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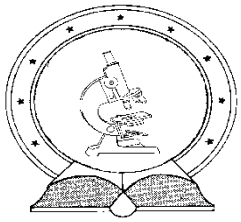

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Parasites, taxonomic composition and ploidy level of the *Pelophylax esculentus* complex

Paraziáltság, fajösszetétel és ploiditás vizsgálata a *Pelophylax esculentus* komplexen

Egyetemi doktori (PhD) értekezés

Herczeg Dávid

Témavezetők:

Prof. Barta Zoltán

Dr. Vörös Judit

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A doktori értekezés betétlapja

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Értekezés a doktori (PhD) fokozat megszerzése érdekében
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Témavezetők:
Prof. Barta Zoltán
Dr. Vörös Judit

A doktori szigorlati bizottság:

elnök:	Prof. Magura Tibor	egyetemi tanár
tagok:	Dr. Rózsa Lajos	tudományos tanácsadó
	Dr. Sramkó Gábor	tudományos munkatárs

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elnök:	……	……
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Brooks DR, Hoberg EP, Boeger WA, Gardner SL, Galbreath KE, Herczeg D, Mejía-Madrid HH, Rácz SE, Tsogsaikhan Dursahinhan A (2014) Finding them before they find us: informatics, parasites and environments in accelerating climate change. *Comp. Parasitol.* 81:155–164 57

Paper II

Herczeg D, Vörös J, Végvári Zs, Kuzmin Y, Brooks DR (2016). Helminth parasites of the *Pelophylax esculentus* complex (Anura: Ranidae) in Hortobágy National Park (Hungary). *Comp. Parasitol.* 83:36–48 68

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Herczeg D, Vörös J, Christiansen DG, Benovics M, Mikulíček P (2017) Taxonomic composition and ploidy level among European water frogs (Anura: Ranidae: *Pelophylax*) in eastern Hungary. *J. Zool. Syst. Evol. Res.* 55:129–137 82

Papers are referred throughout the text by their boldface roman numerals (**I-III**).

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Glossary

Abundance	The number of individuals of a particular parasite in or on an individual host regardless of whether or not the host is infected
Allozyme	Variant forms of an enzyme that are coded by different alleles at the same locus
Cercaria	The larval stages of trematodes, produced asexually by sporocysts or rediae and released from the first intermediate host
Coracidium	Is a ciliated, short-live, weakly swimming first larval stage of pseudophyllidean cestodes with aquatic life-cycle
Core species	Some parasite species tend to colonize more than one host species and be found at high numbers within a host population. These regionally common and locally abundant species are termed as core species
Dioecy	Characteristic of a species, meaning that it has distinct male and female individual organisms
Definitive host	The host in or on which a parasite sexually reproduces
Gonochorism	The state of having just one set of sexual (either male or female) organs in any one individual organism
Helminth	The term helminth refers to worm-like members of the phylum Annelida, Platyhelminthes, Nematoda and Acanthocephala
Heteroxeny	Nematodes that use intermediate host during transmission
Heterozygosity	The probability of nonidentity (*H*), is a measure of genic variation of a population
Infrapopulation	All individuals of a parasite species in a host individual at a particular time
Intermediate host	The host in or on which the parasite undergoes some developmental change, but does not reach sexual maturity
Microsatellite	A tract of repetitive DNA in which certain DNA motifs (approximately 2–5 base pairs in length) are repeated, typically 5–50 times. Microsatellites have a higher mutation rate than other fragments of DNA leading to high genetic diversity. They also known as simple sequence repeats or short tandem repeats
Miracidium	A swimming, sac-like larva. Each carries number of germinal cells from which will arise subsequent generations of organisms
Monoxeny	Nematodes that can infect hosts directly (i.e. they use no intermediate hosts)
Paratenic host	Host in which development does not occur, but which may serve to bridge an ecological gap in a parasite's life cycle

Polymorphic locus	Number of loci with more than one allele, at which the most common form has a frequency not exceeding 0.95 in a given population
Polyploid	Those organisms containing more than two homologous sets of chromosomes
Population bottleneck	Is a sharp reduction in the size of a population due to environmental events or human activities
Prevalence	The number of hosts infected with at least one parasitic individual divided by the number of hosts examined for that parasite species
Proboscis	Apical organ of acanthocephalans, that armed with spines and hooks, which it uses to stick and hold the gut wall of its host
Redia	Larval form that have rhabdocoele-like intestine, bearing a pharynx and birth pore
SNP	Is a variation in a single nucleotide that occurs at a specific position in the genome, where each variation is present to some considerable degree within a population
Sporocyst	Germinal sacs containing germinal cells which have descendent from the original ovum from which the miracidium developed
Symbiotype	A single host specimen from which the type of new parasite species was described
Voucher specimen	Any specimen, usually but not always a cadaver, that serves as a basis of study and is retained as a reference. Specimen means the whole animal or a part of it

Part I

1. General introduction

1.1 *Traditional way of thinking about parasite inventories*

In ecological context, parasitism is a non-mutual symbiotic interaction between species, in which an intimacy of association exists between the parasites and their hosts. Parasite individuals obtain nutrients from hosts and cause harm, which, nevertheless, rarely leads to the death of host individual. Parasite organisms reached an enormous species diversity through evolutionary time and we may classify approximately 50% of the known species as parasites (Price 1980; Windsor 1998). During the past few decades, parasites have become recognized as significant components of biodiversity (Poulin and Morand 2004; Dobson et al. 2008; Kuris et al. 2008) and as excellent model systems for general evolutionary (Brooks and McLennan 1993) and ecological studies (Poulin 1997; Poulin and Morand 2004; Pedersen and Fenton 2007). Herein, I will focus primarily on *helminth* parasites of amphibians, especially their occurrence in anurans.

Inventories of all species are the essential baseline for evaluating, managing and preserving global species diversity. As we have seen above, parasites are key elements of global species diversity and their inventories can provide meaningful results on several scales. Historically, studies of parasitic helminth communities in any host mainly consisted of faunistic studies or species descriptions. Nowadays, however, the attention appears to shift away from descriptive studies toward (i) more quantitative approaches (Crofton 1971; Shaw and Dobson 1995; Wilson and Grenfell 1997) and (ii) the investigation of processes responsible for creating community patterns (Poulin 1995; Huspeni et al. 2004; Brooks et al. 2006a; Hudson et al. 2006; Lafferty 2008).

Detecting and monitoring parasites within their communities requires intensive and extensive sampling of potential hosts and present specific difficulties. Especially, the host should be correctly identified in scientific terms and *voucher specimens* (both host and parasite) deposited in museum collections are required (Frey et al. 1992). The fact that only a few parasitology journals require the *symbiotype* of the host specimen (Brooks 1993) makes it difficult to compare past and present findings. A well-known example for this is the case of the leopard frog complex (*Rana pipiens* complex) which is distributed through North and

Central America. Parasitologists most extensively studied this group of hosts in North America for decades. Recently, it has turned out that instead of a single species, *Lithobates pipiens* (formerly *Rana pipiens*) ranging from near the Arctic Circle to Panama and leopard frogs represent a diverse species complex with of 27 or more extant and recently extinct species (for a review see Hillis 1988; Hillis and Wilcox 2005). These cryptic species of frogs hosted a great diversity of helminth species, often including multiple congeners. But because no host specimen is known to have been deposited in museum collections from the period during which leopard frogs were considered a single species (except Brooks 1976, 1979a) there is no way to ascertain the specific identity of the hosts reported in most surveys and the parasite fauna reported from *L. pipiens* actually connected to various host species.

A further difficulty is that, due to their small size and often cryptic location within hosts, parasites are challenging to find without proper training and experience and it is also hard to determine how many host specimens have to be sampled to list all their parasites (Poulin and Morand 2004). Poulin (1998) suggests that at least 1 or 2 helminth species remain undetected in surveys of vertebrates, when the sample size is lower than 40 or 50 individuals per host species. Therefore, we would expect that the more parasite species inhabit a given host, the more host individuals we would need to examine to find at least the 95% of the parasites. However, this may be offset if many of the parasites are *core species* (for the core-satellite species hypothesis see Hanski 1982) that occur at high *prevalence*. Almost without exception, parasite species richness cannot be measured or directly estimated by observation because the observed number of species is a downward-biased estimator for the total species richness (Gotelli and Colwell 2011). One way is the calculation of sample-based rarefaction curves (e.g. rarefaction analysis; Sanders 1968) to determine whether sampling methods were adequate for detecting parasite species present within a community (i.e. Johnson and Hoverman 2012). As a result, we get rarefaction curves which are a plot of the number of species as a function of the number of samples.

Other complications may arise when the host species is strictly protected by nature conservation laws, and hence regulations minimize the number of host individuals to be sampled. Therefore it can frequently happen that fewer hosts can be collected than sampling effort curves might suggest, especially if a host is rare in the inventory site. If a host is rare and endemic over it is entire geographic range, we may not to perform destructive sampling. In such cases, we may still obtain information depending on what we find in phylogenetically and/or ecologically related host species in the same area (Brooks and McLennan 2002).

