A survey of the distribution of *Proteus anguinus* by environmental DNA sampling

June 28, 2013 – August 31, 2014

CEPF FINAL PROJECT COMPLETION REPORT

Society for Cave Biology, in partnership with

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Organization Legal Name:	Društvo za jamsko biologijo/ Society for Cave Biology
Project Title:	A survey of the distribution of *Proteus anguinus* by environmental DNA sampling
Date of Report:	October 31, 2014
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CEPF Region: Europe & Central Asia/ Mediterranean

Strategic Direction:
2. Establish the sustainable management of water catchments and the wise use of water resources with a focus on the priority corridors of the Southwest Balkans (2.2. Support IRBM policy and legislation development and implementation through capacity building and advocacy at all appropriate levels),

3. Improve the conservation and protection status of 44 priority key biodiversity areas (3.1 Establish new protected areas and promote improved management of existing protected areas by developing and implementing sustainable management plans; 3.3. Raise awareness of the importance of priority key biodiversity areas).

Grant Amount: 16,449.20 USD

Project Dates: 06.28.2013–08.31.2014

Implementation Partners for this Project (please explain the level of involvement for each partner):

- **Društvo za jamsko biologijo/ Society for Cave Biology**, www.tular.si; Gregor Aljančič, project manager; Dr. Špela Gorički, eDNA analysis developer; David Stanković, qPCR analysis developer; Dr. Magdalena Năpăruş-Aljančič, GIS analysis.
- **Oddelek za zootehniko, Biotehniška fakulteta, Univerza v Ljubljani/ Department of Animal Science, Biotechnical Faculty, University of Ljubljana**, Domžale, Slovenia, http://www.bf.uni-lj.si/en/animal-science/department/introduction/; Dr. Aleš Snoj, qPCR analysis supervisor.
- **Jeffery Lab, Department of Biology**, University of Maryland, MA, USA, http://www.life.umd.edu/labs/jeffery/; Prof. Dr. William R. Jeffery, project mentor.
- **Biološki inštitut ZRC SAZU/ Evolutionary Zoology Laboratory of the Institute of Biology at the Scientific Research Centre of the Slovenian Academy of Sciences and Arts**, Ljubljana, Slovenia, http://ezlab.zrc-sazu.si; Dr. Matjaž Kuntner, eDNA analysis supervisor.
- **Biospeleološko društvo Crne Gore/ Biospeleological Society of Montenegro**, Podgorica, Montenegro; Miloš Pavićević, eDNA and speleological survey in Montenegro.
- **Lokalna iniciativa Vitina/ Vitina Local Comunity Initiative**; Zlatko Grizelj, water sampling developer, Ivan Bebek, educational board coordinator.
- **Hercegovačka gorska služba spašavanja Mostar/ Herzegovinian Mountain Rescue Service Mostar**, Bosnia and Herzegovina, http://www.hgss.ba/; Ivan Skaramuca, speleological survey in the Trebižat River Tributary.
- **Centar za krš i speleologiju/ Center for Karst and Speleology**, Sarajevo, Bosnia and Herzegovina, http://www.centarzakrs.ba; Dr. Jasminko Mulaomerović, speleological survey in Bosnia and Herzegovina.

- **Speleološko društvo Herceg Mostar/ Speleological Society Herceg Mostar**, Bosnia and Herzegovina, https://hr-hr.facebook.com/SpeleoloskoDrustvoHercegMostar; Žana Marijanović, speleological survey in the Trebižat River Tributary.
- **Republički zavod za zaštitu kulturno-istorijskog i prirodnog nasljeđa/ Republic Institute for Protection of Cultural, Historical and Natural Heritage**, Banja Luka, Republika Srpska Entity, Bosnia and Herzegovina, http://nasljedje.org/; Goran Panić, Bosnia and Herzegovina nature conservation adviser.

Conservation Impacts

Please explain/describe how your project has contributed to the implementation of the CEPF ecosystem profile.

This project addressed the CEPF Strategic Direction 3 "I*mprove the conservation and protection status of 44 priority key biodiversity areas in the Mediterranean hotspot*" and has contributed to
"*- 3.1 Establishing new protected areas and promoting improved management of existing protected areas by developing and implementing sustainable management plans* and by
- 3.3 Raising awareness of the importance of priority key biodiversity areas, including those that have irreplaceable plant and marine biodiversity"

in the following ways:

1. **Survey of the distribution of a globally threatened cave amphibian *Proteus anguinus* in three CEPF priority key biodiversity areas and adjacent regions**

One of the world's prime symbols of natural heritage and its history of research, known under the vernacular name "human fish" or the olm, *Proteus anguinus* (hereafter *Proteus*), is a blind amphibian strictly endemic to the subterranean waters of Dinaric karst. It is the only European cave vertebrate, and the largest cave animal in the world.

Fig. 1. The olm, *Proteus anguinus*, a blind amphibian endemic to the subterranean waters of Dinaric karst. Photo: G. Aljančič.

Proteus is a globally threatened species. Although the IUCN Red List of Threatened Species defines it as vulnerable, and it is a priority species under both the Bern Convention (Appendix II) and the EU Habitats Directive (Annex II* and IV), no monitoring scheme for *Proteus* exists. Due to its high specialization to a narrow range of abiotic conditions in the subterranean environment, *Proteus* (and other groundwater organisms) is extremely vulnerable to direct and indirect alterations of its habitat. Recent negative human influences come from intensive agriculture, energy production, and unregulated urbanization. In Bosnia and Herzegovina these are reaching catastrophic proportions, but their impact on *Proteus* cannot be determined without first establishing its exact distribution.

The innovative environmental DNA survey developed during the project and conducted in three CEPF priority key biodiversity areas – the Trebižat River Tributary, Hutovo Blato (Eastern Adriatic corridor) and the Skadar Lake (Southwest Balkans corridor Montenegro) with surrounding areas – has

successfully circumvented the critical problem when determining *Proteus* occurrence – inaccessibility of its subterranean habitat.

With new localities of *Proteus* discovered in the present survey, the project doubled the previously known number in the Trebižat River and Hutovo Blato CEPF priority key biodiversity area. *Proteus* was not confirmed to be present in the Skadar Lake area; however, our results show that it is likely present in the Grahovo polje area (Šanik spring). This discovery represents the first indication of *Proteus* presence in Montenegro.
This project has complemented the activities of the governmental sector in BiH, and advocated for protection of *Proteus*: proposal for monitoring methodology and scientific argumentation for the urgent enforcement of full legal protection of *Proteus* in Bosnia and Herzegovina, implemented through a partnership with the nature conservation agency of Republika Srpska Entity. As a result of this project, accurate information on the distribution of *Proteus* and vulnerability of groundwater in the studied CEPF priority key biodiversity areas are available to guide management planning of the Emerald or future Natura 2000 network.

The first physical evidence of *Proteus* in Montenegro discovered during this project (plausible traces of *Proteus* environmental DNA detected in groundwater) indicates that this vulnerable amphibian is very likely present in Montenegro. This discovery is not only scientifically important, but also raises considerable international attention. Further investigations are urgently needed to additionally resolve the question of *Proteus* distribution in Montenegro.

However, with additional evidence of *Proteus* in Montenegro, this country will also confront a challenge to preserve it in the future, an act 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 possible future nature conservation legislation obligations of Montenegro as a consequence of this discovery. The Society for Cave Biology and partners will continue to appeal and counsel the nature conservation authorities in Montenegro to start all necessary legal and practical actions to protect *Proteus* in their territories, as well as to financially support further surveying of *Proteus* distribution in Montenegro.

2. **Long-term protection of groundwater and karst ecosystem through education and public promotion**

Karst areas are among the World's landscapes most vulnerable to environmental impacts. Karst groundwater is not only extremely vulnerable to contamination, but very difficult to remediate once contaminated. Rapidly growing anthropogenic pressure on the unique karst ecosystem of the Trebižat River and Hutovo Blato was encountered during the field-work of this project (see Project Components, section 2), particularly on the groundwater of the Trebižat River Tributary area. Considering the largely unexplored karst underground of both areas, particularly numerous unchecked springs as plausible localities of *Proteus*, the conservation of Trebižat in particular should promptly reach a better level of nature protection than presently enforced in Hutovo Blato. Through a shared dependence on groundwater, the vulnerability of subterranean biodiversity directly predicts the vulnerability of the human population, imposed on ourselves and our descendants by our own irresponsible acts.

However, a long-term protection management may not be reached if imposed only by the law, its importance must be recognized and accepted also by the public. The project addressed this fundamental issue through education on groundwater biodiversity and karst ecosystem services, focused on regional school youth and university students, local communities and NGOs, as well as foreign tourists to the Trebižat River area. In addition to direct awareness raising activities (educational board and brochure), any opportunity was used to clearly explain the value of *Proteus* and groundwater integrity to all persons encountered during the field-work and nature conservation meetings.

Since many of the fast growing socio-economic problems arise from overuse of natural resources, the general public was informed on the importance of sustainable use of groundwater – the only reliable source of drinking water in karst regions of the Mediterranean Basin. In the Eastern Adriatic and Southwest Balkans corridor, *Proteus* is therefore a critical indicator species of groundwater purity, and as such its study is of paramount importance to public health and everyday life in the Balkans.

3. ***Proteus*** **and groundwater trans-boundary alliance**

The project followed the CEPF Strategic Direction 2 *("2.2 Support IRBM policy and legislation development and implementation through capacity building and advocacy at all appropriate levels")*, and has contributed to management planning and wiser use of groundwater resources in vulnerable karst areas through:

- new arguments and successful linking: shared dependence of cave fauna and migrating birds on ecohydrological integrity of karst polje (see Project Components, section 5.2.1);
- Southwest Balkan transboundary alliance and NGO capacity building: training of local NGOs and conservationists on biology of *Proteus* and conservation challenges, transfer of know-how and good practice from Slovenia.

4. This project was implemented **100 years after the first regulation on the protection of karst underground in Bosnia and Herzegovina** (1914), one of the first in the world of its kind. This high anniversary was our best motivation to contribute further towards an efficient legal protection of the subterranean biodiversity and groundwater in the Southwest Balkans.

Please summarize the overall results/impact of your project.

