with multiparameter and spectrophotometric field equipment (Hach-Lange GmbH, Düsseldorf, Germany) following standard procedures. We measured each variable in triplicate subsamples at each sampling site. We omitted data from turbid or temporarily flooded rivers from the analyses.

#### Database and analysis

We converted the locations of the 428 sampling sites into an ArcGIS (ArcMap version 9.3; Environmental Systems Resource Institute, Redlands, California) point shapefile. We added the data on species presence/absence and environmental variables as attributes to each point. We identified limestone areas from 1:200,000-scale geological maps produced by the Romanian Institute of Geology and calculated the distance from each sampling site to the nearest limestone area within the river network (i.e., the distance along rivers). We created the hydrological

network from a 90-m digital elevation model (Farr et al. 2007) with the hydrology tool in *spatial analyst*. We used *network analyst* to connect each sampling point to limestone areas via the hydrological network and to calculate the shortest connection.

We divided the data set into 2 subsets. Subset A consisted of 241 sites from the river basins where the stone crayfish could have occurred according to the local connectivity of the hydrographical network, and subset B contained the remaining 187 sites from river basins currently without stone crayfish (Fig. 1). The statistical analyses consisted of training a series of classifiers on the data from subset A, with the purpose of identifying the main variables influencing crayfish presence. We used the models for prediction on subset B to gain insights into the reasons behind the absence of stone crayfish from the entire eastern part of the country.

The main tool used in the statistical analysis was boosted regression tree (BRT) analysis. For a comprehensive description of the method and applications in ecological modeling, we refer the reader to Elith et al.

(2008). We built all models with R software (version 2.14.0; R Project for Statistical Computing, Vienna, Austria) in the *gbm* package (version 1.6–3.2; Ridgeway 2012). One advantage of R:*gbm* is that the most relevant predictors can be identified easily by means of relative variable influence plots. Partial dependence plots showing the effect of one variable on the response after accounting for the average effects of all other variables in the model also can be obtained.

The 1<sup>st</sup> step in our analysis was aimed at understanding whether the central and eastern parts of the country could be considered suitable habitat for the stone crayfish. We built a BRT classifier using the data from subset A, with altitude, river width and depth, water velocity, and physicochemical variables as predictors to separate locations with and without crayfish. We assumed an interaction depth = 1 (single-split decision trees) and, therefore, no interaction between explanatory variables. This decision was motivated by the fact that previous attempts at modeling using more complex trees had not performed significantly better, and, in each case, the computation of Friedman's *H* statistic (Friedman and Popescu 2008) for every pair of predictors indicated no significant interaction. We chose a small learning rate (0.001), and a bag fraction = 0.5. We determined the optimal number of trees by cross-validation.

In the 2<sup>nd</sup> step, we tested for a connection between crayfish presence and the distance from the sampling site and the nearest limestone area (distance from karst). We trained a new BRT classifier with distance from karst added as a supplementary predictor. Tree complexity, learning rate, and bag fraction were the same as above. After their development and evaluation, we used both models for prediction on the data from subset B. In 2012, we conducted a thorough investigation of sites at which stone crayfish were predicted to occur by visiting 34 additional rivers surrounding the sites and by collecting information from local residents.

To compare stone and noble crayfish ecological preferences, we used Mann–Whitney and 2-sample Kolmogorov–Smirnov tests for differences between species for each ecological variable in the subsets of data corresponding to the study sites where one or the other species was found. When we found evidence of significant differences between species, we visualized the distribution of the respective variable with paired box plots.

## Results

Of 428 sampling sites, 123 were populated by stone crayfish, 101 by noble crayfish, and 205 lacked both species. The distribution patterns highlight the con-

trast between the clustered stone crayfish populations and the apparently uniform spread of noble crayfish (Fig. 1). The stone crayfish was found coexisting with another crayfish species (noble crayfish) at only 1 site. Four populations of stone crayfish and 2 of noble crayfish were found in karstic caves.

The most significant factors contributing to model fit of the first BRT classifier were water velocity, altitude, conductivity, river width, and dissolved O<sub>2</sub> (Fig. 2A). The BRT model had reasonable ability to differentiate between locations where stone crayfish were present and locations where they were absent (the area under the receiver operating characteristic curve [AUC] = 0.81). Of the 187 locations in subset B, 93 (50%) were predicted to be suitable for stone crayfish (threshold value for the predicted probability of presence = 0.5). These 93 locations were uniformly distributed over the study area (Fig. 2B).