Traditional parasitological inventories of helminths aimed to collect as many parasites from as many hosts as possible – especially hosts that have not been examined for parasites previously – gathering both taxonomic, ecological and population data from that extensive destructive sampling. **Paper I** summarized a comprehensive parasite inventory protocol under the acronym DAMA (Documentation, Assessment, Monitoring, Action). We emphasized that the initial phase of documentation involves destructive sampling but that should be minimized according to **Paper I**, however, it is not necessary to kill large numbers of hosts to obtain important information about parasite biodiversity. Information derived from long-term inventories allows the determination of the persistence and stability of parasite diversity (for example, see Bursey et al. 2010). Comparison of data with baselines (i.e., what is already known about the parasites, hosts, and geographic location of an inventory) offers an efficient way to address the question of how current findings relate to previous ones? It requires the understanding and acceptance of that new inventories of habitats and hosts assessed previously are just as important, and possibly even more so, than inventories of new hosts and new places never sampled before (**Paper I** and **II**). The rapid evolution of cheap and efficient molecular methods and molecular data (e.g., DNA barcodes) have opened the door to the possibility of conducting parasite inventories based on non-destructive sampling, once we know what parasite species can occur in the target host(s) and in the target area(s) (Darling and Blum 2007; Ferri et al. 2009; Moszczynska et al. 2009; Darling and Mahon 2011; Locke et al. 2010; Pérez-Ponce de León and Chudbury 2005; Locke et al. 2011; Alcántar-Escalera et al. 2013; Prosser et al. 2013; Van Steenkiste et al. 2015). DNA barcoding is a broadly used tool for specimen identification to species level in a diverse range of animal taxa including helminths (Hebert et al. 2003). We could take advantage of that helminths release their eggs to the environment along with the host faeces or urine. This type of transmission is not only true for those helminth species that parasitize the gastrointestinal tract but species that occur in the lungs, liver or the gallbladder, as well. The faeces of the host are easily and non-invasively collectable, which contains relevant information about the presence of the parasites. Therefore, the extraction of parasitic DNA from host faeces is then match with existing barcodes or material from voucher specimens as it was suggested in **Paper I**.

1.2 *Host-parasite interactions*

In their most fundamental sense, parasitic communities are not different from other biological communities, and the definition of population used in evolutionary biology or ecology are applicable in parasitology with a slight modification. An infracommunity is a community of parasite *infrapopulations* in a single host. All parasitic community data should be collected at this level. A component community refers to all infrapopulations of parasites associated with a subset of a host species or a collection of free-living phases associated with some subset of the abiotic environment (Bush et al. 1997). If we investigate parasites below the community level, they commonly categorised as ecto- and endoparasites based on the location of their occurrence in hosts. Ectoparasites are those organisms that live on the surface of their hosts (e.g. skin, hair, feather etc.) and their existence is not fully parasitic because many of them maintain only periodic contacts with their host. In contrast, endoparasites are living within their host, for example in body cavity, intestines, lungs or other organs. The existence of those organisms is nearly completely parasitic (Smyth 1995). Based on their size parasites are usually classified into two categories. Microparasites are small in size and often live intracellularly, like bacteria or viruses. They multiply within their host and may occur in high *abundance* in the infected host species. In contrast, macroparasites grow but do not multiply within their host. They produce specialized infective stages (eggs, larvae etc.) which can infect new susceptible hosts after their release into the environment. Macroparasites like helminths and arthropods mostly live in the body cavity or on the external surface of their host.

Successful transmission from host to host in order to accomplish life-cycle is the core of parasite survival. With all parasitic organisms, the nature and evolution of transmission strategies should be assessed by the basic characteristics of certain parasite taxa. In the followings I present a brief summary of transmission strategies, life-cycle diversity and evolution in some of the major helminth groups.

Nematodes. The body of nematodes is cylindrical. Nematodes have separate sexes, with females generally larger than males. There are about 16,000 species of nematodes (Poinar 1983) and 40% of them are parasites of animals. Basically, all nematodes pass through 5 developmental stages separated by 4 moults even in the parasitic forms (Anderson 1988). Third-stage larvae of nematodes (i.e. the dauer larvae) can survive for long periods of harsh conditions like scarcity of food and drought.

For their transmission between *intermediate*- and *definitive hosts* several strategies exist. A number of major groups of nematodes are *monoxenous* and infect the definitive host directly through skin or oral penetration. Two ways of monoxeny are defined. Primary monoxeny means that *intermediate host* was never used during transmission. In contrast, secondary monoxeny assumes the loss of the intermediate host during the course of evolution. On the other hand, many nematode superfamilies are *heteroxenous* and use arthropods, oligochaetes, molluscs or even vertebrates as intermediate hosts. Several nematodes also use in transmission a transport or *paratenic host* and that process usually called paratenesis (Baer 1951). Paratenesis occurs in three ways. First, it may transport larval stages from the external environment to the intermediate host, in which larvae develops to the stage infective to the final host occurs. Second, it may move infective larvae between the intermediate and final hosts. Third, it may move larvae from one paratenic host to another.

Cestodes. Because of lacking a gut adult cestode are unable to occupy such diverse environments as the lungs, kidney, or the circulatory system of the host and hence they are restricted to the intestines solely. At first sight, it seems to be disadvantageous to specialize into only one habitat, but a variety of benefits reveals when examined closely. Firstly, the intestines of hosts serve as an unlimited source of nutrients, which has an important evolutionary consequence for those organisms with high fecundity, providing an ideal condition for r-selection strategist (Jennings and Calow 1975). Secondly, inhabiting in the alimentary tract results in a lower risk of a heavy immune attack by the host, thus enable the same host to be continuously exploited over their life. On the other hand, their restricted habitat specificity may strengthen intra- and interspecific competition for space and nutrients (Read 1951; Roberts 2000; Bush and Lotz 2000). For example, a study of cestode parasite communities of elasmobranchs (sharks and rays) revealed species that compete for resources and hence tend to have non-overlapping distributions in the host intestine (Friggens and Brown 2005).

Basically, cestodes lack free-living larval stages (with the exception of *coracidium* larvae) therefore their eggs, and even coracidia, are essentially biological nomads, undirectedly and passively transmitted to potential hosts. Cestode life-cycles can be arranged in three ways: aquatic, amphibious or terrestrial. The aquatic and amphibious cycles largely based on 3-hosts including first-, second intermediate hosts and definitive host. In contrast, in terrestrial ecosystems, a single intermediate host, in other words, a 2-host cycle is dominating. One host (direct) life-cycles are very rare among cestodes and only known in the genus

Vampirolepis from mice and *Archigetes* from aquatic oligochaetes (Calentine 1964; Hickman 1964; Murphy 1988). The rarity of singe-host cycles indicates that involving at least one other host is a key element of cestodes success, largely because by providing a vehicle for transmission it increases the chance of successful dispersal of infective stages from one definitive host to other (Mackiewicz 1988).

Acanthocephalans. This phylum consists of a small group of unsegmented, cylindrical, *dioecious*, endoparasitic worms parasitizing in all classes of vertebrates, especially fish and birds. Like cestodes, acanthocephalans are also lacking an alimentary canal throughout their development. The main diagnostic feature of the group is the presence of an invaginable *proboscis* armed with hooks. Worms that have terrestrial life-cycle usually use insects as intermediate hosts. In contrast, acanthocephalan parasites of fish mainly harbour amphipods (*Gammarus* sp.) and isopods (*Asellus* sp.) as intermediate host species. Their fully developed larvae called cystacanths which infect the definitive host. Habitat specificity is common in the phylum Acanthocephala, and most species inhabit a specific portion of the digestive tract (Kennedy 2006).

Trematodes. The subclass Digenea contains the most diverse and complex life-cycles among helminths, in which intermediate (first or second, or both) hosts are involved (reviewed by Shoop 1988; Poulin and Cribb 2002). Complex life cycles are a hallmark of parasitic trematodes. The typical life-cycle involves three transmission events, hence two intermediate hosts, in order the life-cycle to be completed in the definitive host. First, adult trematodes harbour in vertebrates as definitive host and release eggs to the environment. The *miracidia* larvae hatched from these eggs and seek to find suitable first intermediate host, which in most of the time is a mollusc. After asexual amplification of larval stages inside *sporocysts* (sometimes in *rediae*) the *cercariae* develop. Second, cercariae emerge from the first intermediate host and infect the second intermediate host, which is usually a crustacean, odonate larva or a tadpole. Cercaria has a free, but short-live stage during which it should find a suitable second intermediate host where they encyst as metacercaria. Third, second intermediate host is ingested by the definitive host in which metacercariae develop to adult worms (Poulin and Cribb 2002). A considerable number of alterations of this typical life-cycle occurs (summarised in Fig 1, but the interested reader should refer to Poulin and Cribb 2002).

