JAMSKI LABORATORIJ TULAR
DRUŠTVO ZA JAMSKO BIOLOGIJO

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A SURVEY OF THE DISTRIBUTION OF *PROTEUS ANGUINUS* BY ENVIRONMENTAL DNA SAMPLING

(Istraživanje rasprostranjenja čovječije ribice pomoću okolišnog DNA uzorkovanja)

July 2013 – June 2014

Društvo za jamsko biologijo, u partnerstvu sa

A SURVEY OF THE DISTRIBUTION OF *PROTEUS ANGUINUS* BY ENVIRONMENTAL DNA SAMPLING

PROJECT KEY MEMBERS

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PARTNERS

- The **Evolutionary Zoology Laboratory** of the Institute of Biology at the Scientific Research Centre of the Slovenian Academy of Sciences and Arts, Ljubljana, Slovenia;
- **Jeffery Lab**, Department of Biology, University of Maryland, College Park, MA, USA;
- **Center for Karst and Speleology**, Sarajevo, Bosnia and Herzegovina;
- **Republic Institute for Protection of Cultural, Historical and Natural Heritage**, Banja Luka, Bosnia and Herzegovina;
- **Biospeleological Society of Montenegro**, Podgorica, Montenegro;
- **Herzegovinian Mountain Rescue Service**, Mostar, Bosnia and Herzegovina.

BUDGET

1. Total Project Budget: $26,515
2. In-kind contribution (Society for Cave Biology): $3.000
3. Co-funding:
- Critical Ecosystem Partnership Fund: $16,515
- Jeffery Lab, Department of Biology, University of Maryland, College Park, MA, USA: $6,300
- The Institute of Biology at the Scientific Research Centre of the Slovenian Academy of Sciences and Arts (ZRC SAZU), Ljubljana, Slovenia: $700

4. Non-financial contribution
- voluntary work of all participants;
- Society for Cave Biology: facilities and staff of the Tular Cave Laboratory, field work equipment, IR observation devices, vehicle, use of the Laboratory library, specialized on *Proteus* literature;
- The Institute of Biology ZRC SAZU: facilities and staff of the Evolutionary Zoology Lab, DNA analysis equipment (microcentrifuge, thermocycler, gel electrophoresis apparatus, UV hood, autoclave sterilizer, consumables), morphological analysis equipment (microscopes, deep freezers, image analysis hardware and software), IT system, digital and classical libraries, software license for GIS (ESRI ArcGIS 10 with extensions);
- Center for Karst and Speleology, Republic Institute for Protection of Cultural, Historical and Natural Heritage, Biospeleological Society of Montenegro, Herzegovinian Mountain Rescue Service: database on distribution of *Proteus*, vehicle, field work equipment.

MISSION OF THE PROJECT

1. Surveying the distribution of the elusive Proteus
- data on exact distribution of *Proteus* is the basic step for an efficient conservation action plan

2. Proteus is in need of urgent protection
- flagship species of the Dinaric karst groundwater and its endangered cave fauna
- strictly protected, but facing most serious degradation of its habitat:
  - groundwater pollution: extensive agriculture, industry and sewage disposal
  - change of hydrological regime: water extraction, large scale hydrotechnical works

3. Shared dependence on groundwater
- in studied karst areas, groundwater is the only reliable source of drinking water

4. Dinaric karst countries alliance for conservation of Proteus
- establish contact with institutions along Dinaric karst and exchange knowledge on Proteus and cave fauna conservation;
- promote joint activities and rising competence levels of the participating partners, in a clear mutual interest of all the countries along Dinaric karst.