The inclusion of distance from karst as an additional predictor in the 2<sup>nd</sup> BRT model was well justified. This variable was identified as the one with the greatest relative importance (13.8%) in separating the locations with and without stone crayfish. Other significant predictors were water velocity, conductivity, and altitude (Fig. 3A). The probability of presence clearly decreased with increasing distance from karst (Fig. 4A). Distance from karst >30 km negatively affected probability of presence. Probability of presence was generally constant at water velocities between 0 and 0.5 m/s, increased slightly at values between 0.2 and 0.4 m/s, and declined rapidly at values >0.5 m/s (Fig. 4B). Probability of presence peaked at conductivities between ~300 and 400 µS/cm and at altitudes between 400 and 600 m asl (Fig. 4C, D). The 2<sup>nd</sup> BRT was a better predictor of probability of presence than the 1<sup>st</sup> model for subset A (AUC = 0.86) and subset B for which the number of locations predicted positive decreased to 46 (24.6%). These locations were no longer uniformly distributed across the eastern part of the country, but were grouped in certain areas in the Carpathians (Fig. 3B). None of the 34 locations investigated for confirmation of these positive predictions had stone crayfish.

## Discussion

Our study showed that stream habitat in most of Romania is ecologically suitable for stone crayfish. The 1<sup>st</sup> BRT classifier (Fig. 2A, B) predicted ½ of the locations outside the actual area of occurrence to be inhabitable by stone crayfish. A similar frequency (51%) was calculated inside the area of occurrence, which is a strong argument for a potentially even distribution of stone crayfish over the investigated

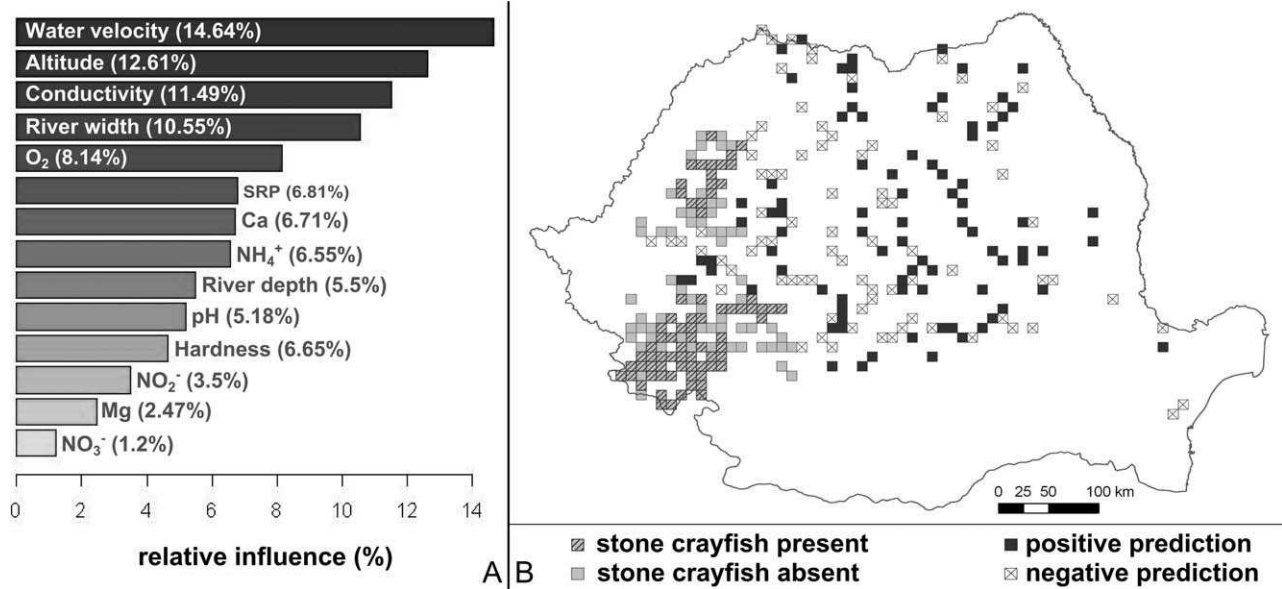


FIG. 2. Relative influence plot of the ecological variables (A) and the predicted spatial distribution of stone crayfish in Romania (B) for the 1<sup>st</sup> boosted regression tree model. SRP = soluble reactive P.

area. In spite of this widespread ecological suitability and the absence of natural or artificial barriers, the populations occur in 2 distinct and compact areas (Fig. 1). The strongest environmental variable explaining this pattern is karst. Thus, the 2<sup>nd</sup> BRT classifier (Fig. 3A, B), which included this variable, halved the frequency of prediction outside the area of occurrence.

