Olm *Proteus anguinus* (Photo: Gregor Aljančič)

Endangered Proteus: combining DNA and GIS analyses for its conservation

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Summary

Proteus anguinus, an endemic amphibian of the subterranean waters of the Dinaric Karst, is restricted to its cave habitat. However, during seasonal flooding, some specimens are washed-out of their subterranean environment. While this may be considered as a highly hazardous way for Proteus to disperse into new habitats, it is obvious that all these individuals present a constant loss to their population. The Tular Cave Laboratory serves as a sanctuary for injured specimens accidentally washed-out of their subterranean habitat during seasonal flooding. Since 2008, 17 cases have been documented in Slovenia, and 7 of these animals were successfully returned to their source populations. Although the periodic loss of individual animals has been well balanced through the evolution of Proteus, a concern is raised when possible effects of climate change, large-scale hydrotechnical works and agriculture intensification are considered: the timing, frequency and magnitude of flood events are expected to be changed within a very short period of time. Here we discuss the risks and propose the actions necessary to halt the loss of these rare and highly endangered animals due to man-induced changes in flood regimes of the karst poljes.

Firstly, before any animal is returned to nature, the veterinary care and a strict protocol should minimize the transmission of potential infection. Secondly, if washed-out individuals are to be returned to nature, their source population must be accurately identified. Screening for DNA markers powerful enough to detect ongoing gene-flow, such as micro-satellites and single-nucleotide polymorphisms (SNPs), should minimize the potential for genetic pollution. Thirdly, the washed-out individuals deposited on karst fields often cannot be returned directly to their local cave system, since only small fragments of Proteus subterranean habitat can be accessed by man.

We are currently developing a method of detection of traces of Proteus DNA in water samples (environmental DNA); when integrated in an accurate distribution Geographic Information System (GIS) model, the potential pattern of its genetic variability within the complex karst landscape will be determined. The resulted database should then be referenced not only to guide the return of washed-out individuals to nature but also to minimize the potential impact of any planned hydrotechnical and water-extraction activities in karst poljes on the genetic integrity of Proteus populations.

Sažetak

Proteus anguinus, endemični vodozemac podzemnih voda dinarskog krša, vezan je za pećinska staništa. No, tokom sezonskog plavljenja, neki primjerci budu izbačeni vodenom strujom iz svog podzemnog okruženja. Sa jedne strane, to se može smatrati veoma riskantnim načinom kojim Proteus naseljava nova staništa, ali je očito i da ovakve jedinke predstavljaju stalni gubitak za populaciju. Jamski laboratorij Tulat služi kao utočište za povrijeđene primjerke koji su slučajno izbačeni iz svog pozdemnog staništa tokom sezonskih poplava. Od 2008, u Sloveniji je zabilježeno 17 slučajeva, a 7 tih životinja je uspješno vraćeno u populacije iz kojih su potekle. Iako je povremeni gubitak jedinki nešto sa čime se ova vrsta sustetala tokom evolucije, postoji zabrinutost kada se u obzir uzmu moguće posljedice klimatskih promjena, hidrotehničkih radova i razvoja poljoprivrede: vrijeme, učestalost i veličina poplava će se vjerovatno promijeniti u veoma kratkom vremenskom periodu. U ovom radu bavimo se rizicima i predlažemo radnje koje su neophodne da se zaustavi gubitak ovih rijetkih i veoma ugroženih životinja zbog promjena u režimu poplava u kraškim poljima, koje je čovjek uzrokovao. Prvo,