1. Survey of *Proteus* distribution

- Innovative non-invasive DNA-based method to detect of traces of *Proteus* environmental DNA in any spring or cave water examined regardless of whether or not the animal has ever been seen there: method developed, tested and ready to use;
- Survey at 59 sites (springs, caves and wells), of which 31 in the Eastern Adriatic corridor (Hutovo Blato and Trebižat River Tributary, Bosnia and Herzegovina), 1 in Southwest Balkans corridor (Skadar Lake, Montenegro) and 21 in adjacent areas (Bosnia and Herzegovina, Montenegro) and 6 in Slovenia;
- The present survey added 9 verified and 9 plausible/suspicious new localities of *Proteus* to the 6 previously known (according to the last published inventory for BiH; Kotrošan, 2002) in the Trebižat River and Hutovo Blato priority key biodiversity area.
- Testing for the presence of *Proteus* in 14 selected sites outside its confirmed range, in Montenegro: a plausible trace of *Proteus* eDNA recorded in two localities represents the first physical evidence indicating the presence of this species in Montenegro;
- GIS database and distribution map of *Proteus* presence documented within the Eastern Adriatic corridor (Hutovo Blato, Trebižat River Tributary, Bosnia and Herzegovina), Southwest Balkans corridor (Skadar Lake, Montenegro) and adjacent areas;
- The data gathered in this survey forms the basis of the urgently needed monitoring scheme for *Proteus* populations in Bosnia and Herzegovina, and Montenegro.
- An updated survey of karst springs and caves on important karst areas of BiH and Montenegro.

2. Public promotion and academic outreach

- Published and distributed educational leaflets (local language and English editions; 3,000 copies) promoting the subterranean biodiversity of Trebižat River Tributary and a wiser water management in the karst landscape.
- Educational board unveiled in Vitina, BiH (additional product of the project) to present biodiversity of Trebižat River and to explain the fragile link between *Proteus,* sustainable use of groundwater and local community; the board is bilingual (local language and English).
- Conducted press conferences in BiH (3), local and national media coverage in BiH and Montenegro (7), short report in CEPF MED RIT Radar (1).
- Other outreach activities: popular science lecture on threats to groundwater and protection of *Proteus* (1), Educational board opening event (1).
- Academic dissemination: scientific and conservation meetings (3 international and 4 regional); published 1 paper and 3 abstracts, 3 lectures and 2 poster presentations.

3. Trans-Balkan *Proteus* conservation alliance

- Trans-boundary *Proteus* conservation alliance established (over 20 NGOs, local communities and GOs).

- NGO capacity building: training in the survey and protection of *Proteus* and groundwater biodiversity, involving our partners and local community in field work, laboratory work process and outreach activities.
- Visit of the Biospeleological Society of Montenegro team in the Tular Cave Laboratory (Slovenia) Conservation authorities in BiH advised to enforce legal protection of *Proteus* and start the urgently needed monitoring.

Please provide the following information where relevant:

Hectares Protected: *I*

Species Conserved: *Proteus anguinus*

Corridors Created: *I*

Describe the success or challenges of the project toward achieving its short-term and long-term impact objectives.

Success
- eDNA method – a novel tool for conservation of subterranean biodiversity – tested and ready to use.
- Previously known number of *Proteus* localities in the Trebižat River and Hutovo Blato priority key biodiversity area doubled.
- First evidence of the globally threatened cave amphibian species in Montenegro: traces of *Proteus* environmental DNA discovered in two karst springs.
- Networking, constant search for contacts and valuable collaboration was the key to success during our field work, as well as in outreach activities and dissemination.

Challenges
- Development of sampling and filtering methodology during field work, in improvised laboratories, and transportation of delicate filters back to Slovenia for eDNA analyses. With the growing network, excellent facilities of a specialized laboratory in Ljubuški, BiH, were introduced by Zlatko Grizelj (Vitina Local Community Initiative), resulting in an improved filtering protocol – and better results.
- Lack of accurate and homogeneous topographical, hydrogeological and geological data (both in BiH and Montenegro) obstructed the field-work and building the distribution maps. In order to address this challenge within the GIS analyses and statistics, we used mainly the European environmental and terrain open data, since most of such data are not publicly available in BiH and Montenegro.

Were there any unexpected impacts (positive or negative)?

The effort put in communication and networking during field-trips and workshops in Bosnia and Herzegovina received high attention of local communities and regional nature conservation NGO sector. This unexpectedly strong support – an indication for a successful long-term protection of *Proteus* and groundwater – stimulated the implementation of an unplanned activity of the project: arrangement of the educational board in Vitina (Trebižat River, BiH).

Project Components

Project Components: *Please report on results by project component. Reporting should reference specific products/deliverables from the approved project design and other relevant information.*

Component 1: **Environmental DNA (eDNA) method development in lab conditions and its optimisation in natural conditions**
Product 1: **Method to detect traces of *Proteus* eDNA in groundwater, ready to use**

Development of *Proteus* environmental DNA method was started already in 2012 by Špela Gorički, in co-operation with Gregor Aljančič, Magdalena Năpăruş-Aljančič, Matjaž Kuntner and William Jeffery, at the Evolutionary Zoology Lab of ZRC SAZU (Ljubljana, Slovenia), employing the least costly, qualitative PCR technique (Figs. 2 & 3). Promising results were obtained on samples from controlled laboratory conditions at the Tular Cave Laboratory with relatively high concentrations of *Proteus* eDNA in pools of known volumes. However, this approach soon proved too unreliable for detection of extremely low concentrations of eDNA in nature.

Fig. 2. Collecting eDNA from spring water by filtering. Photo: Š. Gorički.

Fig. 3. Target DNA fragments separated and visualized by gel electrophoresis. Photo: G. Aljančič.

In the beginning of August 2013, we switched to a more advanced, quantitative or real-time PCR (qPCR) technique, which is approximately 10 times more sensitive (i.e., able to detect *Proteus* eDNA in 10 times more diluted samples). The qPCR approach was developed by David Stanković, in co-operation with Špela Gorički, Gregor Aljančič, Miloš Pavićević, Magdalena Năpăruş-Aljančič and William R. Jeffery, under supervision of Aleš Snoj at the Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Slovenia (Fig. 6–10).

As a result of this switch, we finally managed to isolate first traces of *Proteus* eDNA from nature, when re-examining the sample from a known *Proteus* locality in Slovenia (karst spring of Mahniči), which was negative when using the initial technique (Fig. 2&3). With this first confirmation from a known locality in nature, we have successfully passed the most important milestone of our project. With further development of qPCR technique approach, the karst spring of Vir (the classical locality of *Proteus*) and Kompoljska Jama (both Slovenia) were first localities confirmed for presence of *Proteus* eDNA (Fig. 4 & 5), while the eDNA analysis of Mahniči Spring (the third known locality of *Proteus* examined in Slovenia) showed a plausible trace of *Proteus* eDNA.

Fig. 4 & 5. Collecting eDNA water from the spring of Vir (left) and Kompoljska Jama (right), both Slovenia.
Photos: M. Năpăruş-Aljančič

In October 2013, we started calibrating the method (establishing its standards) - to assess the minimal detectable eDNA concentration, and to estimate the rate of eDNA degradation in water samples. This analysis was conducted at the Tular Cave Laboratory, the Evolutionary Zoology Laboratory of the Institute of Biology at the Slovenian Academy of Sciences and Arts (ZRC SAZU), and at the Department of Animal Science at Biotechnical Faculty, University of Ljubljana, Slovenia (Špela Gorički, David Stanković, Gregor Aljančič, Aleš Snoj, William R. Jeffery and Matjaž Kuntner).

INNOVATIVE eDNA METHODOLOGY

During the course of our project we developed the following methodology for detection of *Proteus* in natural environment by environmental DNA sampling. 10-20 L of water were collected in brand-new plastic containers during the visit of each sampling site (some sites were visited multiple times). Water samples were filtered through sterile 0.45 µm PCTE membrane filters (Sterlitech & Whatman) mounted on Nalgene polysulfone reusable bottle top filter holders or through Nalgene MF75 series disposable bottle top filters with integral 0.45 µm SFCA membrane (Thermo Scientific). Between 0.5 and 10 L of water were filtered per each sample taken from the Tular aquaria and at least 10 L of water were filtered per each sample taken from the field sites. Up to 4 filter membranes were used to filter 10 L of water, depending on the amount of sediment and other particles in the water sample.

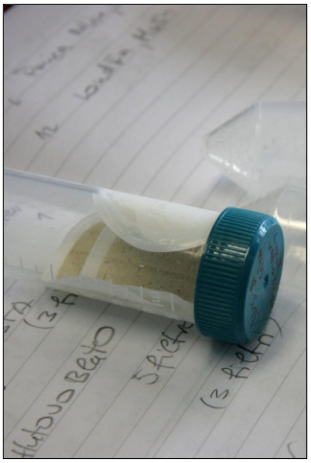

Fig. 6, 7 & 8. Extraction of eDNA with PowerWater DNA isolation kit (MO BIO Laboratories). Photos: G. Aljančič.

After filtration, the filters were rolled up and put into 5 ml tubes provided in PowerWater DNA isolation kit (MO BIO Laboratories) and stored at -20°C until DNA extraction. Up to two filters of the same sample were stored and processed together in a single tube to minimize DNA losses during extraction. When filtration was conducted on site or in local laboratories (Mostar, Ljubuški, Kranj and Podgorica) the filters were transported in cool-box to Ljubljana. DNA was extracted just prior to real-time PCR (qPCR) analysis, as extracted DNA is more prone to degradation. To prevent contamination, DNA extraction was conducted in a building where no post-PCR work or extraction of DNA from *Proteus* tissue samples is conducted. Rigorous controls for preventing and monitoring contamination were employed, including commercial decontamination solutions (DNA-ExitusPlus solution, AppliChem), chlorine-, autoclave- and UV-based sterilization of all re-usable materials, and workspace decontamination with DNA-ExitusPlus solution. Extraction was conducted using bead beating and the PowerWater DNA isolation kit in accordance with manufacturer's instructions, with the following minor adjustment to concentrate the eluted DNA: the final elution volume was 50 µl for all samples.