Our results showed the proximity of karst as the main driver of the current distribution of stone crayfish. However, physicochemical properties of karst (e.g., hardness or dissolved Ca) do not appear among the important predictors in any of the BRT models, and no physiological relationship between water quality associated with this geological formation

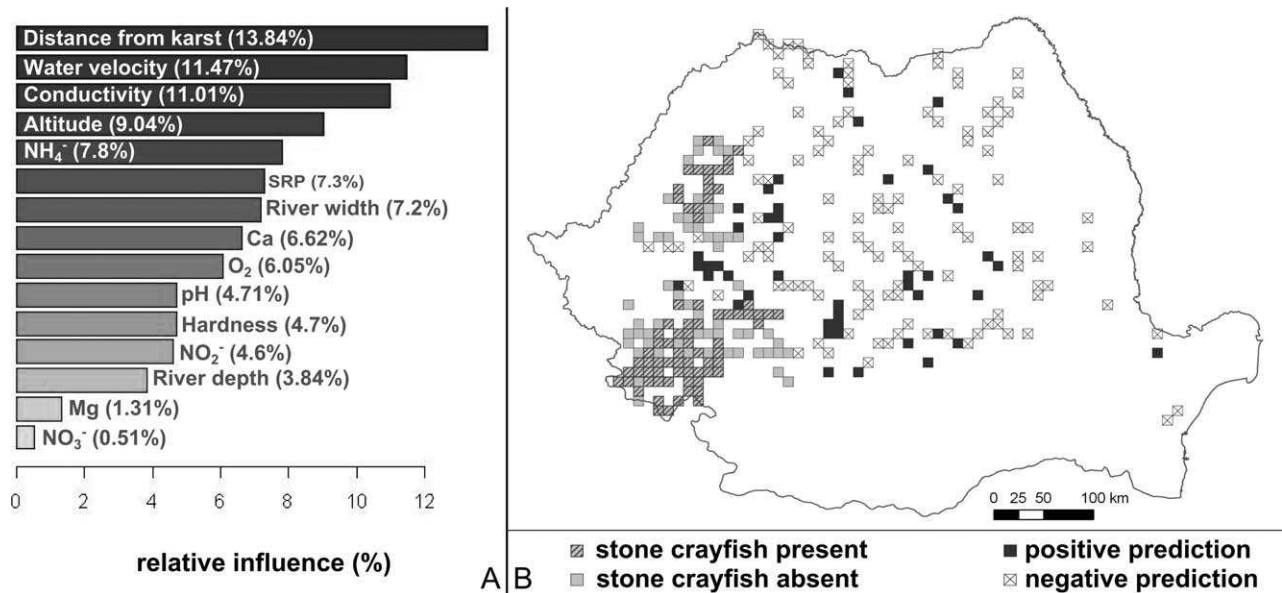


FIG. 3. Relative influence plot after including the distance from karst (A) and the predicted spatial distribution of stone crayfish in Romania (B) for the 2<sup>nd</sup> boosted regression tree model. SRP = soluble reactive P.