prije nego što se ijedna životinja vrati u prirodu, veterinarska njega i strogi protokol treba smanjiti prenos moguće infekcije. Drugo, ako se izbačene jedinke trebaju vratiti u prirodu, treba tačno odrediti iz koje populacije su potekle. Screening DNA markera koji su dovoljno jaki da otkriju protok gena, kao što su mikrosateliti i jednonukleotisni polimorfizmi (SNPs) trebali bi smanjiti mogućnost genetičkog onečišćenja. Treće, jedinke koje su izbačene iz podzemnih staništa i nađene na kraškim poljima se obično ne mogu direktno vratiti u lokalni pećinski sistem, jer je samo malidio podzemnih staništa ove vrste dostupan čovjeku. Trenutno razvijamo metodu za otkrivanje tragova DNA Proteusa u uzorcima vode (okolišna DNA); kada se to ujedini sa stvarnim GIS (Geografski Informacioni Sistem) modelom rasprostranjenja, potencijalni režim genetske raznolikosti unutak kompleksa kraških polja će biti određen. Baza podataka koja će biti rezultat toga bit će korištena ne samo kao vodič za vraćanje izbačenih jedinki u prirodu, nego i za smanjenje potencijalnog uticaja planiranih hidrotehničkih aktivnosti u kraškim poljima na genetički integritet populacija Proteusa.

Keywords: Proteus, Amphibia, environmental DNA, karst ecohydrology, vulnerability map

Introduction

The Dinaric Karst is one of the world's prime heritage landscapes, both for natural and cultural phenomena. Dinaric Karst is also important for the study of evolutionary and ecological interplay. For example, here the endemic subterranean vertebrate fauna meets migrating birds, both animal groups having been affected by the single geological evolution of karst polje and its periodical flooding (sensu Bonacci *et al.* 2008), and subject to natural selection of over ten million years (Trontelj *et al.* 2007). The flagship species of this unique natural diversity is the olm, *Proteus anguinus* Laurenti 1768, a true symbol of karst and its history of research (M. Aljančič et. al 1993). Periodical flooding has probably been an important selective force in the evolution of this cave-dwelling amphibian (Aljančič & Năpăruş 2012), as well as of some populations of endemic surface-dwelling fish (*Telestes*, *Phoxinellus* and *Delminichthys*) (see also Palandačić *et al.* 2012). Periodical flooding in this karst system, however, also influences the occurrence of migratory birds of the Adriatic Flyway; these represent transitory, yet punctual guests that are also synchronized with periodical flooding of the Dinaric Karst (Schneider-Jacoby *et al.* 2006; Stumberger 2010).

Through the centuries, human populations have also adapted to the karst landscape developing their own sustainable strategies. Recently, however, anthropogenic activities are largely negative, coming from intensive agricultural activities (e.g., Slovenia: overuse of biogas slurry fertilizer/ B. Bulog & A. Hudoklin, pers. comm.; Bosnia and Herzegovina: converting pastures into arable land/ B. Stumberger, pers. comm.), energy production (hydro and thermal power plants), and unregulated urbanization. Southeast Herzegovina, in particular, has seen these human activities reaching catastrophic proportions (see the case of Popovo Polje; M. Aljančič 1963, Čučković 1983, Lewarne 1999, Lučić 2013).

The case of Proteus: floods as a constant threat?

Flooding—an important periodic event in caves— is a transport agent for organic matter (also for contaminants) as well as for organisms, affecting their colonization, dispersal, and life cycles. In common with all stygobionts, Proteus is entirely restricted to its cave environment, and no longer able to be ecologically competitive in surface habitats. However, during flooding, a number of specimens get washed-out onto the surface, where they become stranded away from their subterranean environment and become exposed to predation on the open surface of the karst polje. It is reasonable to speculate that the earliest human knowledge of the existence of subterranean fauna was through encounters with such washed-out animals. As early as 1689 J. V. Valvasor encountered Proteus precisely under these circumstances, and provided its first description.

While becoming stranded on the surface may in fact be a part of natural history of Proteus intended to enable dispersal into new habitats, this highly hazardous strategy is, obviously, very costly in terms of loss of individuals from source populations.

Fig. 1: Proteus stranded in Kljunov ribnik near Pivka, Slovenia, 29 December 2008; serious chilblains on dorsal side (Photo: Gregor Aljančič)

While becoming stranded on the surface may in fact be a part of natural history of Proteus intended to enable dispersal into new habitats, this highly hazardous strategy is, obviously, very costly in terms of loss of individuals from source populations. The fate of stranded individuals is quite predictable as the odds to re-enter the underground and thus to survive are minimal. The animals are often deposited on temporarily flooded fields and may survive for up to several weeks and as long as high waters persist; they may suffer sunburns or chilblains, and eventually desiccate (Fig. 1). Others may be carried further into surface streams where they get preyed upon by fish, birds or other predators.