PROJECT APPROACH

I. Environmental DNA (eDNA) analysis

II. Field work (collection of water samples of springs and caves)

III. Geographic Information System (GIS) analysis

IV. Public promotion and academic outreach

THE SOCIETY FOR CAVE BIOLOGY AND TULAR CAVE LABORATORY
(DRUŠTVO ZA JAMSKO BIOLOGIJO IN JAMSKI LABORATORIJ TULAR)

The Society for Cave Biology is a NGO founded in 2002, with its principal mission to carry on the legacy of the Tular Cave Laboratory, Slovenia's only subterranean laboratory. The Tular Laboratory was established in 1960, but reorganized in 2002 into a constituent body of the Society for Cave Biology. Since 2007, the Society and the Laboratory have been led by Gregor Aljančič. All activities are conducted on voluntary basis.

Since only small fragments of *Proteus* subterranean habitat can be accessed by man, most knowledge about this species has been acquired in laboratories, away from its natural environment. One such case is the Tular Cave Laboratory, arranged in 1960 by speleobiologist Marko Aljančič (1933–2007) in a small conglomerate cave Tular in Kranj (Fig. 1). After more than 50 years, almost all animals brought from various localities in Slovenia, maintained in a series of large artificial pools are still alive. On the basis of this long-term experiment we predict the maximum lifespan of *Proteus* to be close to 100 years.

Fig. 1. Main laboratory chamber in 1962. Photo: M. Aljančič.

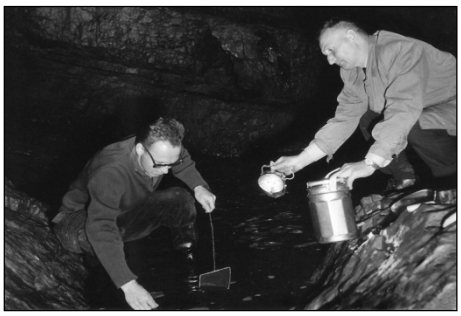

Fig. 2. Collecting cave fauna in Podpeška jama, 1965. Photo: M. Aljančič.

Tular is the only cave laboratory in SE Europe designed for studying *Proteus* and – apart from the subterranean laboratory in Moulis, France – the only place with successful *ex situ* breeding program of this highly endangered cave amphibian (Fig. 3 & 4).

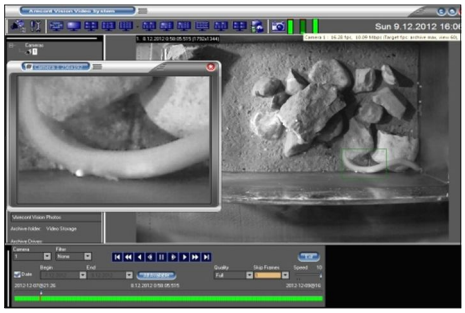

Fig. 3. *TularVirtualLab:* remote monitoring of oviposition. Video: G. Aljančič.

Fig. 4. Hatching of *Proteus,* never seen in nature (larva, 2 cm). Photo: G. Aljančič.

In the laboratory, the ecology and behaviour of *Proteus* (Fig. 5 & 6), mainly its reproduction, are studied. All our studies are based on observations, which are carefully designed not to harm or stress the animals. *Proteus* is observed by high-resolution infra red video cameras and night vision devices. Considerable effort is put into fieldwork – observing the behaviour of *Proteus* in nature, surveying environmental parameters of the habitat, verifying old data on its occurrence, and discovering new localities. The laboratory also serves as a sanctuary for injured *Proteus* accidentally washed out of caves during seasonal flooding, providing veterinary inspection and animal care.

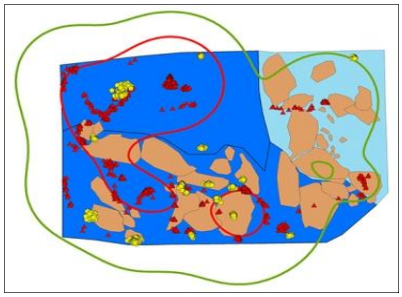

Fig. 5. GIS analysi s of space use in laboratory pools (Aljančič & Năpăruş, 2009).