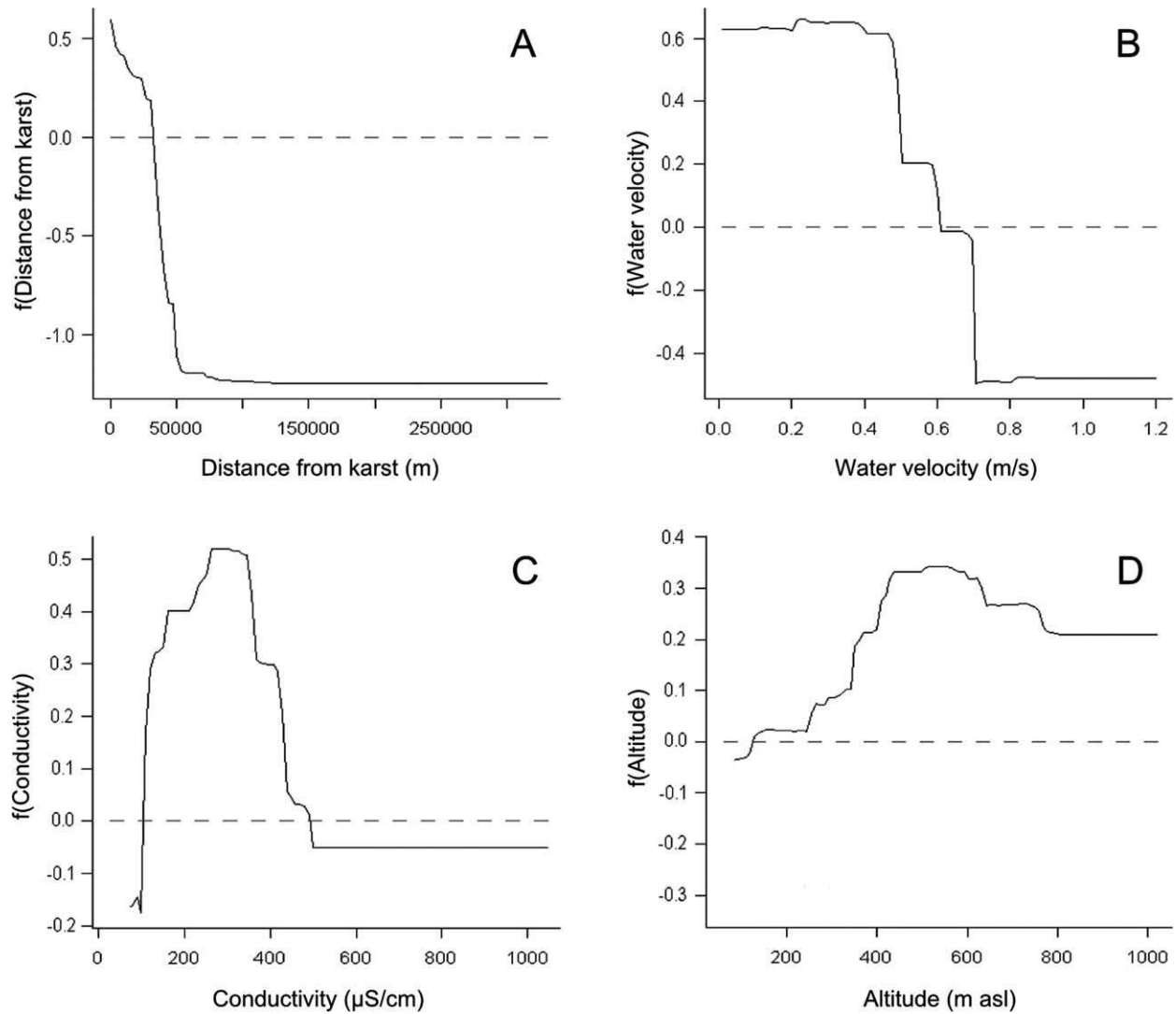


FIG. 4. Partial dependence plots for the influence of the most important variables in the 2<sup>nd</sup> boosted regression tree. A.—Distance from karst. B.—Water velocity. C.—Conductivity. D.—Altitude.

and stone crayfish has been described previously. Some limestone areas develop karstic relief, including underground galleries that support permanent water bodies. The largest and most developed karstic areas are present in the western part of Romania in the Apuseni and Banat mountains (Bleahu and Rusu 1965), where stone crayfish populations are spatially grouped (Fig. 1). Therefore, we hypothesize that underground karst habitats provided climatic refugia for stone crayfish during the Pleistocene.

Dramatic climate changes during Pleistocene glaciation cycles influenced the distributions of many European species because of multiple oscillations between warm and cold conditions (Hewitt 1999). Even if the southern limit of the last glacial maximum (LGM) ice cover did not touch Romania, the higher

Carpathians were covered by glaciers, and most of the lowland areas that are now mainly temperate and boreal forest were then tundra and cold steppe (Hewitt 1996). At the end of the last glacial cycle, reconstructed July temperatures were  $\sim 15^{\circ}\text{C}$  and January temperatures were  $-19^{\circ}\text{C}$ , with a mean annual temperature of  $\sim 6^{\circ}\text{C}$  (Renssen and Isarin 2001). The coldest and longest glacial event was between 0.3 and 0.12 mya, with estimated July temperatures of  $6^{\circ}\text{C}$  and permafrost conditions at altitudes  $< 1646$  m (Urdea 2000). Stone crayfish do not live under such conditions now (Albrecht 1983, Grandjean et al. 2006). For example, water temperature during the summer period has to be  $\geq 8^{\circ}\text{C}$  for this species to develop normally (Füreder 2006). Periglacial conditions probably constituted a survival

TABLE 1. Results ( $p$ -values) of Mann–Whitney (MW) and Kolmogorov–Smirnov (KS) tests comparing the distributions of ecological variables at locations with stone crayfish vs locations with noble crayfish. ns = not significant.