Natural selection has favoured various adaptations in trematodes to increase the chance to complete their life-cycle. Examples include high adult-fecundity, asexual multiplication within the molluscan first intermediate host or parasite-mediated increases in the susceptibility of the second intermediate host to predation. Efficient host-finding mechanisms in miracidia and cercaria are key element of transmission. The miracidia achieve it at least two ways (i) passive penetration through ingestion, herein the miracidia remains in the egg or (ii) active penetration directly the epithelium of the mollusc by the ciliated miracidia (Pearson 1972; Overstreet 1978). The distinct strategies assume different adult fecundity. In those digenetic trematodes where miracidia remain in eggs and await ingestion by the mollusc, are small in size and produced in high numbers. These eggs are capable to survive for extended period of time in the environment while waiting for ingestion. On the other hand, species that produce eggs in low numbers but large in size, the hatching miracidia has a limited time (approximately 48 hours) to find suitable host (Shoop 1988). Henceforward, a series of parthenogenetic events (asexual reproduction of mother sporocysts which give rise to daughter sporocysts or rediae) takes place within the mollusc, following the emergence of the cercarial generation.

The transmission of cercariae is largely dependent on the dietary habit of the definitive host. For instance, in a 2-host system, a mollusc-consumer definitive host are ingesting cercariae all along with first intermediate host and then encyst as metacercariae, following the development into adult helminth or cercariae to adult directly. In a non-mollusc-consumer host, cercariae must exit the mollusc, which assume a brief free-living stage, and ultimately encyst upon plants, animals, or other inorganic objects that may serve as food items for the vertebrate definitive host (for example see the turtle lung fluke *Heronimus* sp.). Transmission to the vertebrate definitive host is facilitated by ingestion, the site of maturation is constantly the gastrointestinal tract and its embryological derivatives (e.g. salivary gland ducts, nasolacrimal ducts, eustachian tubes, lungs, liver, etc.).

In the followings, I present mechanisms that shape the evolution of host-parasite associations, which can be investigated in two ways. The first approach is based upon the comparison of host-parasite phylogenies and identifying points of congruence as examples of co-speciation (Brooks 1979b). Based on this phylogenetic approach, incongruent portions of host-parasite phylogenies falsify the hypothesis of co-speciation at those nodes and thus required investigations into the influence of other factors, like dispersal and host switching on the evolution of the host-parasite association. The second and more recent research program is

called “maximum co-speciation” which assumes that, hosts and their parasites share a specialized and exclusive evolutionary association (Brooks and McLennan 2003; Page 2003; Clayton et al. 2004; Johnson and Clayton 2004). Additionally, the maximum co-speciation assumes that speciation in one lineage causes speciation in the other. Thus, host-parasite phylogenies are expected to be completely congruent, with departures from congruence explained by invoking extinction in one lineage or the other. Because of this, a debate standing between the two approaches, namely the treatment and importance of host switching during the evolution of host-parasite associations. This debate comes from the assumption that parasites are resource specialists with restricted host ranges and host-switching to a relatively unrelated host species considered to be an unlikely event through evolutionary time. This view suggests that the host itself is the key component which defines host-parasite interactions (host-centred view). Contrary, the other approach assumes that resources for parasites are particular attributes of the host species what they are tracking, and not the host species itself (parasite-centred view). Based on that, host-switching is not a rare event. At first sight it seems paradoxical but phylogenetic conservatism in host attributes upon which a parasite is specialized might produce a range of susceptible hosts, whereas restricted geographic distribution of the parasite at any given time would mean that many susceptible hosts are not infected. There are several reasons which entails (e.g. host range, host density, ecological or geographic barriers, abiotic factors etc.) why parasites do not capable to infect all of those susceptible hosts, but when these impediments are disappearing, and parasite enter into a novel environment and encounter with new hosts, then a chance is open to host-switching. The fact that present-day associations might be shaped in part by the distribution of phylogenetically conservative traits is called ecological fitting (for the concept of ecological fitting see Janzen 1985). Ecological fitting, provide substantial opportunities for rapid host switching in changing environments, in the absence of the evolution of novel host-utilization capabilities (Brooks and McLannen 2002; Brooks et al 2006a; Agosta and Klemens 2008; Araujo et al. 2015).

Better understanding of ecological fitting let see the case study of lung flukes of bullfrogs and leopard frogs in Costa Rica, which was the first described event of ecological fitting in helminths under natural circumstances (Brooks et al. 2006a). *Haematoloechus floedae* (Digenea, Haematoloechidae) is a fluke species native to the southeastern United States where it harbours in the lungs of the bullfrog, *Lithobates catesbeianus* (formerly *Rana catesbeiana*). The ancestors of the fluke parasitized leopard frogs (*Rana pipiens* clade) so it is

considered as plesiomorphic hosts for *Haematoloechus*. When bullfrogs were introduced to the southwestern United States, the Yucatán, and Costa Rica, they carried the parasite, and are now found in bullfrogs in those areas, as well as in leopard frogs in the Yucatán and Costa Rica. Although the ancestor of *H. floedae* switched to bullfrogs, the presence of the fluke in leopard frogs indicates that the parasite has preserved its plesiomorphic ability to infect leopard frogs as well (Brooks et al. 2006b). Interestingly, bullfrogs have disappeared from Costa Rica, but the parasite still persists, and survived the loss of its preferred host. This is the first demonstration that parasites, like phytophagous insects (Janz et al. 2001) might display ancestral host preferences under certain circumstances.

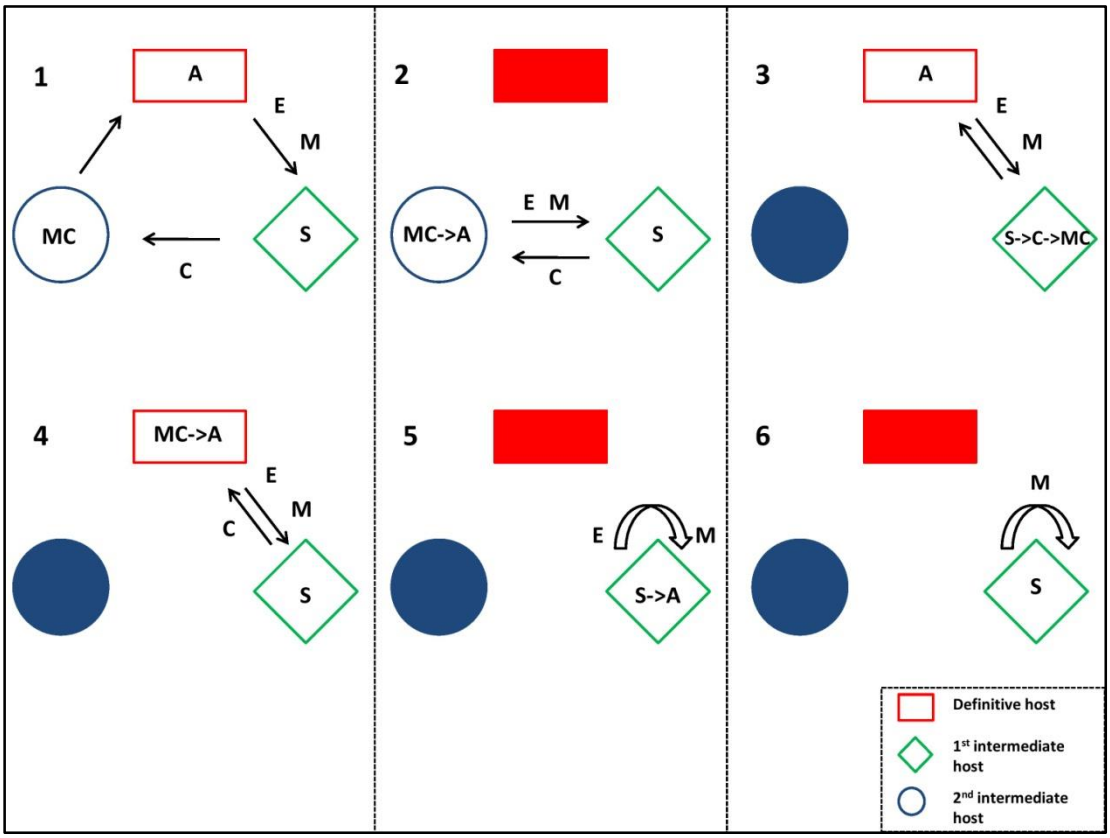

Fig 1 Typical (1) and abbreviated (2-6) trematode life cycles. Five different types of life cycle truncation are: (2) progenesis in the second intermediate host; (3) first intermediate host used as second intermediate host; (4) second intermediate host also used as definitive host (sequentially); (5) sexual adult develops in first intermediate host; and (6) sporocysts in the first intermediate host produce miracidia directly. A: adult parasite; E: egg; M: miracidia; S: sporocyst; C: cercaria; MC: metacercaria. Filled objects indicate the absence of particular host in the abbreviated life cycle. Redrawn after Poulin and Cribb 2002.