Four sets of primers unique to *Proteus* were designed that would result in amplicons ranging between 90 and 200 bp in size, based on the alignment of mitochondrial 16S rDNA sequences of 51 individuals from 32 localities and of mitochondrial control region of 172 individuals from 38 localities along the Dinaric karst (Gorički & Trontelj 2006 and unpublished data). Based on the results of preliminary PCR amplifications of DNA isolated from the tanks in the Tular Cave Laboratory, two primer pairs were selected for further analysis. Performance of both primer pairs was confirmed against tissue samples of two different lineages of *Proteus* (black *Proteus* from Bela Krajina and white *Proteus* from Postojna-Planina cave system, both Slovenia).

Fig. 9 & 10. Prepearing the mixture for qPCR (left), analysing the qPCR data (right). Photo: G. Aljančič.

To increase the sensitivity and specificity of *Proteus* detection we used real-time PCR approach using SYBR chemistry. The specificity of both primer pairs was tested against trout, newt and human DNA. In the majority of cases there was no DNA amplification; if non-specific amplicons were present (primer dimers, homologous sequences), however, they could easily be distinguished from amplified DNA of *Proteus* by the shape of the melting curve. Melting curve analysis is an assessment of dissociation characteristics of double-stranded DNA during heating. As the temperature is raised, the double helix begins to dissociate, which is detected by the drop of fluorescence of the SYBR Green intercalating dye. The temperature at which 50% of DNA is denatured is known as the melting point. As different nucleotides have different physical and chemical characteristics, different sequences reach the melting point at different temperatures.

Next, the efficiency of SYBR chemistry-based real-time PCR (qPCR) was tested under controlled conditions as follows. In *Proteus* husbandry environment of the Tular Cave Laboratory a single adult specimen was placed into a new water tank holding 100 L of fresh tap water (no *Proteus* eDNA present). 10 L of water were sampled in weekly intervals for five weeks, replacing the water removed with fresh tap water. The water sample was diluted into a twofold dilution series (1:0, 1:1, 1:2, 1:4 and 1:8) and 0.5 L of each dilution was filtered. Additional sampling and a dilution series (1:8, 1:16, 1:64, and 1:128) was done after eight weeks. eDNA attached to the filters was extracted as described above. The experiment was performed in two parallels. qPCR was performed using 6 µl of template DNA in a 20 µl reaction mix with SYBR® Green PCR Master Mix (Life Technologies). To avoid inhibition, 1:5 and 1:25 dilutions of the templates were used. Two independent qPCR replications were performed for each sample. DNA of *P. anguinus* was first detected after two weeks, while the detection limit after 8 weeks corresponded to the dilution of 1:64 (Fig. 11). This coincides with up to one animal per 256 m^3 of water, depending on the volumne of sampled and filtred water (in our survey we sampeled up to 20 L of water).

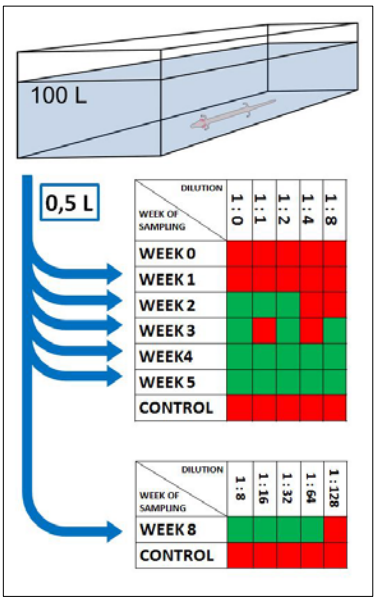

Fig. 11. Calibration of the eDNA method. Plot: D. Stanković.

For *Proteus* detection from natural sites we developed the following protocol. We used 15 µl reactions containing 7.5 µl of SYBR Green Real-time PCR master-mix, 2x 0.15 µl of 100 µM primers, 1.2 µl of sterile DNA grade water and 6 µl of diluted sample (4x, 16x). All samples were analysed with both primer pairs (on separate plates) and each combination of the diluted sample and the primer pair was run in three-plicate. One qPCR 384-well plate contained between 4 and 12 samples distributed over the plate, so each sample was always separated by at least one empty row. In addition, each plate included 6 negative controls and two positive controls (tissue DNA and eDNA extracted from Tular Cave Laboratory water tanks), for a comparison of melting curves. Samples were scored positive if at least two out of three parallels (wells) of at least one combination (dilution-primer pair) were positive by qPCR. If one of the three wells tested positive for *Proteus*, the sample was rerun using the same primer combination. Again, if at least two of the three parallels in the rerun were positive by qPCR, the sample was scored positive. On the other hand, if only one of the three wells tested positive, the sample was considered as plausible. However, if none of the wells tested positive in the rerun the sample was considered as suspicious. For a simplified presentation of this report (Fig. 20, 28 & 29), we joined the eDNA plausible and suspicious localities into a single category.

Component 2: **FIELD-WORK (COLLECTION OF WATER SAMPLES FROM SPRINGS AND CAVES) AND eDNA ANALYSIS**

Product 2: **Confirmation of the presence of *Proteus* and possible discovery of new localities within and outside its confirmed range**

1. **WATER SAMPLING IN BOSNIA AND HERZEGOVINA**
(Trebižat River, Hutovo Blato & adjacent areas of BiH)

Trebižat River *s.lat.* (including segments of the river known as Tihaljina, Mlade and Trebižat) is the right-hand tributary of the Neretva River, collecting waters from a complex karst system of western Herzegovina (BiH) and nearby Dalmatia (HR). Trebižat represents the last surface section of a multiple-sinking river, i.e. the final 51 km from the spring of Tihaljina to the confluence with the Neretva. Across the Neretva lies Hutovo Blato (Deransko-Svitavsko polje), a flooded karst polje at the altitude of only 2 m. Numerous karst springs all around the edge of Hutovo Blato receive a larger part of their waters from Popovo Polje.

Fig. 12. Trebižat River at Ljubuško-Vitinsko Polje. Photo: I. Bebek.

The distribution of *Proteus* in the Trebižat and Hutovo Blato areas is both grossly underexplored and presumably underestimated. The residents of Hutovo Blato knew *Proteus* already in the Middle Ages, as indicated by the oldest representation of *Proteus* in BiH, observed on a tombstone from the necropolis Boljuni by Stolac. However, the first scientific record of *Proteus* in BiH dates to 1895, from the Topoljak spring in the Trebižat River Tributary. In the most recent list of *Proteus* localities in BiH by Dražen Kotrošan (published in 2002), only 7 are known in the project area. Many reports on washed-out *Proteus* after floods are found in local media, often not reaching the experts. One of such finds happened in the village of Vitina in 2006, when during works on the water supply system, a previously unknown,

exceptionally large colony of *Proteus* was discovered. Thirty animals were counted, 12 of which were rescued to the nearby spring of Vrioštica by the conscious residents.

Fig. 13. Thirty *Proteus* found during works in Vitina, 2006. Photo: I. Bebek.

Fig.14. Local news on *Proteus* finds were investigated to record new localities. Source: Hercegovina info.

Serious and rapidly increasing negative anthropogenic pressure on the unique karst ecosystem of the Trebižat River and Hutovo Blato was encountered during the field-work. The major existing and potential stressors include:

- Trebižat River: plans to construct five small hydroelectric power plants on the Trebižat (MHE Modro oko, Klokun, Koćuša, Kravica and Stublica);
- Hutovo Blato: after construction of the reversible hydroelectric power plant Čapljina, karst springs on the edge of Hutovo Blato lost most of its groundwater, resulting in a possible *Proteus* habitat lost, as well as in a negative socio-economic context (limited water supply for residents);
- undeveloped infrastructure (sewage system, wastewater treatment, public landfills and illegal dumps) and extensive agriculture in the immediate hinterland of the spring; an example: touristic Medjugorje and large vineyards behind the springs of Studenčica (public water supply);
- fish farming: organic pollution, introduction of alien fish species and diseases affecting endemic fish in Trebižat River Tributary (Grab, Trebižat);
- land reclamation, excessive water pumping directly from springs along the Trebižat River.

Considering the rapidly increasing pressure on the unique karst ecosystem of the Trebižat River Tributary CEPF priority key biodiversity area, threatening one of the most vital populations of *Proteus* (and other endemic aquatic fauna), the conservation management should promptly reach a better level of nature protection than presently enforced in Hutovo Blato.

Fig. 15 & 16. Forest above Studenčica springs transformed into high production vineyards (left), illegal dumps in caves (right). Photos: M. Năpăruş-Aljančič.

BiH field-work was organized in two rounds, 25 days spent in total:

- March 31 – April 8, 2014: Gregor Aljančič and Magdalena Năpăruş-Aljančič (Society for Cave biology), Miloš Pavićević (Biospeleological Society of Montenegro), Ivan Skaramuca and Zdenko Marić

(Herzegovinian Mountain Rescue Service Mostar), Ivan Bebek and Zlatko Grizelj (Vitina Local Community Initiative);
- April 27 – May 10, 2014: David Stanković (Society for Cave biology), Zlatko Grizelj and Ivan Bebek (Vitina Local Community Initiative), Ivan Skaramuca (Herzegovinian Mountain Rescue Service Mostar), Žana Marijanović (Speleological Society Herceg Mostar), Jasminko Mualomerović (Center for Karst and Speleology);
- June 19 – 20, 2014: Gregor Aljančič and Magdalena Năpăruş-Aljančič (Society for Cave biology), Ivan Bebek and Zlatko Grizelj (Vitina Local Community Initiative).



Fig. 17, 18 & 19. Water samples collected from springs of Bregava (left), Tihaljina (center) and from a well in Gornji Trebižat (right). Photos: G. Aljančič.

One of the important tasks during the first sampling round was to solve numerous technical problems of water sampling and filtering in field-work conditions. Each day after the water samples were collected (as described in Chapter 1 of this report), they were filtered in a make-shift laboratory, set up at the Herzegovinian Mountain Rescue Service facility in Mostar. By the end of this sampling round, Zlatko Grizelj, an expert on hydrology of the Trebižat River joined the project team, and offered excellent facilities at the Laboratory for Waters in the town of Ljubuški. This facility was used with benefit during the second round of sampling in BiH.