Variable	MW	KS
Distance from karst	<0.001	<0.001
Altitude	ns	ns
River width	0.008	0.005
River depth	ns	ns
Water velocity	ns	ns
pH	0.013	0.032
Dissolved O <sub>2</sub>	ns	0.004
Conductivity	ns	0.001
Hardness	<0.001	<0.001
Dissolved Ca	<0.001	<0.001
Dissolved Mg	0.022	0.020
NO <sub>3</sub> <sup>-</sup>	<0.001	<0.001
NO <sub>2</sub> <sup>-</sup>	ns	ns
NH <sub>4</sub> <sup>+</sup>	ns	ns
Soluble reactive P	ns	ns

bottleneck for the species. During the colder periods of the Pleistocene, probably only populations living in or near thermal refugia (e.g., underground water bodies) avoided extirpation. Karstic areas provided refuge for aquatic animals during glaciations (e.g., Sket 1999, Verovnik et al. 2005). Crayfish would have fed on terrestrial organic matter collected within these open-system karstic habitats (Hogger 1988, Ewald 2003). Warmer Pleistocene interglacial periods were accompanied by intense ice melting, which severely destabilized substrates (van Weert et al. 1997). However, crayfish ensconced in karst habitats would have largely avoided this physical disturbance. The absence of populations adapted to cave conditions (lesser pigmentation, loss of photoreceptors, etc.) can be explained by the reversibility of these adaptations (Beatty 1949).

The northern limit of the noble crayfish during the glaciations was estimated to have been south of the permafrost border (Albrecht 1983), i.e., south of the Romanian Carpathians (Hewitt 1999). Molecular investigations reveal that most of the central and northern European noble crayfish populations arose from late interglacial or postglacial colonization (Schulz and Grandjean 2005, Schrimpf et al. 2011). Therefore, noble crayfish would have been a competitor of the stone crayfish populations in colonizing the Carpathians. The main differences between locations occupied by each species were distance from karst, river width, pH, water hardness, Ca and Mg dissolved ions, and NO<sub>3</sub><sup>-</sup> concentration (Table 1). Noble crayfish occupy sites having a broader range of

all of these variables except NO<sub>3</sub><sup>-</sup> concentration (Fig. 5), indicating that it is a more competitive species. Eleven of the 46 sites where stone crayfish were predicted to be present were inhabited by noble crayfish. We hypothesize that after the Pleistocene glaciations, stone crayfish populations began to recover and slowly expanded from their karstic refugia. At the same time, the noble crayfish was colonizing most of Europe via the Danube drainage system (Albrecht 1983). Stone crayfish can defend their shelters even against larger invasive crayfish (Vorburger and Ribí 1999) and are more sedentary than noble crayfish (Kadlecová et al. 2012). Therefore, stone crayfish were able to persist in face of competition from noble crayfish, but have been unable to expand their range enough to lose the spatial association with karst.

We considered several alternative explanations for the present-day distribution of stone crayfish. A scenario in which the stone crayfish, driven by the preference for colder water, colonized the Romanian territory in the direction of glacial retraction cannot explain why the species is missing from nonkarstic cold streams (e.g., flowing from glacial alpine lakes). An hypothesis of extirpation of stone crayfish populations from anthropogenic causes cannot explain why population losses would have been restricted to eastern Romania. According to natural and historical conditions, the anthropogenic development was relatively uniform across the country (Giurcăneanu 1970), and so were the associated disturbances. The available literature concerning this region (1908 to present) and museum collections do not confirm the species' presence in the central or eastern Romanian Carpathians. Moreover, the distribution of the noble crayfish includes all of Romania and shows no evidence of severe degradation.