We presume that Proteus has developed several responses to reduce the danger of being washed out of its subterranean habitat (Aljančič & Prelovšek 2010) as well as adaptations of its feeding and reproductive behaviour. Due to the extremely long lifespan on the one hand (animals may survive nearly 100 years in captivity) and long reproduction cycles on the other (Proteus reproduce approximately every 8 years in captivity), each individual loss may be fairly detrimental for the size of its population. Although this periodic loss has presumably been an annual constant through its evolution, more rapid changes due to human induced climate change, which are expected to change the timing, frequency and magnitude of floods dramatically, will most probably exceed the ability of Proteus to cope with environmental changes.
The Tular Cave Laboratory has extensively studied this neglected phenomenon since 2008, documenting 17 cases of stranded Proteus in Slovenia. All animals were found by chance and reported by locals. Through this research we unexpectedly became involved in a rescue mission: seven of these animals were saved and returned to their source population (Aljančič & Năpăruş 2012), while injured animals found sanctuary in the Tular Cave Laboratory.

Why location matters

When an individual found on the surface cannot be returned directly to the cave or spring from which it was washed out by flood, the data on the exact distribution of its source population can guide its return. Analyses of mitochondrial DNA sequences revealed distinct lineages of Proteus from the Dinaric Karst of (1) Istria, (2) Dalmatia-Herzegovina, (3) Bosanska Krajina, (4) Lika, (5) SW Slovenia and (6) SE Slovenia (Gorički 2006; cf. Fig. 4 in Gorički & Trontelj 2006; Trontelj *et al.* 2007, 2009; and unpublished data). A study of highly variable nuclear DNA sequences is still lacking, but will, hopefully, resolve the complex network of relationships on a more local scale, i. e. within the populations that mitochondrial DNA indicated. To clearly delimit populations with on-going gene flow, intensive sampling coupled with datasets of karst features and their spatial relationships within the karst system, datasets with geological and hydrogeological features and their spatial relationships, a high resolution digital elevation model of the area, and spatial statistical modelling are needed. This is especially significant in the case of populations with very small ranges or which inhabit areas that have received little attention in the past.

The Tular Cave Laboratory is developing an indirect, forensic approach (which will be published elsewhere) to facilitate the search for Proteus in even the most inaccessible locations. Namely, during the process of skin growth and regeneration, fragments of epidermal cells, along with the DNA they contain, are constantly shed from the skin of aquatic vertebrates and carried away by water. Such DNA dissolved in water is called environmental DNA (eDNA). In its most basic form, the methodology aims at detecting traces of Proteus DNA released in water; as new genetic markers are found, it can be expanded for fast and routine genotypization of water samples. DNA extracted from the mucous of a washed-out individual is then compared to the integrated DNA-GIS database. In this way it will be possible to determine the origin of the washed-out animal, and the appropriate site to release the animal can be identified.

Further implications of combined DNA and GIS analysis for Proteus conservation

Due to their high specialization to a narrow range of abiotic conditions in the subterranean environment, all groundwater organisms are extremely vulnerable to direct and indirect alterations of their habitats. In Southeastern Bosnia and Herzegovina, large scale hydrotechnical activities are the main cause for disturbances to the natural flooding regime of caves and karst poljes, reduction of the catchment area, land amelioration, and water pumping (Ozimec 2011). Through reduction or loss of aquatic habitats in caves, or microclimatic and ecological changes such as temperature increase, reduction of dissolved oxygen, and reduction or increase in the quantity of organic matter (Ozimec 2011) these activities may have a detrimental impact on the density of groundwater fauna, including its flagship species Proteus (e. g., the case of Popovo Polje; M. Aljančič 1963, Čučković 1983, Lewarne 1999, Lučić 2013). In particular, 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 considerably reduce the flow of groundwater towards the lower Neretva River and its delta (J. Mulaomerović, pers. comm.). Furthermore, a substantial portion of waters from Popovo polje is being diverted towards Ombla spring, while only a small part is directed to the reversible hydroelectric plant "Čapljina", located on the eastern side of the Svitavsko-Deransko polje. The reduction of input of groundwater from karst poljes of Eastern Herzegovina into the Neretva River's lower course and the delta results in an increase of saltwater penetrating further upstream. The observed increase of salinity may have a direct adverse effect on the localities of Proteus in the Neretva River delta and Hutovo Blato. Changes in the direction of watercourses may also affect the gene pool of Proteus populations (Sket 1997).