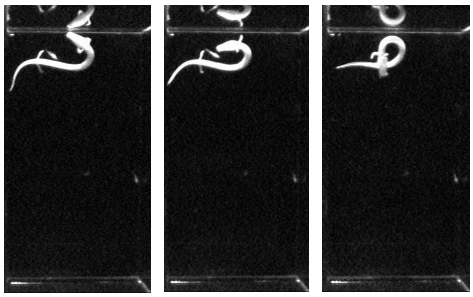

Fig. 6. Prey-capture experiments: *Proteus* feeding on cave shrimps (with J. Jugovic et al., 2010).

Since 2002, a colony of extremely rare darkly pigmented subspecies *Proteus anguinus parkelj* (Fig. 7) is also maintained in this laboratory. In case of black *Proteus,* even a local pollution incident could have a devastating impact on the entire population, and thus its global existence. This morphologically most distinct of all *Proteus* populations, is limited to the cave system behind of only four springs in Bela krajina, SE Slovenia, an area encompassing less than 50 km^2 (Fig. 8).

Fig. 7. The black *Proteus* (*Proteus anguinus parkelj*), discovered in 1986. Photo: G. Aljančič.

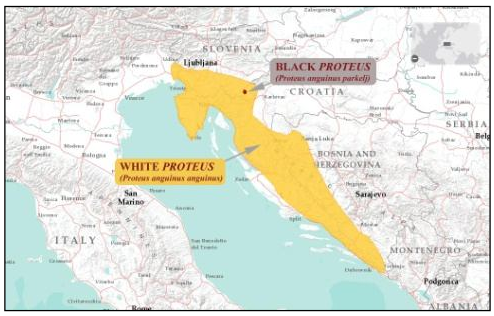

Fig. 8. Distribution of *Proteus* (after Arntzen *et al*., 2009, IUCN Red list of Threatened Species, V 2012.2)

The Society for Cave Biology is aware that protection of *Proteus* and its habitat may truly be effective only by constant public promotion of the species as one of the world's prime symbols of natural heritage and subterranean biodiversity, as well as by emphasizing its vulnerability and karst groundwater conservation issues. Besides addressing the general public across Europe, the Society particularly focuses on nature conservation education in local communities where *Proteus* is present. Through a program of regular lectures and by involvement in construction of local natural heritage information centers, the necessity of the protection of *Proteus* and groundwater is presented as a unique opportunity for sustainable development.

An anecdote

In 1968, the Tular Cave Laboratory and its *Proteus* became the subject of international politics. Despite its opposition for conservation reasons, the Laboratory had to provide six animals for President Tito, to pass them as a precious gift to the Japanese Emperor Hirohito, a distinguished marine biologist. The fate of these animals remained unknown for the next 40 years, when the Laboratory, with the support of the Slovenian Ministry of Foreign Affairs and the Embassy in Tokyo, launched a delicate diplomatic inquiry. The initial fear was confirmed: Tokyo animals had survived for only a few months. Consequently, before his visit to Japan in 2008, the Slovenian Prime Minister J. Janša asked for a *Proteus* as a gift to the Emperor Akihito, who is a renowned expert on fish taxonomy. In democratic circumstances of today's Slovenia, the nature conservation dedication of our Laboratory finally prevailed. For a brief moment, the strict protocol of the Imperial Palace harkened to the story of the endangered *Proteus*. As a symbol of Dinaric karst, only a bronze model of this unique animal was presented to HIM Akihito – a testimony of Laboratory's dedication to preserve *Proteus* in future.

PROTEUS IN SURVEYED AREAS OF BOSNIA AND HERZEGOVINA: DISTRIBUTION AND THREATS

Knowledge on the distribution of *Proteus* in this Bosnia and Herzegovina is critically lacking, with only around 60 localities recorded (Sket, 1997; Kotrošan, 2002). Many of these populations were reported to be decimated or destroyed, mostly as a consequence of extensive damming of karstic rivers in Herzegovina (Culver and Pipan, 2009).The proposed project directly addresses the need to raise awareness of the importance of three priority key biodiversity areas in Bosnia and Herzegovina: Trebižat, Hutovo blato and the Neretva delta. The results of the proposed project will provide a justification and scientific foundation to improve their conservation and protection status.