During the field-work in BiH, in total 38 localities were visited (karst springs, caves and wells), of which 29 were sampled, and additional 10 new reports on finds of *Proteus* were documented:
- Trebižat River Tributary: 17 sampled localities (+8 reports)
- Hutovo Blato: 6 sampled localities (+1 report)
- adjacent areas in BiH: 6 sampled localities (+1 report)

RESULTS OF BiH *PROTEUS* eDNA SURVEY

- The springs Londža (Čapljina), Nezdravica and Izvor Bregave showed weak signals (one positive signal in three replicates) on only one of the genes and were negative in the rerun; they are therefore categorised as suspicious for the presence of *Proteus* (possibly on the limit of detection, although we cannot completely exclude contamination despite all the precaution mechanisems put in place).
- Vakuf Spring and Private well in Gornji Trebižat showed a week signal (one positive signal in three replicates) on the CR gene only, and the signal (one positive signal in three replicates) was again observed in the rerun; this sample is categorized as plausible to contain traces of *Proteus* eDNA. Here contamination is less likely as the signal was observed in independent runs.
- Perića Vrelo and Kajtezovina were positive on one gene while the samples from Bunar Mehmedalije Dizdarevića and Česma Izpod Pogledovače were positive on both genes; these localities were interpreted as positive for presence of *Proteus* eDNA.

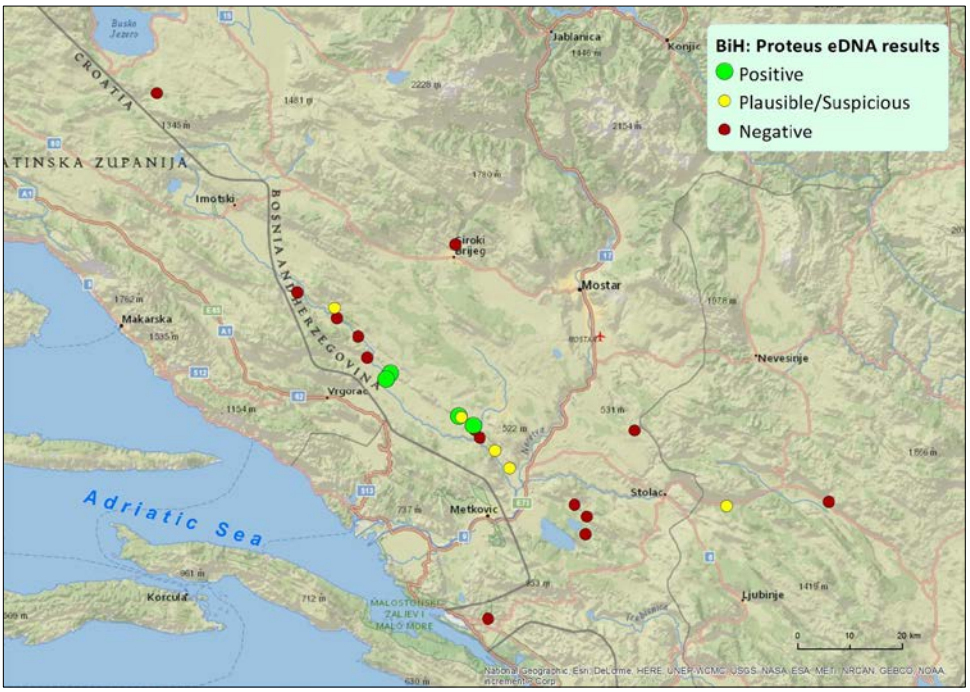

Fig. 20. Locations of *Proteus* eDNA water sampling in Bosnia and Herzegovina. Map: M. Năpăruş-Aljančič.

2. **WATER SAMPLING IN MONTENEGRO**

The sampling in Montenegro, as initially conceived, was to be limited to the cave Obodska Pećina (Rijeka Crnojevića). However, because *Proteus* has not yet been documented in Montenegro, we believed that it was important to extend the sampling towards the southeastern Herzegovina (Trebinje area) – the southernmost known localities of *Proteus*. Namely, a hypothetical continuation of *Proteus* distribution into Montenegro is strongly indicated by geology and the presence of specific Dinaric cave invertebrate taxa. With the approval of CEPF, we therefore expanded the Montenegro survey outside the Skadar Lake priority key biodiversity area, and tested 11 additional locations.

Montenegro field-work was organized in three rounds, 11 days spent in total:
- October 3–4, 2013: Gregor Aljančič, Luka Vodnik and Špela Gorički (Society for Cave biology), Miloš Pavićević (Biospeleological Society of Montenegro) and Slavko Polak (Notranjska Museum);
- November, 2013: Miloš Pavićević and Leonid Merzlyakov (Biospeleological Society of Montenegro);
- June, 2014: Miloš Pavićević, Leonid Merzlyakov and Vladimir Novović (Biospeleological Society of Montenegro).

Water samples collected during the first and second field trips were transported to Slovenia and filtered in Ljubljana after a considerable travel delay. During the last field-work in June 2014, water samples were filtered daily by Miloš Pavićević in an improvised laboratory of the Biospeleological Society of Montenegro in Podgorica.

In total, 15 localities from a wide area of the Dinaric Karst in Montenegro were visited (karst springs and caves), of which 11 were analyzed:
1. Grahovo Polje with and territory and Banjan (6 samples)
2. Nikšić region (1 sample)
3. Skadar Lake region and Cetinje (1 sample)
4. Springs in Boka Kotorska Bay (3 samples).



Fig. 21, 22 & 23. Water samples collected from springs of Sopot (left), Grahovo 2 (center) and Vrela (right). Photo: G. Aljančič and M. Pavićević.

In the second round of sampling it was decided to explore in greater detail the springs in Grahovo Polje and the parts of Banjan whose groundwaters gravitate towards the hydropower accumulation of Bilećko Lake and the submerged valley of the Trebišnjica River.

This sampling zone (No. 1) was chosen due to its nearest location to the sites in Bosnia and Herzegovina, namely the Trebinje area and along the famous Popovo Polje, known for a long time to harbour *Proteus*. The north and north-western sides of Mount Orjen, Bijela Gora and the western side of Golija feed the above mentioned water flows via the Zaslapnica and Jazina rivers and other small permanent or periodical streams. From looking at the hydrogeological characteristics of this region, the presence of *Proteus* is very likely. During the preparation of field-work, the hydrological systems of the selected areas were studied in detail with the assistance of hydrologist Mag. Neda Dević at the Geological Survey of Montenegro. Valuable information was also provided by Dr. Andrej Mihevc, Karst Research Institute ZRC SAZU, Slovenia. It was decided to split the area into two parts:

1. Grahovo polje and the springs along Zaslapnica River.
2. The western area of Banjan.

Fig. 24. Zaslapnica Valley. Photo: V. Novović.

Fig. 25. Interviewing residents. Photo: V. Novović.

The main problem during the field-work planning phase was the absence of a detailed hydrological map and outdated data. The only accessible map of the area was an old military map (1:25,000) created at the end of the 1970s, from which approximately ten sampling sites were selected. Numerous springs and other water sources shown on the map were either incorrectly identified, not located on site or were only temporarily active after heavy rains. The latter were dry during our field-work, so the sampling was impossible. Further potential sampling sites are diverted into pipelines for water supply and therefore inaccessible for sampling. In the Banjan area there were no active springs besides Sopot (Petrovići settlement), which is pipelined. The region has not been completely explored by cavers yet. So far no caves with a constant groundwater flow have been identified.

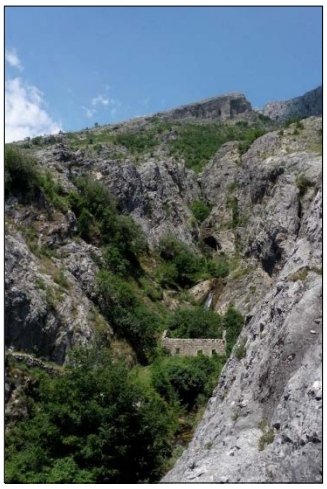

Fig. 26 & 27. Montenegro field-work at Zaslapnica (left & center) and Šanik springs (right). Photo: V. Novović and M. Pavićević.

RESULTS OF MONTENEGRO *PROTEUS* eDNA SURVEY

- The springs Sopot (Boka Kotorska) and Izvor Grahovo 1 (Grahovo Polje) showed weak signals on (one positive signal in three replicates) only one of the genes and were negative in the rerun; they are therefore interpreted as suspicious for the presence of *Proteus* (possibly on the limit of detection, although we cannot completely exclude contamination)
- Šanik spring showed a week signal on the 16S rDNA gene only, and the signal was observed again in the rerun; this sample is interpreted as plausible to contain traces of *Proteus* eDNA. Here contamination is less likely as the signal was observed in independent runs.

In spite of many speculations on the existence of *Proteus* in Montenegro, a plausible trace of *Proteus* eDNA recorded in two localities represents the first physical evidence indicating presence of this species in Montenegro. This discovery is not only scientifically important, but also raises considerable international attention. However, further investigations are urgent to additionally resolve the question of *Proteus* distribution in Montenegro.

Nevertheless, with *Proteus* on the IUCN Red list of endangered species (VU), in Bern Convention (Appendix II) and being a priority species of EU Habitats Directive (Annex II* and IV), Montenegro will also confront a challenge to preserve it in the future as a 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 future nature conservation legislation obligations of Montenegro as a consequence of this discovery.

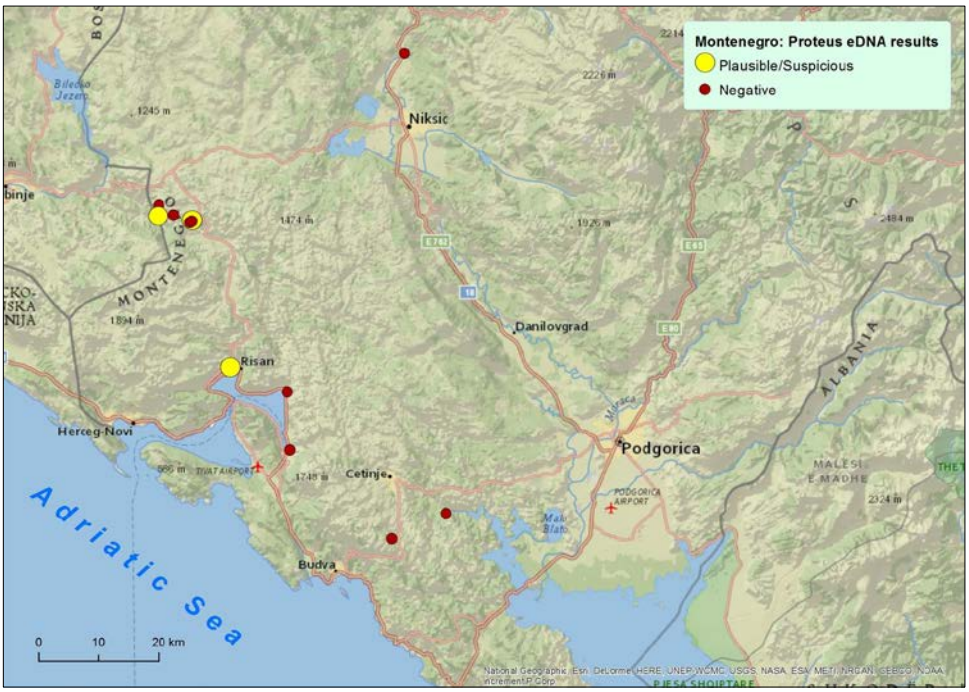

Fig. 28. Locations of *Proteus* eDNA water sampling in Montenegro. Map: M. Năpăruş-Aljančič.