1.3 *Helminth parasites of amphibians*

The widespread decline of amphibians has been viewed as an early warning of problems in the environment (Niemi and McDonald 2004). While most attention has been

narrowly focused on the effects of chytrid fungus, *Batrachochytrium dendrobatidis* (Berger et al. 1998; Daszak et al. 1999, 2003; Briggs et al. 2005; Johnson 2006; Van Rooij 2015) and a few viruses (Mao et al. 1997, 1999; Miller et al. 2011; Kik et al. 2011; Stöhr et al. 2013), amphibians host an enormous array of other – mainly protistan and metazoan – parasites that, together with their transmission dynamics and hosts constitute unparalleled sources of information about organized bio-complexity (Prokopic and Krivanec 1975; Brooks 1976; Pérez-Poncé de León et al. 2002; Zelmer et al. 2004). Amphibians invaded a multitude of habitats and exhibit a striking diversity of life history patterns, reproductive modes, body size, foraging modes, and trophic relations (Wells 2007). The associations between anurans and their helminth parasites hence provide a novel model system for investigating evolutionary relations between hosts and parasites. The majority of helminths require water for the development and transmission of infective stages, while most, but not all, major groups of anurans have a reproductional and developmental connection to aquatic habitats. The fact that many parasites are trophically transmitted enables them to provide important ecological information about the host and its interactions in the ecosystem (Hoberg 1996; Marcogliese and Cone 1997; Marcogliese 1995; Overstreet 1997) and to be useful indicators of ecosystem stability (Marcogliese and Cone 1997). The diversity of life cycle patterns among the helminths of anurans makes their parasite communities excellent indicators of trophic complexity. For example, a digenetic trematode usually infect one – or at least some closely related – species of molluscan host, and harbour a variety of second intermediate and definitive hosts. If any change happens in the environment, by the result of pollution, habitat degradation or human mediated fragmentation, it could have a direct or indirect effect on the dynamics of the susceptible host population (i.e. decrease in population density, availability, change in host behaviour, shifting transmission window, etc.) which is mirrored in the characteristics of parasite populations as well. For instance, when certain parasites are absent we can get insight into what might have been happening in the ecosystem. **Paper II** describes the result of an inventory of the endoparasitic helminth community of water frogs (*Pelophylax* sp., Ranidae) in Hortobágy National Park. Before reporting those findings, I give a brief review of the helminth macroparasites of amphibians, especially their occurrence in anurans (for an overview see Table 1).

Helminths are the most common and extensively sampled group of parasites in amphibians. Nematodes occur in the lungs, small intestine, large intestine, rectum and mesenteries of their hosts. The members of the family Rhabdiasidae (about 90 nominal

species) are common parasites of amphibians and reptiles. These species have an alternation of two generations (heterogony) in their life histories. One generation is hermaphroditic and inhabits the lungs of amphibians and some reptiles, while the other one is *gonochoristic* and dwells in the hosts faeces (Kuzmin et al. 2003; Tkach et al. 2014). A few decades ago macroparasites were thought to cause little pathology in amphibians (Prudhoe and Bray 1982) but recent studies has revealed this may not always be the case (for a review see Koprivnikar et al. 2012). In line with this the representatives of this family are disease causing agents and show pathology like granulomatous inflammation, nodules, impaired performance, reduced survival and decreased growth of their anuran host (Goater and Ward 1992; Goater et al. 1993; Green and Muth 2005; Kelehear et al. 2009; Pizzatto et al. 2010; Kelehear et al. 2011; Pizzatto and Shine 2011). Anuran tadpoles can serve as intermediate or paratenic hosts of spirurid nematodes (e.g., *Spiroxys* sp.), which are likely gained by ingesting a variety of other intermediate or paratenic hosts (Anderson 2000) and which are adults in turtles (Hedrick 1935).

Adult cestodes are not common parasites of amphibians, nor do they represent much diversity. They usually occur in low prevalence in the host population, but they can persist for a long time in their anuran host (James and Ulmer 1967; Bursey et al. 2010; Halajian et al. 2013). The genus *Ophiotaenia* (Eucestoda: Proteocephalidae) contains more than 70 species and 23 of them are known to parasitize amphibians (Chambrier et al. 2006). Other well-known cestode parasites of frogs are the members of the genus *Nematotaenia*, *Cylindrotaenia* and *Distoichometra* (Eucestoda: Nematotaeniidae) which usually occur attached to the wall of intestines in their frog host (Prudhoe and Bray 1982; Jones 1987).

Acanthocephalans mainly harbour in the small intestine of their definitive host, which are mostly vertebrates, including amphibians (Nickol 1985). Acanthocephalan diversity of amphibians is also low and species from the genus *Acanthocephalus*, *Centrorhynchus*, *Nematotenia*, *Pomphorhynchus*, *Pseudoacanthocephala*, *Sphaerechinorhynchus* are known to infect amphibians. Basically, most of the cestodes and acanthocephalans use arthropods as their first intermediate hosts, which are then ingested by amphibians in the life cycle.

The monogenean taxon contains helminth species which have direct life-cycles and they are the most host-specific of parasites in general. They are typically external parasites of fishes, but some of them, notably the members of the Polystomatidae family have radiated into anurans (Boeger and Kritsky 2001; Bentz et al 2003). The representatives of the genus

Polystoma, are specialised to live in the urinary bladder of frogs, and is widely distributed across all continents, with the exception of the arctic and desert areas (Bentz et al. 2006).

Finally, the most diverse group of amphibian parasites are digeneans. Anurans serve as intermediate, paratenic and definitive hosts for several digenetic trematode species. The mode of penetration of cercariae (or metacercariae) into the body of their anuran host serves as a key moment during transmission. By all means, the simplest way, when the metacercaria is ingested along with the second intermediate host. Other possibility when for instance, cercariae of *Opisthioglyphe ranae* (Digenea, Plagiorchiidae) encyst in the skin of their frog host. Frogs and toads shed their skin frequently (Weldon et al. 1993) and when this epithelium is shed, it is swallowed together with the encysted metacercariae by the frog itself and opens an opportunity for the parasite to enter the intestines and develop into adult helminths (Grabda-Kazubska 1976). Other species, like *Haplometra cylindracea* (Digenea, Plagiorchiidae) target the buccal cavity of the frog host where they encyst as metacercaria and then migrate to the lungs to mature into adult helminth. Furthermore, trematodes possess diverse armoury to find the way to their next host in the life-cycle. Namely, an interesting case of manipulation occurs through the control of host development by the parasites. For instance, *Ribeiroia* sp. (Digenea, Psilostomatidae) naturally infect different amphibian species as second intermediate host and cause limb abnormalities (missing, malformed or extra limbs) to increase the chance to become victims of bird predation and hence ease their transmission into their next host. This, clearly reduce survival in the metamorphs (Johnson et al. 1999; 2002; 2004). The metacercariae of these parasites penetrate through the skin of the tadpoles after released from the snail first intermediate host. After metamorphosis the metacercariae migrate to the tissue around the pelvic girdle and hind limbs and cause malformations.

Table 1 Notable helminth groups that parasitize anurans

Species	Phylum	Parasite life-stage	Host	Anurans role in life-cycle	Site of infection	Pathology	References
Acanthocephalus ranae	Acanthocephala	Adult	*Bufo bufo*	Definitive host	Intestines	Reduced survival	Elkan 1960
Rhabdias ranae	Nematoda	Adult	*Lithobates pipiens*	Definitive host	Lungs	Decreased splenocyte cellularity and phagocytosis	Christin et al. 2003
Rhabdias bufonis	Nematoda	Adult	*Bufo bufo*	Definitive host	Lungs	Reduced survival, decreased growth, impaired performance	Goater and Ward 1992; Goater et al. 1993
Spyroxis contortus	Nematoda	Third stage larvae	*Lithobates clamitans*	Paratenic host	Encapsulated on the mesentery	Not examined	Anderson 2000
Nematotenia dispar	Platyhelminthes	Adult	*Bufo bufo*	Definitive host	Intestines	Gastrointestinal lesions and obstruction	Elkan 1960
Cylindrotaenia jaegerskioeldi	Platyhelminthes	Adult	*Amietophrynus gutturalis, Schismaderma carens*	Definitive host	Intestines	Not examined	Halajian et al. 2013
Polystoma interregnum	Platyhelminthes	Adult	*Pelophylax esculentus*	Definitive host	Urinary bladder	Not examined	Popiolek et al. 2011
Opisthioglyphe ranae	Platyhelminthes	Adult	*Pelophylax esculentus, Pelophylax lessonae*	Definitive host	Intestines	Not examined	Popiolek et al. 2011
Ribeiroia ondatrae	Platyhelminthes	Metacercariae	*Hyla regilla*	Second intermediate host	Tissue around the pelvic girdle and hindlimbs	Reduced survival, decreased growth, limb malformation	Johnson et al. 1999
Haplometra cylindracea	Platyhelminthes	Adult	*Rana camerani, Rana macrocnemis*	Definitive host	Lungs	Not examined	Düsen 2007