South of the Danube (Bulgaria) in an area with less glacial influence, populations of the stone crayfish are widespread and interwoven with noble crayfish populations (Holdich et al. 2006). We conclude that the distribution pattern of stone crayfish in Romania is a consequence of the species' biogeographic history. The hypothesis we advanced might be useful in explaining the stone crayfish distributions in other countries with similar glacial influences. For instance, a visual inspection of the distribution map by Holdich et al. (2006) suggests clustering of stone crayfish populations around karstic areas in Hungary and Slovakia. This hypothesis should be coupled with molecular investigations to elucidate the biogeographic history of stone crayfish. However, the conclusions of such studies should be interpreted carefully because Pleistocene events most probably



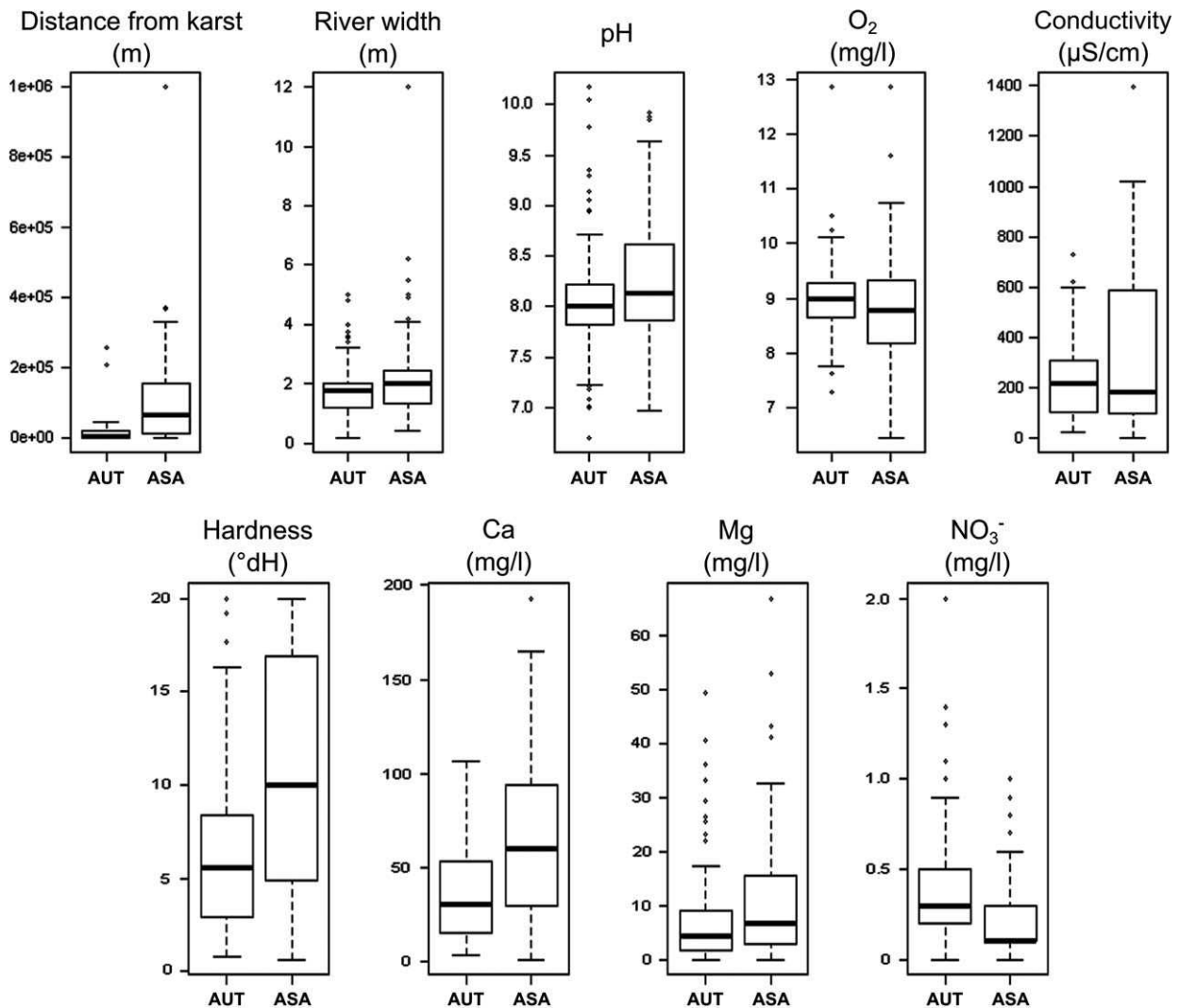


FIG. 5. Box-and-whisker plots of the variables that differed between sites with stone crayfish (AUT) and sites with noble crayfish (ASA). Lines in boxes show medians, box ends show quartiles, whiskers show 1.5 times the interquartile range from the lower and upper quartiles respectively, and dots show outliers.

decreased the genetic diversity of the remaining populations in the Carpathians.

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