When interpreting the results of the Montenegro survey, we should also take into consideration a possibility that the hypothetical Montenegro population has gradually become isolated and genetically distant to all other known populations to the extent that our specific PCR primers could not bind efficiently any more.

SUMMARY OF *PROTEUS* SURVEY IN BiH, MONTENEGRO AND SLOVENIA

1. **eDNA analyses**

Country	Visited Localities	Analyzed localities	eDNA positive	eDNA plausible/ suspicious	eDNA negative
BiH	38	25	4	5	17
MNE	15	11	0	3	9
SLO	6	4	3	1	0
TOTAL	**59**	**40**	**7**	**9**	**26**

2. **New localities of *Proteus***

	***NEW VERIFIED OR PLAUSIBLE LOCALITIES OF* PROTEUS**			***1. documented by eDNA survey***			***2. documented from past reports on finds****	
Country	visited localities	**verified new localities**	**plausible new localities**	analyzed localities	**eDNA verified**	**eDNA plausible/ suspicious**	**reports verified**	**reports plausible**
BiH	38	9	8	25	4	5	6	4
MNE	15	0	3	11	0	3	0	0
SLO	6	0	0	4	3	1	0	0
TOTAL	**61**	**9**	**11**	**40**	**7**	**9**	**6**	**4**

*** New *Proteus* localities documented from past reports on finds:**
- verified: *Proteus* photographed or recently seen by an expert;
- plausible: past reports by residents, with no serious reason to doubt.

The list of localities is available at the Society for Cave Biology upon request.

Component 3: **GEOGRAPHIC INFORMATION SYSTEM (GIS) ANALYSIS**
Product 3: **GIS database and maps of *Proteus* distribution in key areas**

The GIS analysis was carried out in four phases, and was contributing to the project field work planning, data visualization and results assessment. The database and the maps were produced progressively in ArcGIS 10.1 software by Magdalena Năpăruş-Aljančič.

3.1 Inventory of existing data on distribution of *Proteus* and hydrogeology

The main guideline in choosing the eDNA sampling sites in BiH and Montenegro was to include springs, wells or caves with a high potential of hosting *Proteus,* but not yet scientifically documented. We collected GIS data from our partners from BiH and Montenegro. The data collected, especially those on the hidrogeological systems, as well as the topographical and geological data and maps, helped us build a GIS database and prepare a coherent plan for our field-work and sampling (see also p.16 for problems with lack of accurate and homogeneous topographical, hydrogeological and geological data). We constantly updated the database with new information on water regimes of the springs as well as new springs and localities documented in this survey.

3.2 Build GIS database to guide fieldwork investigation

The locality data contain the basic information including the name of the locality, WGS coordinates expressed in decimal degrees, type of locality (cave, spring, well), elevation, annual mean temperature, annual mean precipitation, land cover data, water temperature, status of locality (sampled, not sampled), eDNA results (**++** positive on both genes tested; **+** positive on one gene; **?** plausible on one gene; **(-)** suspicious on one gene; **-** negatives results), *Proteus* status (**V**: verified (*Proteus* recently seen by a reliable person or photographed); **P**: plausible (local reports or near verified localities); **U:** unknown).

We used the following GIS schema:

Feature classes (FC):	**Geometry type**	**Attribute name**	**Value type**	**Predefined value**
A. Proteus	Point	Species	string	
		LocalityName	string	-
		Country	string	-
		LocalityType	string	cave
				spring
				well
		LatitudeWGS	double	-
		LongitudeWGS	double	-
		LocalityStatus	String	Sampled
				Not sampled
		ProteusStatus	String	**V**: verified
				P:plausible
				U: unknown
		eDNAresults	Integer	**4**= positive
				3= plausible
				2= suspicious
				1= negative
				0= not analzyed
		LC2009	integer	-
		TMA	float	-
		WaterTemp	float	-
		PMA	integer	-
		eDNA	string	positive
				negative
		ALT	float	

B. Land Cover (2009)	Polygon	LC2009	integer	
C. Annual mean temperature (°C)	Polygon/raster	TMA	float	
D. Annual mean precipitation (mm)	Polygon/raster	PMA	integer	
E. Geology	Polygon/raster	GEOL	integer	
F. Hydrogeology	Polygon/raster	HDG	integer	
G. Elevation (m)	raster	ALT	float	

3.3 Enter results of eDNA analysis into GIS database and build distribution map of *Proteus*

We built an overview map of the localities visited and of the localities sampled in the three countries - Bosnia and Herzegovina, Montenegro and Slovenia, indicating also the eDNA results (Fig. 29). Separate maps with results of the eDNA analyses for BiH and Montenegro were also built (see Figs. 20 and 28).

3.4 Assess geographical range of *Proteus* through spatial statistical modelling

Using several surface ecological parameters including elevation, annual mean temperature, annual mean precipitation and land cover data, we assessed the patterns and relationships beneath the eDNA results within BiH and Montenegro areas.

We built the scatterplot matrix of surface ecological parameter correlations with respect to eDNA positive and plausible/suspicious results (Fig 30). For a better understanding and data visualization of results, we matched the analyzed data with the same colours we used to illustrate the results of the eDNA analyses (green=positive, yellow=plausible/suspicious). The resulted scatterplots and histograms do not show any specific pattern in data distribution related with ecological surface parameters, most probably due to the small sample dataset and a large geographical extent. A larger dataset is needed for a significant statistical analysis. We believe that past and present subterranean hydrological network would have a significant predictory value. However, due to the absence of detailed hydrogeological data for all areas of interest, we could not perform such an analysis.

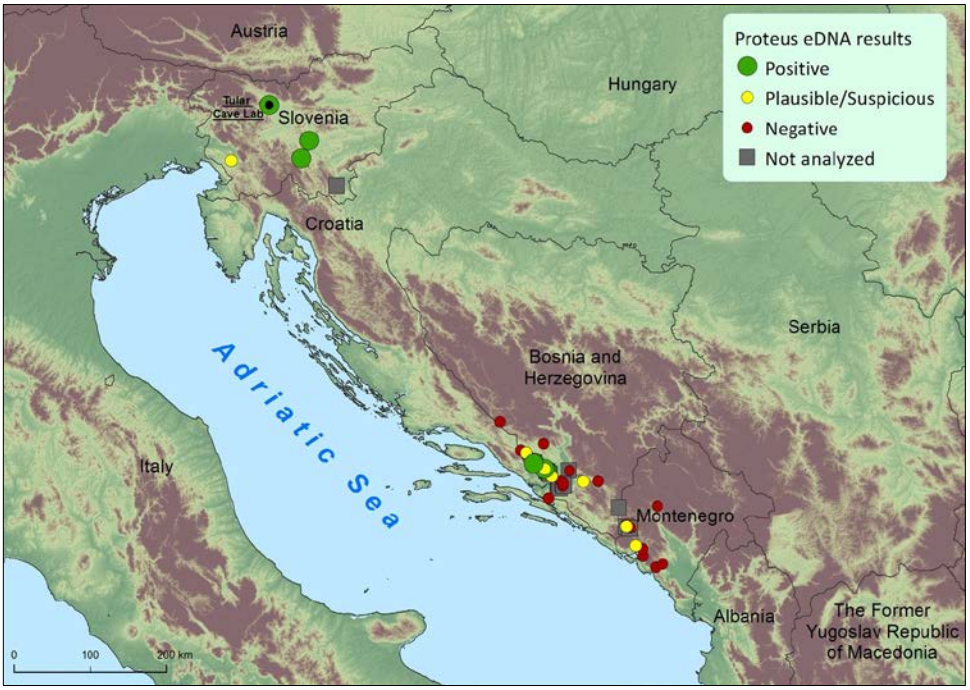

Fig. 29. Map of the *Proteus* eDNA results for the three areas of sampling: Slovenia, BiH and Montenegro. Map: Magdalena Năpăruş-Aljančič

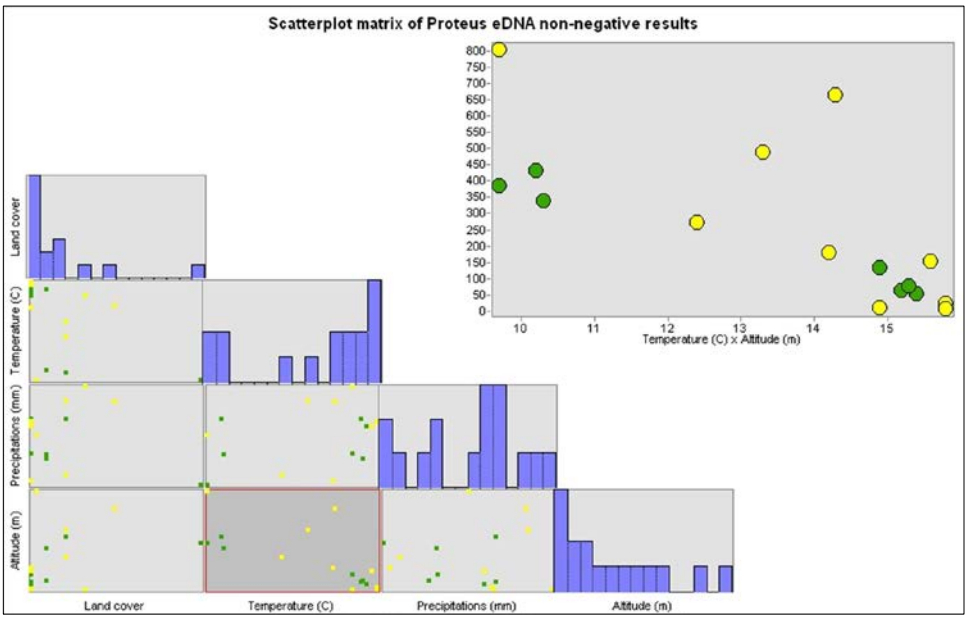

Fig. 30. Scatterplot matrix do not to show any specific correlation among environmental factors related with the localities data. Plot: Magdalena Năpăruş-Aljančič

Component 4: **GRANT REPORTING**

Product 4: **Financial documents and Project reports to CEPF and SCB**

- Members of the project team conduct monthly meeting on the Project's progress. Gregor Aljančič, project manager, submitted summaries of expenses to SCB Executive committee on a monthly basis.
- Regular reports at milestones to CEPF Regional Implementation Team.
- The Mid-term Progress and Financial Reports were submitted on December 31, 2013. The results were collated and submitted in this Final Project Report for CEPF.
- The external audit report was prepared by audit firm Grgorinič d.o.o., Kranj,Slovenia.