Existing legal acts – with Bosnia and Herzegovina being the only political entity within the range of Proteus that lacks any legislation enforcing its conservation – do not entirely protect aquatic cave animals and their habitats from negative human impacts on groundwater integrity. A decline of several populations of Proteus has been reported, and in some localities Proteus has already become extinct (Sket 1997). The IUCN Red List of Threatened Species defines *Proteus anguinus* as vulnerable, and recommends urgent measures to revert its population decline (Arntzen *et al.* 2009). The extent of its decline, however, cannot be estimated without an extensive survey of its distribution. Current knowledge on the distribution of Proteus in Bosnia and Herzegovina (Kotrošan 2002) is particularly scarce in the area along the lower course of the Neretva Rivier and its tributaries. In the area of Hutovo blato, only a single Proteus locality is known: a well in the village Čore near the Babino oko spring. Undoubtedly, Proteus can also inhabit other caves in the area. This is supported by the oldest known depiction of Proteus in Bosnia and Herzegovina, on a 'stećak' (monumental gravestone typical for Medieval Bosnia) from ca. 1477, found in the Boljuni necropolis near Stolac (Mulaomerović & Hodžić 2012), which conveys Proteus as having a long symbolic presence.

Conclusions

The Tular Cave Laboratory has started utilizing the eDNA to infer the presence of Proteus in the most threatened sites. The results of this intensive survey will provide a scientific basis for enforcing the long-term protection of Proteus populations and its habitats, and will help to mitigate current and future threats. The highly efficient, non-invasive, DNA-based method to detect Proteus from water samples coupled with a set of spatial data will provide a vulnerability map of Proteus which will visualize zones most threatened by human impacts along with the most active threats. When integrated in an accurate Geographic Information System (GIS) distribution model, the patterns of the genetic variability of Proteus within the complex karst landscape will emerge. The DNA-GIS database will further provide the scientific basis for the return of washed-out individuals to their source cave system. Furthermore, it will also help to prepare urgently needed vulnerability models for assessing potential impacts of hydrotechnical and water-extraction activities in karst poljes on the

The highly vulnerable Proteus has a huge potential to become a symbol of a successful balance between conservation and sustainable management of the karst environment.

genetic structure of Proteus populations. The accuracy of the resulting distribution and vulnerability models will be validated by spatial statistical analysis (Năpăruș & Kuntner 2012; Meleg et. al 2013). This analytical tool is designed to implement future conservation action plans needed for building sustainable strategies for landscape management and ecological forecasting. Future activities may even reach beyond local geography and specifics of the distribution of Proteus in the Dinaric Karst – with the present survey serving as a model for future assessments of the vulnerability of aquatic cave fauna worldwide – and, thus, offer the possibility to implement science based conservation strategies for a sustainable management of the karst environment and its biodiversity.

The present study has strengthened our belief that a complex ecosystem of Dinaric Karst poljes (above and underground) can only be preserved through multidisciplinary scientific efforts and with the support of international nature conservation organizations. An active alliance of organisations committed to nature conservation of the Dinaric Karst is urgently needed in order to embark on an ambitious task to promote, share and implement an advanced action plan to save Proteus, Europe's only cave vertebrate, along its fragmented range in the Western Balkans. The highly vulnerable Proteus has a huge potential to become a symbol of a successful balance between conservation and sustainable management of the karst environment.