Current knowledge on the distribution of *Proteus* around the lower course of the river Neretva is scarce. Located on the eastern side of the Neretva are Svitavsko-deranska depresija and Hutovo blato, which drain via a small stream, the Krupa, into the Neretva, while the river Trebižat drains into the Neretva from the western side. In the area of Hutovo blato, only one *Proteus* locality – a well in the village Čore near the Babino oko spring – is known. Undoubtedly *Proteus* can be expected to inhabit other springs in the area as well. Waters draining towards Hutovo blato and Svitavsko-deranska depresija derive from Popovo polje, in which numerous *Proteus* localities have been recorded. On the other hand, waters from Nevesinjsko polje drain via Dabarsko polje towards the Bregava river, which at Čapljina flows into the Neretva from its left side. The presence of *Proteus* has been confirmed on Dabarsko polje as well. In the Neretva delta itself, *Proteus* was recorded thirty years ago in several springs at the village of Gabelo on the border with Croatia.

During the recent years, intensive engineering works to divert the waters from Dabarsko polje towards Fatničko polje and further towards Bileća, Trebinje, and the Ombla hydroelectric plant near Dubrovnik have been undertaken. The flow of groundwater towards the lower Neretva and its delta is thereby considerably reduced. Furthermore, a substantial portion of waters from Popovo polje is being diverted towards Ombla, while only a small part is directed to the reversible hydroelectric plant "Čapljina" located on the eastern side of the Svitavsko-deranska depresija (the depression serves only as a compensational reservoir). Large scale hydrotechnical works unfavorably affect the ecological characteristics of the areas through the reduction of the catchment area, land amelioration, disturbances to the natural flooding regime of karst poljes, changes in direction of watercourses, flooding of caves, and water pumping (Ozimec, 2011). All these may have a detrimental impact on the density or even existence of *Proteus* populations (Čučković, 1983). The reduction of input of groundwater from karstic poljes of Eastern Herzegovina to the Neretva lower course and the delta results in an increase of saltwater penetrating further upstream. The observed increased salinity may have a direct adverse effect on the localities of *Proteus* in the Neretva delta and Hutovo blato. Moreover, changes in the hydrographical connections might affect gene pools of relevant populations (Sket, 1997).

The river Trebižat, which flows into the Neretva from its right side, is an intermittent stream, sinking and reappearing several times and sporting several names (it originates in the area of Imotsko in Croatia). In the Ljubuški region and downstream near the village of Studenci, several localities of *Proteus* are known. A possible threat to these has appeared with the construction of a highway (the "Vc" corridor), which is planned to meet the highway from Croatia in this area. Local residents report on the sightings of *Proteus* in additional springs in the area. A confirmation of these accounts would considerably increase the number of localities in this part of Herzegovina, as well as provide a prospect of locating new sites to the north, in the area of Tribistova, Rakitna.

I. ENVIRONMENTAL DNA (eDNA) ANALYSIS

The project will introduce a forensic method in the field of subterranean biology: detection of traces of *Proteus* DNA in any spring or cave water examined regardless of whether or not the animal has ever been seen there. The highly efficient, non-invasive, DNA-based method to detect *Proteus* from water samples will be implemented to test for the presence of *Proteus* in new potential localities as well as to verify selected old data on the distribution of *Proteus.*

eDNA method description

During the process of skin growth and regeneration, fragments of epidermal cells, along with the DNA they contain, are constantly shed from aquatic vertebrates into water (Fig. 9). Such DNA dissolved in water is called environmental DNA (eDNA). DNA degrades in the environment, but the process is not instantaneous. DNA can be collected from water samples by filtering (Fig. 10).

Fig. 9. Fragments of cells are constantly carried away by water flow. Photo & drawing: G. Aljančič.

Fig. 10. DNA can be collected from water by filtering. Photo: Š. Gorički.