- **CEPF Supervision Mission to the Balkans sub-region** (Hutovo Blato, BiH; April 1, 2014)

The meeting was attended by Gregor Aljančič, Magdalena Năpăruş-Aljančič (Society for Cave biology) and Miloš Pavićević (Biospeleological Society of Montenegro). Gregor Aljančič presented the project's rationale, approach, activities challenges and results.

Aljančič, G., Gorički, Š., Năpăruș, M., Stanković, D., Pavićević, M., Kuntner, M. and Jeffery, W. R. (2014). A survey of the distribution of *Proteus anguinus* by environmental DNA sampling, an ongoing CEPF project.

Fig. 31 & 32. CEPF Supervision Mission to Hutovo Blato. Photo: M. Năpăruș-Aljančič.

Component 5: PUBLIC PROMOTION AND ACADEMIC OUTREACH

Product 5.1: **Increased awareness on vulnerability of *Proteus* and its subterranean habitat, shared dependence on groundwater and opportunity for sustainable development.**

Product 5.2: **Introduction of a novel forensic method in subterranean conservation biology.**

Product 5.3: **Promotion of joint Western Balkans *Proteus* conservation initiative and networking.**

5.1.1 **Conduct press conference and media coverage**
During field-work and outreach assignments in Bosnia and Herzegovina, we organized 3 presentations for local and regional media:
- Čapljina, October 3, 2013
- Hutovo Blato, April 1, 2014
- Vitina, August 20, 2014

Fig. 33 & 34. Interviews on conservation *Proteus* and wise use of karst groundwater in BiH. Photo: M. Năpăruș-Aljančič and Radio Ljubuški.

Media coverage on the project

1. Perić, I. (2014). Speleologija u Crnoj Gori: Tek predstoje pionirski koraci [Speleology in Montenegro: pioneer steps are still to be done]. Pobjeda, Podgorica, Montenegro; September 8, 2014. http://www.pobjeda.me/2014/09/08/speleologija-u-crnoj-gori-tek-predstoje-pionirski-koraci/#.VFTkZBZrv9y
2. Medić, B. (2014). 10 novih nalazišta čovječje ribice [Ten new localities of *Proteus*]. Radio Ljubuški, interview; aired on August 20, 2014. http://www.radioLjubuski.ba/rlj-audio/8610-Ljubuski-10-novih-nalazista-covjecje-ribice-audio-i-foto.html#.VDzveBap7Dv
3. Musa, D. (2014). Otvaranje informativnog panoa o čovječjoj ribici, Trebižatu i pitkoj vodi [Unveiling of the information board on *Proteus*, Trebižat River and drinking water]. Radio Čapljina, interview; aired on August 20, 2014.
4. Musa, D. (2014). Otkriven Edukativni pano o životu u podzemlju krša [Unveiling of an Information board on life in subterranean karst]. Večernji list/ Bosnia and Herzegovina edition, Zagreb, Croatia, August 21, 2014.
5. Musa, D. (2014). O napretku CEPF projekta u BiH [On the progress of CEPF projects in BiH]. Radio Čapljina, interview; aired on April 1, 2014.
6. Musa, D. (2013). Slovenski istraživači na području Hercegovine tragaju za čovječjom ribicom [Slovene researchers survey for *Proteus* in Herzegovina]. Večernji list/ Bosnia and Herzegovina edition, Zagreb, Croatia, October 14, 2013.
7. Rubinić, B. (2013). Environmental DNA Breakthrough for Endangered Cave Amphibian. MED RIT Radar 4. http://us4.campaign-archive1.com/?u=17ff123d369db9f424d486492&id=30588f3716&e=6d5f081288
8. Musa, D. (2013). Ugožena čovječja ribica u Hercegovini [Threats on Olm in Herzegovina]. Radio Čapljina, BiH, interview, aired on October 3, 2013.

5.1.2 **Conduct meetings with local administration (present goals & results; promote Western Balkans *Proteus* conservation initiative and contribute to sustainable livelihood practices)**

Proteus is recognized as a species in need of strict protection by both the Bern Convention (Annex II) and by the EU Habitats Directive (Annexes II and IV), while it is also protected by the national legislations. While BiH obtained the first regulation on the protection of caves and their fauna already in 1914, the protection on *Proteus* in BiH today is not fully implemented. In such an unfavourable situation, a partner from the governmental sector – the Republic Institute for the Protection of Cultural, Historical and Natural Heritage (Banja Luka), was particularly important.

Through 3 meetings (October 1 and 5, 2013; June 21, 2014) and numerous correspondences, we promoted arguments for a legal and practical protection of *Proteus* in BiH, while the partner informed us about their organisation, limitations and latest activities to improve *Proteus* conservation legislation and practice. Since the jurisdiction of the institute is limited to the Republika Srpska entity of BiH, they could not directly participate in project activities on Trebižat and Hutovo Blato. However, the conservation of *Proteus* will benefit from results of the project beyond these areas, because the growing pressure on karst and groundwater, particularly from the energy industry, does not stop at political borders.

5.1.3 **Perform popular science lectures on threats to groundwater and protection of *Proteus,* nature heritage as a unique drive of green tourism and sustainable use of groundwater resources**
Gregor Aljančič gave a lecture on vulnerability of *Proteus*, sustainable use of groundwater and CEPF project, to students at Gymnasium Kranj, Slovenia (April 10, 2014). The meeting was concluded with a practical presentation of the eDNA method, where students learned how to detect *Proteus* eDNA in water samples.

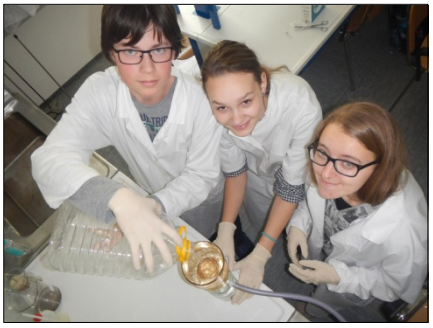

Fig. 35 & 36. Use of innovative forensic methods to protect *Proteus* attracts students of Gymnasium Kranj towards a research or nature conservation career. Photo: M. Năpăruș-Aljančič.

5.1.4 **Publication and distribution of educative leaflets "Svi ovisimo od podzemnih voda" and "Shared dependence on groundwater"**
The educative leaflets were designed to summarize the information of the Information board (see below), co-edited by our partner Miloš Pavićević from the Biospeleological Society of Montenegro. We published 1,500 copies in the local language and 1,500 copies in English.
First, leaflets were distributed in Vitina, at the launching event of the Information board (August 20, 2014). Half of the leaflets were distributed by all project members and partners in Slovenia, BiH and Montenegro, the other half will be given to visiting school groups (Local edition), and distributed at local info points (EN edition).

5.1.5 Additional product: **Information Board** "**Svi ovisimo od podzemnih voda – Shared dependence on groundwater**" (Vitina, BiH, August 20, 2014)
Besides brochures on conservation of groundwater and *Proteus*, an additional product, a bilingual information board was prepared as a more durable medium to promote the goals of the project. In this way, we intend to prolong the impact of the project through reinforcement of a most needed karst conservation awareness in local communities, particularly among school youth, by explaining the biodiversity and vulnerability of their home karst landscape. Furthermore, accurate information must be available to a growing number of visitors to these key biodiversity areas.

Fig. 37. Information board "Svi ovisimo od podzemnih voda – Shared dependence on groundwater", Vitina, BiH.

As a result of this project, the information board "**Svi ovisimo od podzemnih voda – Shared dependence on groundwater**" was unveiled in Vitina near Ljubuški, BiH, on August 20, 2014. Written at a solid popular-science level, the board explains the vulnerability of the Trebižat River karst ecosystem and its endemic biodiversity, with a special focus on *Proteus* and the shared dependence on groundwater. The metal construction was prepared by volunteers of the Vitina local community (size 2000 x 1000 mm), the board was designed in the Studio Solvej d.o.o., Mostar, printed by InterActive d.o.o., Široki Brijeg, and is expected to be renewed in 5 years.

Fig. 38 & 39. Unveiling the Information board "Svi ovisimo od podzemnih voda – Shared dependence on groundwater", Vitina, BiH. Photos: M. Pavićević and Z. Grizelj.

The board was edited by Gregor Aljančič. Texts, photos and other material for the board were contributed from 18 NGOs and GOs (Society for Cave Biology, Biospeleological Society of Montenegro, Slovenian Academy of Sciences and Arts, Center for Karst and Speleology, Herzegovinian Mountain Rescue Service, Speleological Society Herceg Mostar, Devon Karst Research Society, Caudata Hungarian Cave Research, Speleological Society Zelena Brda, Faculty of Civil Engineering and Architecture at University of Split, Republic Institute for Protection of Cultural, Historical and Natural Heritage, Croatian Herpetological Society HYLA, Herpetological Association of Bosnia and Herzegovina ATRA, Croatian Biospeleological Society, Ornithological Society Naše ptice, Ecological Society Ljepa Naša Čapljina, Society for Biological Research and Protection of Nature).

- Aljančič, G. et al. (2014). Svi ovisimo od podzemnih voda – Shared dependence on groundwater. Society for Cave Biology, Information board; Vitina, BiH.