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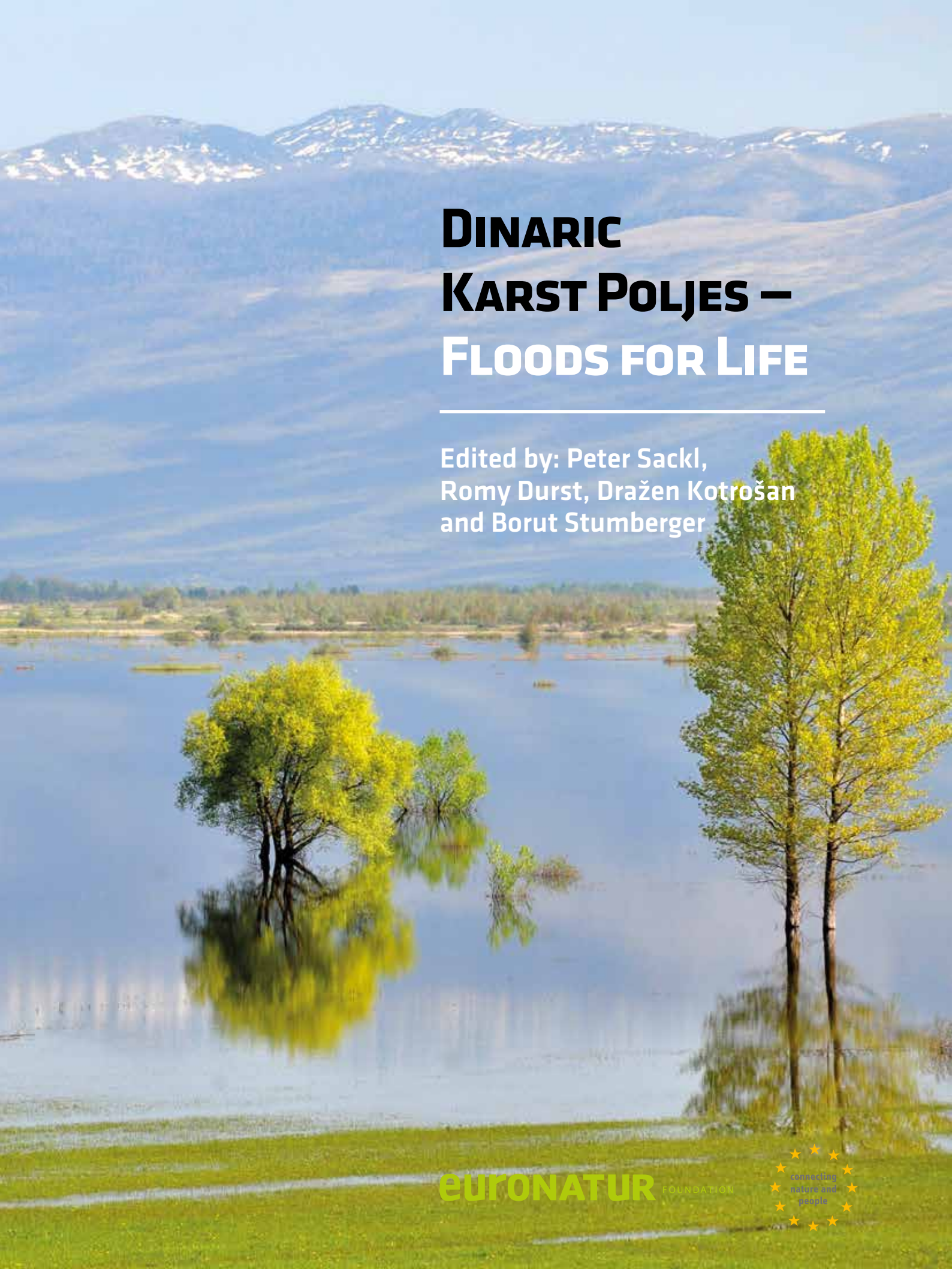

Dinaric Karst Poljes – Floods for Life

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Cover photo: Flooded Livanjsko polje, Bosnia-Herzegovina, 4 May 2010 (Photo: Martin Schneider-Jacoby)

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