Species can then be detected by extracting and amplifying short fragments of DNA by polymerase chain reaction (PCR) using specific primers (Fig. 11). Target DNA fragments of different lengths are separated and visualized by gel electrophoresis (Fig. 12). The presence of a fragment of a specific length confirms the presence of a particular species or population in a sampled location. Or *vice versa*, if the species in question is not present in the examined sample of water, no matching DNA will be amplified.

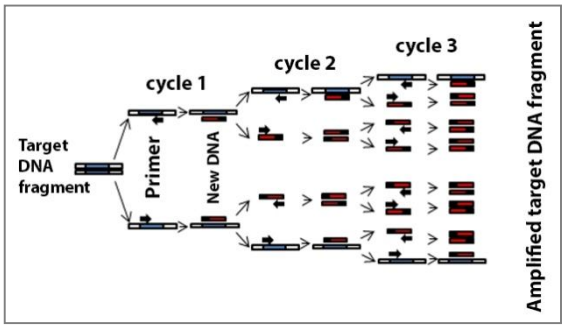

Fig. 11. Short target DNA fragment is amplified (*PCR*). Drawing: Š. Gorički.

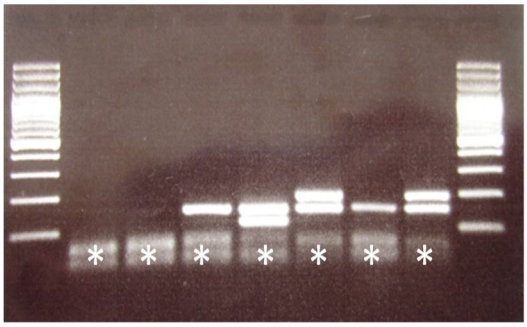

Fig. 12. Target DNA fragments separated and by visualized by gel electrophoresis (*artifacts).

Prior to the application in the project, the method will be optimized and rigorously tested utilizing controlled conditions of *Proteus* husbandry in the Tular Cave Laboratory followed by testing in nature. Four karst springs in Slovenia will be chosen for testing the technique on field samples, two with verified presence of *Proteus* and two outside its range to represent negative controls.

All eDNA analyses will be conducted in the **Evolutionary Zoology Laboratory of the Institute of Biology at the Slovenian Academy of Sciences and Arts (ZRC SAZU)**, Slovenia.

II. FIELD WORK (COLLECTION OF WATER SAMPLES OF SPRINGS AND CAVES IN SURVEYED AREAS)

1. Water sampling in Bosnia and Herzegovina

- Hutovo blato
- Neretva River
- Trebižat River Tributary

Based on the existing distribution data for *Proteus* and the latest spatial data on hydrogeology of the surveyed areas in Bosnia and Herzegovina will be intensively tested for the presence of *Proteus.* Approximately 30 springs will be sampled. At each site, two samples will be taken and analyzed using the eDNA method developed during the first phase of the proposed project. The negative sites will be re-sampled to increase the probability of detection. Field work will be conducted in partnership with the **Center for Karst and Speleology** (Centar za krš i speleologiju), Sarajevo, the **Republic Institute for Protection of Cultural, Historical and Natural Heritage** (Republički zavod za zaštitu kulturno-istorijskog i prirodnog nasljeđa), Banja Luka, and the **Herzegovinian Mountain Rescue Service** (Hercegovačka gorska služba spašavanja), Mostar.

2. Water sampling in Montenegro

- Obodska pećina/ Rijeka Crnojevića

The other country in focus will be Montenegro, where *Proteus* has yet to be documented, despite many unverified reports (Kaležić & Džukić, 2001). Considering the continuation of the Dinaric karst in Montenegro and the vicinity of known localities just across the border in Dalmatia and Herzegovina, Montenegro has a reasonable possibility to actually be inside the distribution range of *Proteus*. In cooperation with the **Biospeleological Society of Montenegro** (Biospeleološko društvo Crne Gore), we will be able to test this hypothesis using the proposed method. Water from Obodska pećina near Rijeka Crnojevića and three springs in the vicinity of known localities (in Dalmatia and Herzegovina) within the Southwest Balkans corridor will be sampled.