5.2.1 **Presentation of the Project at scientific and nature conservation meetings**
The objectives and proceedings of the project were communicated to international and local nature conservation organisations and the global scientific community at the following meetings:

1. Workshop "Dinaric Karst Poljes as Wetlands of National and International Importance";
October 1–3, 2013, Livno, Bosnia and Herzegovina, organized by EuroNatur and Naše Ptice (http://www.euronatur.org/Karst-Workshop-Livno.1534.0.html).

The workshop discussed the importance of the karst polje ecosystem (i.e., seasonally flooded karst fields) in Herzegovina as one of the last large wetlands on the Adriatic Flyway. In our opinion, this was one of the most important meetings in the region in 2013. Not only do the topics of this workshop match the priorities of CEPF, but our lecture on the effect of seasonal floods on *Proteus* (http://www.euronatur.org/fileadmin/docs/docs_english/Karst-workshop_October2013/Gregor_Aljancic_Proteus_Livno.pdf) rounded the debate on the loss of biodiversity in karst poljes by numerous devastating human activities in Herzegovina (e.g., extensive hydrotechnical works in karst poljes, dams, tunnels, construction of drainage and irrigation systems, urbanization, groundwater over-pumping, etc.). These temporary lakes are huge karst systems, divided into a hypo- and epigean part only through our misinterpretation - migrating birds and narrow endemic cave fauna share the same dependence on the hydro(geo)logical integrity of karst polje. The workshop was concluded with the opening of a permanent exhibition in Tomislavgrad (Karst poljes of Bosnia and Herzegovina – the Wetlands of National and International Importance), and the premiere of the documentary film "Karst poljes of Bosnia and Herzegovina, the wetlands of national and international importance" by Ilhan Dervović.

Fig. 40. Extensive hydrotechnical works: destruction of Popovo Polje (BiH). Photo: G. Aljančič.

Fig. 41. Shared commitment to conservation of karst poljes. Photo: G. Aljančič.

The abstract of our presentation is published in the Abstract book of the workshop http://www.euronatur.org/fileadmin/docs/docs_english/Karst-workshop_October2013/Kraska_polja_BiH_-_Knjiga_sazetaka_EN.pdf).

2. The 22nd International Karstological School, Postojna, Slovenia, June 16–20, 2014
(organized by the Karst Research Institute at ZRC SAZU; http://iks.zrc-sazu.si/en/index.html)

The annual karstological school in Postojna, Slovenia, is an important scientific event gathering karstologists and students from all continents. David Stanković presented a poster on the CEPF *Proteus* eDNA project:

- Stanković, D., Aljančič, G., Gorički, Š., Năpăruş, M., Pavićević, M., Skaramuca, I., Mulaomerović, J., Kuntner, M., Snoj, A., Grizelj, Z., Jeffery, W. R. (2014). A novel survey of the distribution of *Proteus anguinus* by environmental DNA sampling. Abstract book of 22nd International Karstological School, Postojna, Slovenia, June 16–20, 2014: 61–62.
  http://iks.zrc-sazu.si/datoteke/GuideBook_2014.pdf

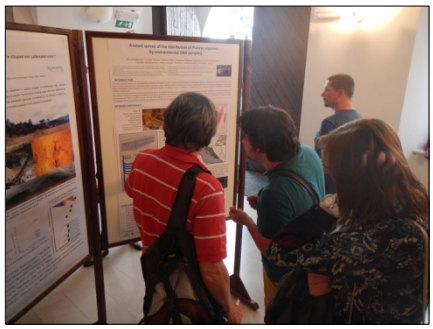

Fig. 42 & 43. The 22nd International Karstological School, Postojna, Slovenia. Photos: M. Năpăruș-Aljančič.

3. Additional activity: **Workshop "The Mediterranean Basin Biodiversity Hotspot: ongoing and potential small and large grants in BiH"**; June 28, 2013, Čitluk, Bosnia and Herzegovina (organized by the Center for Karst and Speleology; http://www.centarzakrs.ba/bats/aktuelnosti/33-radionica-cepf-the-mediterranean-basin-biodiversity-hotspot-ongoing-and-potential-small-and-large-grants-in-bih.html)

The workshop presented some of the ongoing and future CEPF projects in the area of Hutovo Blato, Neretva River and the Trebižat River Tributary, aiming to share the most important issues that are the focus of these studies, and to involve local stakeholders in the process of implementation of CEPF results in the lower Neretva River. SCB used this opportunity to introduce the eDNA survey and its future contribution to preserve *Proteus* in
Bosnia and Herzegovina (see presentation,
http://www.centarzakrs.ba/bh/images/CEPF/eDNA%20prezentacija%20Citluk.pdf).

4. Additional activity: **Regional workshop on conservation of *Proteus***; July 27, 2013, Ogulin, Croatia (organized by the Croatian Herpetological Society Hyla)

Gregor Aljančič presented the project through a lecture "*Proteus* and floods".

Fig. 44 & 45. Regional workshop on conservation of *Proteus*, Ogulin, Croatia. Photos: M. Năpăruș-Aljančič.

5. Additional activity: **Symposium "Frontiers in Amphibian Biology: Endangered Species Conservation and Genome Editing",** Hiroshima, Japan, March 27–28, 2014
(http://home.hiroshima-u.ac.jp/amphibia/FronAmphBiol/FronAmphBiol/English_top.html)

The symposium in Hiroshima was organized by the Institute for Amphibian Biology, Hiroshima University (IABHU), focusing on the cutting edge conservation technologies - the use of molecular forensic methods to trace the presence of rare and endangered amphibians, and also on amphibian breeding in captivity. By attending the symposium we were able to test the scientific value and partial results of the method developed during the project. A very positive response from the Hiroshima meeting is an important external evaluation of the scientific part of the project.

Fig. 46 & 47. Symposium "Frontiers in Amphibian Biology: Endangered Species Conservation and Genome Editing", Hiroshima, Japan, March 27–28, 2014. Photo: D. Stanković and IABHU.

David Stanković received a travel grant from the organizer, and presented a poster on the development of the eDNA method:

- Stanković, D., Gorički, Š., Năpăruş, M., Kuntner, M., Snoj, A., Aljančič, G. (2014). A novel method for detection of the highly endangered cave amphibian *Proteus anguinus* using environmental DNA and real-time PCR. International Symposium "Frontiers in Amphibian Biology: Endangered Species Conservation and Genome Editing", March 27–28, 2014, Hiroshima, Japan: 41–42. http://home.hiroshima-u.ac.jp/amphibia/FronAmphBiol/FronAmphBiol/English_top_files/program_fron_amph_biolX1a.pdf

6. Additional activity: **Conference on the promotion of Trebižat River as an Ecotourism destination** (organized by the Regional Environmental Center, Office BiH/CEPF; Čapljina, June, 19–20, 2014)

7. Additional activity: **Regional workshop "Underground of Dinaric karst"**, June 20–22, 2014, Trebinje, BiH (organized by the Croatian Herpetological Society Hyla, the Herpetological Association of Bosnia and Herzegovina Atra and the Association of Ecologists of Montenegro)
The aim of the workshop was to gather regional experts on *Proteus*, cave fauna and karst, to discuss conservation plans and establish further collaboration. We prepared 2 lectures, both of which directed the attention of the participants towards the molecular era of *Proteus* distribution surveys.
http://www.proteus.hibr.hr/public_html/proteus/en/2-uncategorised/16-reg-rad-treb)

Fig. 48 & 49. Regional workshop "Underground of Dinaric karst", Trebinje, BiH: presentations, excursion and networking. Photos: G. Aljančič.

Gregor Aljančič presented the Project through a lecture:

- Aljančič, G., Stanković, D., Gorički, Š., Năpăruş, M., Pavićević, M., Mulaomerović, J., Kuntner, M., Snoj, A., Grizelj, Z., Jeffery, W. R (2014). A survey of the distribution of *Proteus anguinus* by environmental DNA sampling.

Miloš Pavićević presented the Montenegro activities of the project through a lecture:

- Pavićević, M. (2014). Dosadašnja istraživanja čovječje ribice u Crnoj Gori [Overview of the research on *Proteus* in Montenegro].

5.2.2 **Disseminate scientific results in open access journal**

1. Aljančič, G., Gorički, Š., Năpăruş, M. and Stanković, D. (2014). Endangered *Proteus*: combining DNA and GIS analyses for its conservation. In: P. Sackl, R. Durst, D. Kotrošan and B. Stumberger (eds.), Dinaric Karst Poljes - Floods for Life. EuroNatur, Radolfzell: 70–75. http://afc2014.euronatur.org/uploads/tx_encolumnboxes/KRASKA_POLJA.pdf

2. Stanković, D., Gorički, Š., Năpăruş, M., Kuntner, M., Snoj, A., Aljančič, G. (2014). A novel method for detection of the highly endangered cave amphibian *Proteus anguinus* using environmental DNA and real-time PCR. International Symposium "Frontiers in Amphibian Biology: Endangered Species Conservation and Genome Editing", Hiroshima, Japan, March 27–28, 2014: 41–42. http://home.hiroshima-u.ac.jp/amphibia/FronAmphBiol/FronAmphBiol/English_top_files/program_fron_amph_biolX1a.pdf

3. Stanković, D., Aljančič, G., Gorički, Š., Năpăruş, M., Pavićević, M., Skaramuca, I., Mulaomerović, J., Kuntner, M., Snoj, A., Grizelj, Z., Jeffery, W. R. (2014). A novel survey of the distribution of *Proteus anguinus* by environmental DNA sampling. Abstract book of 22nd International Karstological School, Postojna, Slovenia: 61–62. http://iks.zrc-sazu.si/datoteke/GuideBook_2014.pdf

5.3 Establish a network of NGOs (promotion of joint karst biodiversity and groundwater protection initiative)

- **Bosnia and Herzegovina and Montenegro Trip (Sep. 30 – Oct. 4, 2013)**

During this field trip we conducted meetings with our partners in BiH, and, alongside promoting goals of the project at the First International Workshop on "Dinaric Karst Fields as Wetlands of National and International Importance", Livno, BiH (EuroNatur and Naše Ptice), met many supporters of the project and planned future activities. We also scouted a good part of the project area - the Trebižat River Valley and Hutovo Blato, accompanied by the Herzegovinian karstologist Dr. Ivo Lučić. On the way through the Trebižat River Valley, we stopped in the village of Vitina to inquire about a poorly documented "mass find" of *Proteus* in 2006 (reported in local media but never reached the *Proteus* experts), interviewed Mr. Ivan Bebek who returned these animals back to the Spring of Vrioštica, and started a successful co-operation with the local community.