1.4 *Pelophylax esculentus complex*

A main conclusion from the studies of host-parasite associations can be drawn namely, the precise identity of host species is prerequisite for further studies. This is especially true for species complexes formed by hybridisation of parental species. As it was demonstrated previously (see chapter 1.3), anurans harbour a great diversity of helminth parasites but it is not the only reason we choose them for the model host taxa for our inventory. The western Palearctic water frogs (WPWF) in the genus *Pelophylax* (since Frost et al. 2006; formerly genus *Rana*) include twelve species with partially overlapping morphological features. Some of these species are able to form hybridogenetic species complexes (for reviews see Graf and

Polls-Pelaz 1989; Günther 1990; Plötner 2005). Hybridogenesis (Schultz 1969) is a unique way of reproduction resembles parthenogenesis in many aspects. While in parthenogenetic animals, females clonally produce diploid eggs, which give rise to a new generation of daughters, hybridogenetic individuals (i.e. hybrids) transmit clonally only one chromosome set (maternal genome); the other set of chromosomes (paternal genome) is eliminated during gametogenesis and is restored in each generation by mating (Tunner and Heppich 1981; Fig 2). Among WPWF one of the hybridogenetic complexes is the *Pelophylax esculentus* complex formed by the two parental species, *Pelophylax ridibundus* (Pallas, 1771), genotype RR; *Pelophylax lessonae* (Camerano, 1882), genotype LL; whose primary hybridization leads to hybridogenetic lineages of *P. esculentus* (Linnaeus, 1758), genotype LR, which acts as a sexual parasite that restores hybridity by mating with the respective parental species whose genome was excluded (Fig 2). In hybridogenetic taxa, fertilization takes place but normally there is no recombination between the parental genomes. Without genetic recombination via sexual reproduction, hybrids have to face evolutionary costs of clonal reproduction. The hybrids may have insufficient ability to defence against fast-evolving parasites (Hamilton 1980) and because the lack of beneficial mutations reshuffling of genes caused by genetic recombination (Colegrave 2002; Cooper 2007). Finally, and most importantly, genetic recombination eliminates the deleterious mutations from the population (Muller 1932; Vrijenhoek 1994).

In the *P. esculentus* complex, the type of breeding system determines which chromosome set gets transmitted clonally, and which is excluded from the germ line prior to meiosis (Fig 2). To restore the LR genotype hybrids must backcross with one of the parental species. The most frequent type of breeding system in central Europe is the LE system. Here the *P. lessonae* forms sympatric populations with *P. esculentus*. In the LE system the L genome is excluded in hybrids during gametogenesis and the R genome is transmitted clonally, hence mating with *P. lessonae* is required to restore the hybrid genotype. In the RE system, hybrids eliminate the R genome, transmit L gametes clonally and backcross with *P. ridibundus*. This type of mating system is less common and has been found only in eastern Germany and Poland (Uzzell and Berger 1975; Berger 1977; Uzzell et al. 1980). *Pelophylax esculentus* is also able to maintain pure hybrid populations which are reproductively isolated from the parental species (EE system). In these all-hybrid populations, hybrids are either diploid (LR), or triploid (LLR or LRR). LR frogs make LR and/or R gametes, LLR frogs make L gametes and LRR frogs make R gametes (Graf and Polls-Pelaz 1989; Christiansen

and Reyer 2009; Jakob 2007). Moreover, it seems that in Scandinavian populations, LLR triploids recombine their homospecific genomes, thus resembling sexual reproduction (Christiansen et al. 2005; Christiansen and Reyer 2009). These all-hybrid systems frogs of different genotypes are dependent on mating with each other for reproduction and the propagation of both parental genomes, as well as any recombination within them, must be undertaken by hybrids alone. This type of breeding system of water frogs is found mostly in north-western Europe, and less in central and eastern Europe (Günther 1973; Ebendal 1979; Berger 1988; Hoffmann et al. 2015).

In the previous paragraphs I outlined that the taxon composition and distribution of water frogs strongly depend on their mode of hybridogenesis, but they are also affected by environmental variables, as the parental species largely differ in their habitat preferences (Günther 1974). *Pelophylax lessonae* can usually be found in smaller water bodies, like marshes with low oxygen levels (Rybacki and Berger 1994; Pagano et al. 2001a; Plenet et al. 2005). In contrast, *P. ridibundus* prefers larger water bodies like lakes, river backwaters, oxbows and canals where the water is continuously refilled with oxygen. It often occupies alluvial habitats and shows a higher tolerance for floods (Pagano et al. 2001a,b). Hybrids occupy similar habitats as *P. lessonae* like marshes, sandpits or oxbows, but are found also in fishery ponds and gravel pits, which are mainly preferred by *P. ridibundus* (Pagano et al. 2001a; Mikulíček et al. 2015). Therefore, the taxon composition (not equal with the breeding system) of water frog populations is highly influenced by the characteristics of the habitats, which could then directly affect their breeding system. Therefore, the hybrid habitat preferences might be linked to the hybridogenetic system and reproductive dependence of hybrid on their parental species.

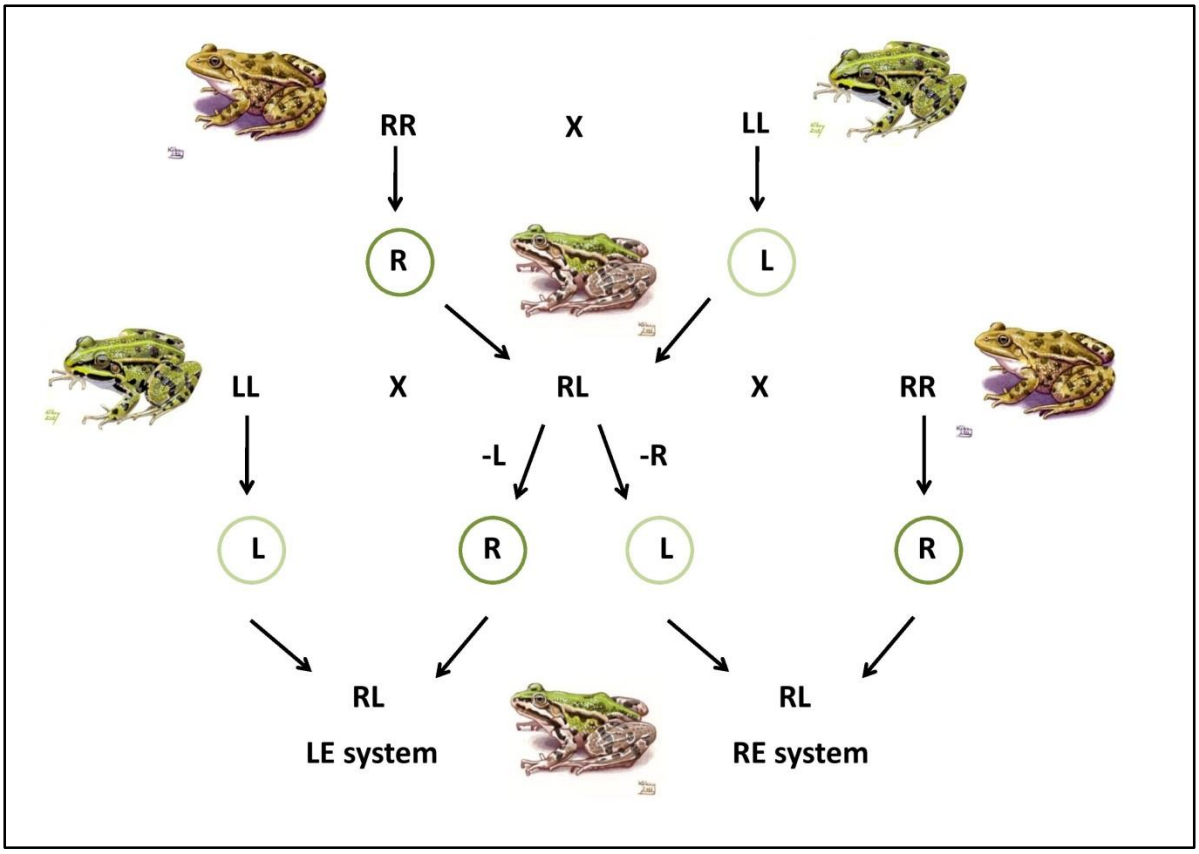

Fig 2 Interspecific mating between *Pelophylax ridibundus* (RR) and *P. lessonae* (LL) leads to hybridogenetic lineages of *P. esculentus* (RL). Most of hybrid lineages exclude *lessonae* (L) genome during gametogenesis, produce unrecombined *ridibundus* (R) gametes and backcross with syntopic *P. lessonae* (LE system). In a RE system, hybrids eliminate predominately *ridibundus* (R) genome, produce *lessonae* (L) gametes and mate with syntopic *P. ridibundus*. Redrawn after Mikulíček et al. 2015.