III. GEOGRAPHIC INFORMATION SYSTEM ANALYSIS

GIS (Geographic Information System) represents the best platform to organize, analyze, visualize and model data from the environment, guiding proper decisions based on this data in order to better preserve natural resources and protect the biotic diversity. Karst system functionality and biodiversity is particularly difficult to map and model. Here, GIS provides the tools and the framework to map the current status of *Proteus* within the complex karst landscape.

1. GIS database,

containing:

- dataset with both the existing and plausible georeferenced localities of *Proteus*;
- dataset with karst features and their spatial relationships within the karst system;
- dataset with geological and hydrogeological features and their spatial relationships;
- high resolution digital elevation model of the area;
- the updated locality dataset obtained from the eDNA analysis, with the complementary attributes (eDNA response).

2. Guidance of DNA investigation

- current map of the localities of *Proteus* with the extent of its distribution
- plausible localities and functionality of the karst system in surveyed areas

3. Distribution map of *Proteus*

- updated localities of *Proteus* detected in eDNA analysis
- map of the accurate boundary of *Proteus* habitat

4. Spatial statistical modeling

- the data will be integrated in a predictive GIS model to determine the potential range of *Proteus* using spatial statistic (linear regression): the Ordinary Least Squares (OLS), Geographically Weighted Regression (GWR) and test for spatial autocorrelation (Moran's I)

IV. PUBLIC PROMOTION AND ACADEMIC OUTREACH

In the countries of Dinaric karst, projects on *Proteus* are traditionally received with considerable public interest. This public attention will be focused on the vulnerability of *Proteus* and its subterranean habitat, groundwater protection and the promotion of natural history through nature conservation.

1. Bosnia and Herzegovina

Since the ancient times, people of Bosnia and Herzegovina have occasionally met *Proteus* while searching for drinking water in caves, karst springs and wells, or found this cave animal on fields after seasonal floods – just like anywhere else along the Dinaric karst. Without any material evidence, we may only speculate on their relation to this unique creature. A rare example, the oldest known representation of *Proteus* from the territory of Bosnia and Herzegovina, engraved on a "stećak" from ca. 1477, was discovered in the necropolis Boljuni near Stolac (Mulaomerović & Hodžić, 2012). This is just the most precious among many historical and cultural bonds between the local people and *Proteus,* which will provide a conceptual basis for a promotion of an integrated approach to sustainable livelihood practices and serve as guidelines for improving the negative human impact on *Proteus* and groundwater.

Planed activities in BIH:

- meetings with partners, local community and administration (present goals & results; promote Dinaric countries *Proteus* conservation initiative and contribute to sustainable livelihood practices);
- Perform popular science lectures on i) threats to groundwater and protection of *Proteus, ii)* nature heritage as a unique drive of green tourism and sustainable use of groundwater resources;
- Publish and distribute educative leaflet on protection of *Proteus* and groundwater;
- press conferences (Ljubljana and Sarajevo) and other media coverage in BIH, ME and SLO.

2. Montenegro

A possible discovery of the first locality of *Proteus* in Montenegro would not only be scientifically important. Most of all, Montenegro would also confront a challenge to preserve it in the future, as an example of true commitment to its natural heritage. In this respect, the Society for Cave Biology has already informed the Embassy of Montenegro in Slovenia about the nature conservation consequences of the possible discovery of *Proteus* in Montenegro. If the existence of Proteus in Montenegro will indeed be confirmed during this project, the Society for Cave Biology and Biospeleological Society of Montenegro will continue to appeal and counsel the nature conservation authorities in Montenegro to start all necessary legal actions to protect Proteus in their territories.

3. Academic outreach

- Presentation of the project on scientific meetings (First International Workshop on "Dinaric Karst Fields as Wetlands of National and International Importance", Livno, BiH; 22nd International Karstological School, Postojna, SLO);
- Disseminate scientific results in open access journal.

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