- **Visit of the Biospeleological Society of Montenegro team in Tular Cave Laboratory**
(Miloš Pavićević, Olga Kreshchenova and Leonid Merzlyakov; November 24 – 30, 2013)

Fig. 50. Visit of Biospeleological Society of Montenegro in Tular Cave Laboratory. Photo: L. Merzlyakov.

Fig. 51. Descend to *Proteus* habitat in Habečkovo Brezno, Slovenia. Photo: M. Pavićević.

Results of the BSM visit:
- delivery of 6 new water samples from Montenegro;
- presentation of the Tular Cave Laboratory;
- meeting with SCB project team: visit of the Evolutionary Zoology Laboratory at IB ZRC SAZU, presentation of eDNA analysis method;
- visit of the Department of Biology at Biotechnical Faculty, University of Ljubljana;
- meeting with Mr. Vojislav Kovač, the Honorary Consul of Montenegro;
- visit of *Proteus* localities in Slovenia (Postojnska Jama and Habečkovo Brezno).

- **Networking at workshops**

- 4 regional workshops and 2 international symposia (see Section 5.2.1)

- **Photo and video contributions in other publications**

- photo of Proteus, in educational leaflet:

Pašić, J. *et al.* (2014). Pećinski biodiverzitet [Cave Biodiversity]. Brochure; Center for Karst and Speleology, Sarajevo, 8 p. http://www.centarzakrs.ba/biodiversity/images/PDFovi/Brochura_biodiverzitet.pdf

- short video clip of *Proteus*, in a documentary film on importance of Natura 2000 in BiH:

Dervović, I. *et al.* (2014). Priča o saradnji za Naturu 2000 [Story on cooperation for Natura 2000]. Documentary film; Production Visoki, 27:21'. https://www.youtube.com/watch?v=uLc11dQF1Eo

Were any components unrealized? If so, how has this affected the overall impact of the project?

No.

Please describe and submit (electronically if possible) any tools, products, or methodologies that resulted from this project or contributed to the results.

(see Project Components)

Lessons Learned

Describe any lessons learned during the design and implementation of the project, as well as any related to organizational development and capacity building. Consider lessons that would inform projects designed or implemented by your organization or others, as well as lessons that might be considered by the global conservation community.

Project Design Process: (aspects of the project design that contributed to its success/shortcomings)

Specifically, the project taught us how to address the risk management issues - related with the sampling, filtering and transporting filters to be analysed, as well as to prevent the data loss by an active networking and interaction with local community. The network, created during the field work activities and work meetings, helped to successfully address in a short time most of the problems we encountered and to produce very good results.

The initial project design was changing and growing in a favorable way as a result of our in-house expertise in state-of-the-art molecular genetic techniques, an excellent cooperation among the project partners, other NGOs and experts in various fields of research and conservation, and the local community (see Additional Comments).

Project Implementation: (aspects of the project execution that contributed to its success/ shortcomings)

Overall, the implementation of the project and project execution were successful. However, the constructive networking on site, the re-sampling field work sessions in BiH and Montenegro, and the necessity to build a sustainable educational tool in the Trebižat area as a statement of excellent cooperation with the local community forced us to extend the initial project timeline for two months over the original plan. The effectiveness of these additional tasks was not possible without permanent

communication and feedback from our main donor, CEPF. Furthermore, our participation in April at the workshop organized by CEPF to assess the progress of the funded projects gave us a clearer image of the importance of networking, and also of the importance of getting in direct contact with the project evaluators for addressing issues and searching for solutions.

Other lessons learned relevant to conservation community:

The main lessons we learnt during this project were that dissemination and practical educational tools are crucial and that in order for the project to be effective, the partners and other NGOs, local community and other institutions had to be involved in the field-work process, laboratory work, and in writing and preparing educational materials (info board, flyers, presentations and events). Such actions result in high credibility and trust in the project organizers and provide sustainable and long-lasting knowledge and practice to the conservation community involved in the project.

Additional Funding

Provide details of any additional donors who supported this project and any funding secured for the project as a result of the CEPF grant or success of the project.

Donor	Type of Funding*	Amount	Notes
SCB, in-kind	Project co-financing	27,201 USD	Evaluated voluntary work
Jeffery Lab, UMD	Project co-financing	8,000 USD	eDNA lab material, travel costs
ZRC SAZU	Project co-financing	1,000 USD	eDNA lab material

Additional funding should be reported using the following categories:

A) Project co-financing (Other donors contribute to the direct costs of this CEPF project)

B) Grantee and Partner leveraging (Other donors contribute to your organization or a partner organization as a direct result of successes with this CEPF project.)

C) Regional/Portfolio leveraging (Other donors make large investments in a region because of CEPF investment or successes related to this project.)

Sustainability/Replicability

Summarize the success or challenge in achieving planned sustainability or replicability of project components or results.

- Successful development and implementation of the eDNA method in nature will drive the studies on *Proteus* in the next decade, and provide the impetus for the use of the forensic method in studies of rare and endangered aquatic cave fauna worldwide.

- The highly vulnerable *Proteus* has become a symbol of a successful nature conservation management linkage. The Society for Cave Biology has promoted joint activities and rising competence levels of the participating partners, in a clear mutual interest of all the countries along its range.

- EEA and Norway Grants (2015): A new grant obtained to adapt the eDNA method to the extremely rare black *Proteus* (*Proteus anguinus parkelj*) in Southeast Slovenia.

Summarize any unplanned sustainability or replicability achieved.

1. **eDNA water sampling method already replicated in Bosnia and Herzegovina**
Although the applicability of the eDNA method is a long-term output of this project, the know-how of the water sampling method to discover new *Proteus* localities in BiH was transferred to the Center for Karst and Speleology and the Herpetological Association of Bosnia and Herzegovina Atra already before the end of the project.

2. **The Western Balkans *Proteus* conservation aliance**
- The network of the *Proteus* conservation aliance was initially conceived to link only 4 partners of the project, however, by the end of its implementation the number reached 24 comprising NGOs and GOs from BiH, Slovenia, Montenegro, Croatia, Romania, Hungary, GB, Germany and USA.
- Information Board unveiled in Vitina, BiH (Trebižat River Tributary): bilingual (local language and English) educational communication channel, focused on target groups (regional school groups, local communities and foreign visitors to the CEPF priority key biodiversity area on the Trebižat River).

3. **Replicability through trans-hotspot collaboration on rare aquatic vertebrates**
Though the project was designed to serve as a model for future assessment of the vulnerability of subterranean aquatic fauna worldwide, its relevance was recognised already during the Supervision Mission meeting in Hutovo Blato, through a quick assistance of Pierre Carret and Jack Tordoff, who introduced our innovative forensic method to the global conservation community. As a result of this unplanned initiative, we started an exchange of experience and new techniques between Mediterranean and Indo-Burma hotspots projects. Urgent conservation actions on two species of IUCN critically endangered freshwater turtles Zhou's Box Turtle (*Cuora zhoui*) and Yangtze Giant Softshell Turtle (*Rafetus swinhoei*) will benefit through the established co-operation. Both species are increasingly rare, and thus quick identification of the last areas still harbouring lone individuals may help save the target species.

Safeguard Policy Assessment

Provide a summary of the implementation of any required action toward the environmental and social safeguard policies within the project.

The project respected all fundamental ethical principles and requirements valid in Slovenia, Bosnia and Herzegovina and Montenegro, including those under the Charter of Fundamental Rights of the European Union. The care of animals was in accordance with the Animal Welfare Act (7 U.S.C. 2131 et. seq.) and other applicable federal laws, guidelines, and policies of the United States of America. The care of captive animals in the Tular Cave Laboratory was in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of The National Institutes of Health of the United States of America. No human subjects were used in this project.

During the project, specimens of *Proteus anguinus* were observed in nature, as well as in captivity of the Tular Cave Laboratory, from a distance with infrared observation devices, with no physical interference to disturb them. These animals were not involved in any experiments. Housing permits are held by the Tular Cave Laboratory (Slovenian Environment Agency, permit #35601-79/2009-2 and permit #35601-79/2009-4).

Additional Comments/Recommendations

All project activities described in this report were performed in its entirety by a voluntary work.

Members of the project team, contributing to the implementation of the project:
Gregor Aljančič, Dr. Špela Gorički, David Stanković, Dr. Magdalena Năpăruş-Aljančič, Luka Vodnik, Prof. Marija Aljančič, Tatjana Kordiš, Matej Umek, Katarina Vodnik, Andreea Guțu and Primož Vodnik (Society for Cave Biology); Prof. Dr. William R. Jeffery (University of Maryland); Prof. Dr. Matjaž Kuntner, Ren-Chung Cheng, Mag. Nina Vidergar and Tjaša Lokavšek (Institute of Biology ZRC SAZU); Prof. Dr. Aleš Snoj (Department of Animal Science, Biotechnical Faculty, University of Ljubljana); Miloš Pavićević, Leonid Merzlyakov, Olga Kreshchenova and Vladimir Novović (Biospeleological Society of Montenegro); Zlatko Grizelj and Ivan Bebek (Vitina Local Community Initiative); Dr. Jasminko Mulaomerović, Simone Milanolo, Ilhan Dervović and Dr. Ivo Lučić (Center for Karst and Speleology); Ivan Skaramuca, Zdenko Marić and Ana Božić (Herzegovinian Mountain Rescue Service Mostar); Žana Marijanović, Ema Voloder and Danijel Buntić (Speleological Society Herceg Mostar); Goran Panić, Dejan Radošević and Sara Todorović (Republic Institute for Protection of Cultural, Historical and Natural Heritage, Banja Luka).

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For its dedicated contribution to this project, the Society for Cave Biology recommends the Biospeleological Society of Montenegro as a trust worthy and reliable partner.

Information Sharing and CEPF Policy

CEPF is committed to transparent operations and to helping civil society groups share experiences, lessons learned, and results. Final project completion reports are made available on our Web site, www.tular.si, www.cepf.net, and publicized in our newsletter and other communications.

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