Identification of water frogs is sometimes challenging and taxon determination based solely on morphology or call of individuals may lead to species misidentification (Lode and Pagano 2000). This is especially apparent in the case of juveniles. Recent papers have shown that *lessonae* haplotype has a greater influence on morphology than *ridibundus* haplotype, all the hybrids being morphologically more similar to *P. lessonae* than to *P. ridibundus* (Kierzkowski et al. 2011, 2013). Furthermore, it is impossible to distinguish between hybrids of different ploidy levels (LR, LLR, LRR) by morphological means. Although, the colouration pattern of different genotypes shows some trends, there are numerous exceptions, with the most prominent being much higher variability of colouration of *P. lessonae* individuals (Kierzkowsi et al. 2013). Nevertheless, various molecular methods may give reliable results (for a brief overview of molecular methods used see Borkin et al 2004;

Hauswaldt et al. 2012) and it is expected that their combined use can provide strong evidence about the taxonomical composition and level of ploidy in the hybrid populations.

In Hungary, the *Pelophylax esculentus* complex has the most frequent occurrence in lowland aquatic habitats of natural, semi-natural or urbanized areas (Puky et al. 2005; Solomampianina and Molnár 2011; Herczeg et al. 2012). Our understanding of the taxonomic and genotypic composition of Hungarian water frog populations is limited: only a few studies have been published (Mészáros 1973; Mészáros and Bartos 1978; Berger et al. 1988; Lőw et al 1989; Gubányi 1990; Gubányi and Pekli 1991; Gubányi and Korsós 1992; Uzzell and Heppich-Tunner 1992; Gubányi and Creemers 1994) and solely Berger et al. (1988) and Tunner and Heppich-Tunner (1992) examined their breeding system. Historical data is scarce and publications from the early and mid-20th century did not distinguish between *P. lessonae* and *P. esculentus* (Bolkay 1909; Fejérváry 1921; Fejérváry-Lángh 1943; Dely 1953). **Paper III** aimed to contribute to the knowledge of the distribution, taxon composition and breeding system of water frogs in Hungary.

1.5 *Polyploidy, hybridogenesis and population genetics*

Polyploidy has played a major role in the evolution of many eukaryotes (Sidow 1996; Spring 1997). The recent discoveries in speciation are that many *polyploid* species formed recurrently from natural populations of their progenitors (for a review see Soltis and Soltis 1999). Molecular genetic studies of extant clonal vertebrates have shown that they are often polyploids and most have genomes combined from at least two ancestral sexual species (Lamatsch and Stöck 2009; Kearney et al. 2009).

In vertebrates, shifts from sexual reproduction to clonal are acquired in three reproductive modes. First, in parthenogenesis, offspring develop from unreduced eggs without any male contribution. Second, in hybridogenesis, one of the parental genomes is discarded during the initial steps of meiosis, followed by the production of clonal gametes containing the other parental genome. Third, in gynogenesis, in which unreduced egg need to contact with sperm to trigger the development, but do not incorporate the paternal genetic material, i.e., the sperm only activates the egg and do not fertilize it (for a review see Bullini 1994). The common features of most asexual forms are their hybrid origin, which is tightly linked with polyploidy, the disruption of meiosis, closely associated with no or limited recombination and the transmission of maternal (rarely paternal) genome to the next

generation (Schön et al. 2009; Choleva et al. 2012). The mode of hybridogenesis in water frogs were detailed in chapter 1.4, herein I briefly refer the population genetic approaches which may help to understand the genetic mechanisms that shape the evolution of clonality.

In the past decade the first generation DNA sequencing by chain termination and fragmentation (e.g. Sanger et al. 1977) has been replaced by next generation sequencing (NGS) techniques in molecular biology and biotechnology (reviewed by Morozova and Marra 2008; Shendure and Ji 2008). The enormous cost of the Human Genome Project (which developed with Sanger genome sequencing in 2004) stimulated the emergence of the cost-effective NGS technologies like Roche 454, Illumina/Solexa, ABI SOLiD and Helicos tSMS and their application rapidly spread among molecular ecologists (for example see Ekblom and Galindo 2010; Gardner et al 2011). The main advantages of NGS techniques are the capability to sequence thousands-to-many-millions reactions simultaneously and electrophoresis does not need to detect the sequence output. In contrast, the main disadvantage is the required infrastructure, such as computer capacity and storage for huge data, and also the personnel expertise in bioinformatics required to comprehensively analyse and interpret the results. NGS and whole genome sequencing (WGS) tools are also valuable for the discovery, validation and assessment of genetic markers in populations. The technological background of *microsatellite* development has recently undergone a revolution with these technologies allowing large numbers of DNA sequences to be searched for microsatellite repeats (e.g. Csencsics et al. 2010; Davey et al. 2011; Gardner et al. 2011; Fernandez-Silva et al. 2013; Králová-Hromadová et al. 2015).

The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular biology (Allendorf 2017). Molecular markers represent a powerful tool for identification of morphologically similar species and their hybrids. An ideal marker is species-specific, with alleles alternatively fixed in the species of interests. This rule, however, does not hold in many species complexes and shared *polymorphic loci* may obstruct its straightforward application for species and hybrid identification. The fact that two species share the same genetic marker could be a result of introgressive hybridization, when a DNA fragment of one species is transferred to a gene pool of another species via hybrids, or ancestral polymorphism, when two species carry the same sets of alleles from their common ancestor (e.g. Babik et al. 2005; Mims et al. 2010; Nadachowska-Brzyska et al. 2012).

With the implementation of population genetic approach we study evolutional mechanisms in the level of populations, i.e., how the frequency of alleles changes over time and the reasons for these changes. This means that we are interested in the evolution of polymorphisms. Genetic diversity among populations occurs if there are differences in allele and genotype frequencies between them and can be measured using several different metrics that are all based on allele frequencies in populations. One of these is the measure of genetic diversity based on allelic richness (AR) which is considered important in conservation genetics (Schoen and Brown 1993; Bataillon et al. 1996) and the indication of *population bottlenecks* in the past (Nei et al. 1975). The application of allelic richness is also relevant in a long-term perspective, as selection limits are determined by the initial allelic composition more than by *heterozygosity* (Petit et al. 1998). Heterozygosity (*H*, or gene diversity after Nei 1973) can be calculated in several ways and values of these measures will range from zero to nearly one. If the values close to 0 that indicates a low level of heterozygosity, consequently low genetic variability in the population. On the other hand, when the values close to 1 that shows the population contains a large number of equally frequent alleles and high genetic diversity. Most of the time we compare the observed level of heterozygosity (H_0) to what we expect under Hardy-Weinberg equilibrium. If the observed heterozygosity is lower than expected heterozygosity (H_E), we seek to attribute the discrepancy to forces such as inbreeding or a bottleneck event. If H_0 is higher than H_E, we might suspect an effect which mixes previously spatially or genetically isolated populations together.

Genetic markers such as *allozymes*, microsatellites, *single nucleotide polymorphisms* (*SNP*s) or mitochondrial and nuclear DNA sequences can be used widely to estimate many parameters of interest in population genetics and conservation biology (Allendorf 2017). The metrics mentioned in the previous paragraph can be calculated after microsatellite analysis. Microsatellites have emerged as one of the most popular genetic markers for studies dealing with population size, bottlenecks or even migration rates (reviewed by Selkoe and Toonen 2006). A common feature of such genetic studies is that no information can be gained on population structure if the markers implemented are not polymorphic. In contrast to the slightly outdated allozyme methods, DNA-based techniques, such as microsatellites, use PCR approach to amplify the marker of interest from a tissue sample. Furthermore, microsatellite markers are usually shorter in length than sequenced loci so they can amplify with PCR despite DNA degradation (Taberlet et al. 1999). This trait allows microsatellites to be used such DNA extraction methods, like ancient DNA, or DNA from hair or even faecal samples

used in non-invasive sampling (Taberlet et al. 1999). Finally, because microsatellites are species-specific, cross-contamination by non-target organisms is much less of a problem compared with methods which applicate universal primers that sensitive for any species (Selkoe and Toonen 2006). Microsatellite markers generally have high-mutation rates resulting in high standing allelic diversity which makes them ideal markers for population genetics, evolutionary and conservation biology (Hedrick 1999).

If microsatellite data set is put together we can relatively easily calculate indices like multilocus genotypes (MLG) and multilocus disequilibrium (or multilocus linkage disequilibrium, $\bar{r}_d$). In the *Pelophylax esculentus* complex one of the parental genomes in hybrid individuals transmitted clonally. In that way, we can determine the level of genome clonality calculating these indices from microsatellite data (e.g. Pruvost et al. 2015; **Paper III**). Multilocus genotypes are the number of identical combinations of alleles found in microsatellite datasets. Few combinations and high frequencies of these combinations may indicate clonal reproduction. Because parental genomes virtually do not recombine (see e.g. Hoffmann et al. 2015) in hybridogenetic lineages in *P. esculentus*, frequent MLGs would represent hemiclones (i.e. clonally transmitted haploid genomes sensu Vrijenhoek 1979). Besides the detection of genome clonality MLG is also effective to study introgressive hybridization (Barilani et al. 2007) or even human migration (Rannala and Mountain 1997).

Processes like population differentiation and isolation by distance, asexual reproduction, linkage, and natural selection can cause $\bar{r}_d$ (Agapow and Burt 2001). Multilocus disequilibrium is often quantified using statistics of association between allelic states at pairs of loci. This index is a score of non-random association of alleles at microsatellite loci and values varies on a scale from 0 to 1. Low $\bar{r}_d$ values are related to high recombination rate and thus would indicate low levels of clonality. Contrarily, high $\bar{r}_d$ values might be associated with clonal inheritance, as clonal genomes are inherited *en bloc*, without recombination.

2. Objectives and major results

In this section, I specify the objectives of the studies, and also briefly refer the core method used. Furthermore, the new scientific findings are outlined. Note, that details could be found in **Papers I-III** in Part II.

2.1 Paper I *Proposed new parasite inventory protocol* (Brooks et al. 2014)

Objectives

Parasites occupy a central role in efforts to develop proactive protocols for monitoring changes in ecosystem structure and for detecting the potential for emerging disease in resident and colonizing host species, be they human, livestock, or wildlife. Traditional parasitological inventories of parasites aimed to collect as many parasites from as many hosts as possible especially hosts that have not been examined for parasites previously. This may request extensive and destructive sampling of hosts which we would like to restrict into the initial documentation stage in our proposed DAMA (documentation–assessment–monitoring–action) inventory protocol. Our intention was to:

(i) propose a general parasite inventory protocol which can serve as a blueprint for many different collaborative efforts; and

(ii) provide a broad platform for essential information about the evolution, ecology, and epidemiology of parasites across host groups, parasite groups, geographical regions, and ecosystem types.

Results

DAMA is a comprehensive proactive action plan for dealing with climate change and emerging diseases. Baseline studies (i.e. faunal surveys) of parasites must be brought back to centre stage, but within a novel conceptual framework. DAMA approach suggests that it is not necessary to kill large numbers of hosts to obtain important information about parasite biodiversity. The implementation of DNA barcoding has the potential to supply quick and cost-effective ways to monitor parasite species and their transmission dynamics and thus can be used as a baseline for future non-destructive study within the monitoring phase of DAMA. Our protocol also suggest that all inventory information should be stored in a form that can be updated in real time and that is freely and readily available on the world wide web (for example see species web pages in the Encyclopaedia of Life or Global Biodiversity Information Facility database).

2.2 Paper II *Parasite survey based on DAMA protocol – initial steps: documentation and assessment* (Herczeg et al. 2016)

Objectives

In this study we wanted to depart from classical parasite inventories aim to collect as many parasites from as many hosts as possible. Hence, we followed the DAMA protocol (**Paper I**), presenting findings documenting the helminth fauna of water frogs in eastern Hungary and initial efforts in assessing that fauna with respect to previous studies. We have chosen the Hortobágy National Park (HNP) for inventory site and surveyed the members of the *Pelophylax esculentus* complex as host species. DAMA protocol suggests that destructive sampling of hosts ends with the initial documentation phase and non-invasive molecular taxonomic methods (i.e DNA barcoding) will replace it on the later monitoring stage. The faeces of the host contain eggs of parasites which are a good indicator of helminth species inhabiting in the gastrointestinal tract or the respiratory organs. In line with this, we collected parasite material not just for classical morphology-based taxonomic identification, but also a portion of the helminth specimens were preserved in 96% EtOH for development of DNA barcodes. With the application of DNA barcodes of the helminth species examined previously will make the parasites easily and non-invasively detectable for future monitoring. Our goals were to:

(i) determine the macroparasite fauna (i.e. helminth community) of the *Pelophylax esculentus* complex in HNP using morphological species identification of parasites species;
(ii) compare our faunistical findings with previous studies; and
(iii) collect DNA samples from helminths to develop DNA barcodes of each parasite species for future non-destructive survey within the monitoring phase of DAMA.

Results

The examined host population composed of *Pelophylax ridibundus* and *Pelophylax esculentus*. The overall prevalence of endoparasitic helminths was 76.2% in the examined water frog population. The overall mean intensity was 2.44 helminth species per host. We find that the macroparasite fauna changed significantly during the past 40 years. Our

faunistical findings were set up into three categories: persistent core species, missing species and species new to HNP. The helminth fauna of water frogs in HNP consists of the loss of 5 species of digenean trematodes, 1 species of nematode, and the addition of 2 new species for HNP. We also found that *P. ridibundus* individuals inhabiting canals had a significantly greater overall mean intensity of helminth species per host than did the same species in still water (χ^2 = -4.782, df = 1, P = 0.002). As it was suggested in **Paper I** we developed species home pages for each parasite species in *Encyclopaedia of Life* database which contains all of the relevant inventory information i.e. images of parasites (uploaded to *flickr*), reported hosts, distribution and references.

2.3 Paper III *Taxonomic and genotypic composition of water frogs* (Herczeg et al. 2017)

Objectives

In this study, we focused on the *Pelophylax esculentus* complex, which consists of two sexual species, *Pelophylax ridibundus* and *Pelophylax lessonae* and their hybridogenetic hybrid, *Pelophylax esculentus*. We investigated the taxonomic composition and ploidy level of water frogs sampled in three different types of wetland habitats in eastern Hungary at HNP. We applied population genetic approach by calculating allelic richness, gene diversity, multilocus genotypes and multilocus disequilibrium from microsatellite data to determine the type of gametes produced by hybrids and to infer the breeding system of water frogs. Specifically, our aim was to:

(i) determine the taxonomic composition of the *P. esculentus* complex using molecular markers;

(ii) specify the ploidy level of hybrid individuals; and

(iii) examine the effect of population structure on genetic diversity and the degree of clonal inheritance in water frogs using population genetic indices.

Results

Allele size polymorphism in serum albumin intron-1 gene fragment and microsatellite data detected all members of the *P. esculentus* complex in the localities studied. We identified two types of populations occurring in HNP. In two sites we found *P. ridibundus* and *P. esculentus* individuals (RE population) and a population with all three taxa, but dominated by *P. esculentus* occurred at the third site (LER population). The microsatellite data showed that no triploid (LLR or LRR) frogs were present in the sample. All hybrid individuals were indicated to have diploid (LR) genotype. Our data demonstrated that at least in two populations hybrids form gametes with clonally transmitted *P. ridibundus* genome and can produce a new hybrid generation by mating with *P. lessonae* (LE system).

3. General discussion

In the studies presented in Part II, I investigated a complex system both in the level of parasites and their hosts, respectively. The proposed DAMA protocol (**Paper I**) differ from parasite inventories suggesting that destructive sampling of hosts ends with the initial documentation phase. This will require comprehensive taxonomic inventories. We need to gather species-associated information, and discover as much as possible about the natural history of each parasite species, especially with respect to its geographic origin, transmission dynamics, microhabitat preferences, and host range. For that, essential to get access to voucher specimens of parasites and hosts in properly curated archival collections (Frey et al. 1992; Haverkost et al. 2010). The role of archival collections held in museum repositories are essential and collections developed from earlier inventories provide important historical data, and predictive baselines for understanding the patterns, distribution and evolutionary processes of organisms in the biosphere (Holmes et al. 2016, Dunnum et al. 2017). The infrastructure for collections must be regarded as an integral component of any developing programs for survey, inventory, and documentation of global biodiversity resources (Brooks and Hoberg 2000, 2001; Scholz 2001).

Basically, in the initial, documenting stage we need to answer the following questions. What parasite species are present in the inventory site? When are they there in the season? In what hosts, and in what parts of the hosts are they found in, and how are they transmitted? If these questions are answered, then we can step into the assessment phase of DAMA. Species-specific information about ecology, behaviour, and geographic distributions, examined in an evolutionary (i.e., phylogenetic) context, provides more information than a simple list of species and their known properties (Brooks and McLennan, 2002) and this fundamentally Darwinian perspective is amplified in the case of parasites. According to Manter (1966) who stated that parasites always tell not just the stories of their own ecology, but also that of their hosts and of the geographic distributions and complex ecosystems in which they live and evolve. **Paper I** and **Paper II** seek to follow that assertion in their own way. The importance of classical parasitological inventories was highlighted but in a new perspective.

I followed the DAMA protocol and carried out a new type of parasite inventory (**Paper II**) in eastern Hungary at Hortobágy National Park (HNP). As suggested by **Paper I**, assessment in the DAMA protocol is a two-part process. The first of these is comparison of data with baselines (i.e., what is already known about the parasites, hosts, and geographic location of an inventory) to address the question of how current findings relate to previous

ones. At HNP a baseline inventory was conducted 40 years ago with a few but essential deficiencies (Edelényi 1972; Murai et al. 1983). First, in these studies no voucher specimens (hosts or parasites) were fixed, thus making comparisons with recent studies difficult. Second, the authors did not provide any prevalence or mean intensity data of helminth infection in anuran hosts and all water frogs were identified as either *Rana ridibunda* (=*Pelophylax ridibundus*) or *Rana esculenta* (=*Pelophylax esculentus*). Information derived from such long-term studies allows the determination of the persistence and stability of parasite diversity (Radomski and Pence 1993; Bursey et al., 2010). In our case at HNP the helminth community of water frogs significantly changed during the last four decades. We detected the loss of six species and the emergence of two new species to the area (**Paper II**). The species previously reported in the baseline inventory but absent from our, seem to have little in common with respect to their basic ecology: as a group they live in the rectum, small intestine, urinary bladder, and lungs. Their first intermediate hosts include both gastropods and bivalves, and their second intermediate hosts range from the surface of plants and the exoskeleton of crustaceans to odonate larvae and tadpoles. One thing the missing species may have in common is their water frog host.

Of the three members of the *Pelophylax esculentus* complex occurring in HNP, Edelényi (1972) and Murai et al. (1983) listed only *P. ridibundus* and *P. esculentus* as hosts, and all the missing helminth species were reported from these two species. In **Paper II**, we collected only *P. ridibundus* and *P. esculentus*, so it is tempting to suggest that the missing parasites still occur in HNP, but that they are restricted to *P. lessonae*, which we have not collected in this study. Unfortunately no research paper focused on the spatial distribution of *P. lessonae* at HNP, and we can only refer to the presence or the absence of the species in eastern Hungary (Mészáros 1973; Mészáros and Bartos 1978; Gubányi and Korsós 1992; Mester et al. 2015; Béla Mester pers. comm.). Our results in **Paper III** confirmed the presence of *P. lessonae* in HNP, but in low abundance.

Paper III aimed to determine the taxon composition of water frogs in HNP through the examination of three distinct populations related to different habitat characteristics. The 164 frogs examined comprised 100 *P. ridibundus*, 1 *P. lessonae* and 63 *P. esculentus* individuals. We found only a single juvenile *P. lessonae* during the three years of collecting. Two alternative explanations were proposed for the low detection probability of *P. lessonae* individuals. First, *P. lessonae* may be more abundant in HNP (we found only in Egyek-Pusztakócs marsh system, a habitat that fulfils *P. lessonae* habitat preferences) and our results affected by small sample size in Egyek-Pusztakócs marsh system (N=21). Second, the species

suffered a severe population decline and now is become rare in HNP. Mészáros and Bartos (1978) confirmed the presence of *P. lessonae* in HNP, but they did not provide sample size data of collected specimens, so it is hard to make any conclusions about their past abundance. On the other hand, Mester et al. (2015) reported higher frequencies of *P. lessonae* at Egyek-Pusztakócs marsh system based on morphological and call monitoring data. This may support our first explanation about the effect of our low sample size.

The genomic composition of the examined hybrid specimens analysed using microsatellites showed the occurrence of diploid (LR) genotypes only. Tunner and Heppich-Tunner (1992) reported triploids in a Hungarian water frog population in northwestern Hungary and they described a population system comprising *P. ridibundus* of both sexes, diploid *P. esculentus* of both sexes, and exclusively male triploid (LLR) *P. esculentus*. This was the only report of a triploid population in Hungary; all others (Mészáros and Bartos 1978; Berger et al. 1988; Gubányi and Korsós 1992; **Paper III**) encountered only diploid hybrids.

The most appropriate way to find a type of gametes produced by hybrids and to determine breeding system of water frogs is to implement crossing experiments (Christiansen 2009). In the lack of crossing experiments, we aimed to assess the breeding system with the application of population genetic approach (calculating allelic richness [AR], genetic diversity [*H*e], multilocus genotypes [MLG] and multilocus disequilibrium [$\bar{r}_d$] from microsatellite data). **Paper III** was the first study attempting to use $\bar{r}_d$ to infer breeding systems of water frogs. The three localities analysed produce different results. In two populations, as expected the L genome was indeed less clonal, and had the higher level of genetic diversity than the R genome. This suggests that those populations belong to the LE system typical in central Europe, in which hybrids produce R gametes and can only produced hybrids by mating with *P. lessonae*. The third population may suggests the presence of an RE system (L genome had a lower number of MLG's, high score of $\bar{r}_d$ and a lower level of genetic diversity than the R genome) but we suspect that a lower number of *P. lessonae* parents might produce a genetic signal in the hybrid offspring that is indistinguishable from clonal reproduction (i.e. a low number of multilocus genotypes and high value of $\bar{r}_d$). This could explain why the third population could also belong to the LE system.

Multilocus disequilibrium analysis didn't provide ideal results in regard of the breeding system of water frogs in **Paper III**. Although, it doesn't mean that $\bar{r}_d$ is not potentially useful and not hiding additional information for breeding system determination studies. Christiansen and Reyer (2009) used it successfully – but slightly differently – for a many population study in Scandinavia. Basically, their paper suggests that there is often much

scatter in $\bar{r}_d$ data so that several populations are needed for a clear trend to show. Actually, **Paper III** and Christiansen and Reyer (2009) may point in the same direction, namely $\bar{r}_d$ might be more suitable for investigating trends in a handful of populations than to draw conclusions for a single populations. In fact, 2 studies are not adequate to draw such conclusions. To fully evaluate this method for breeding system determination, ideally: it should test this new idea with microsatellite data from a large sample of populations from different mating systems, in which the mating system had already been determined from crossing experiments. Second best option is to test it in at least 4 typical LE-system populations with a handful of both *P. lessonae* and *P. esculentus* frogs, and 4 typical RE system populations with a handful of both *P. ridibundus* and *P. esculentus* individuals in the sample. Afterwards, to examine the limitations of the method, it should be tested in less typical/ideal populations with fewer parental species or both parental and hybrids in the sample. In such populations the results might be expected less clear as it was presented in **Paper III**. From these less typical/ideal populations crossing experiments needed to show if the mating system is clear or there are some kind of admixture of the typical LE system and RE system gamete patterns.

All in all, the way **Paper III** applied $\bar{r}_d$ in relation to breeding system is novel and potentially useful for future water frog studies aiming to determine breeding system without the implementation of crossing experiments. More and more researchers put together microsatellite data sets to tell apart LL, LLR, LR, LRR and RR genotypes in *P. esculentus* complex (Christiansen 2005; Christiansen et al. 2005; Som and Reyer 2006; Daum et al. 2012; Hoffmann et al. 2015; Pruvost et al. 2013; Mikulíček et al. 2015). This is an extensive work, so afterwards it is desired to gain more information out of the microsatellite data set than just genotype, genetic diversity or the isolation by distance. The measure of $\bar{r}_d$ is such an additional information, which is easy to calculate with the program Multilocus 1.3 (Agapow and Burt 2001) once the microsatellite data set is put together but it need to be gather experience on how to interpret the results.

4. Conclusions and the way forward

The studies of this thesis examined a host-parasite system in Hortobágy National Park, in eastern Hungary. In addition, new approach of parasite inventory protocol was proposed and also applied on that system. The growing focus on parasites in the past few decades revealed that parasitic organisms cover a great majority of known biodiversity and they started to recognize them as significant linkage components between climate change, biodiversity dynamics, and emerging infectious diseases (Brooks and Hoberg 2006; Agosta et al. 2010; Daszak et al 2000; Weaver et al. 2010; Altizer et al. 2013; Hoberg and Brooks 2013). We documented and assessed the macroparasite fauna of *Pelophylax esculentus* complex and collected helminth material not just for classical morphology-based taxonomic identification, but the majority of the samples were preserved for the development of DNA barcodes (Herczeg et al. in prep). DNA barcoding emerged as a cost-effective method for rapid species identification (Hebert et al. 2003). The information derived from DNA barcoding is far more beyond than just simply tag organisms with a species ID. It has the potential to accelerate the discovery of new species and improve the quality of taxonomic information. Additionally, it offers a unique way to perform non-destructive monitoring of hosts for parasites. This creates the potential to shift from logistically challenging field collections, necropsy, and morphological characterization based on assessments of a few hosts to more geographically extensive, site-intensive, and near-simultaneous sampling across ecosystems, thus linking landscape to regional scales for assemblages of host species and populations (e.g., Jennings et al., 2005; Kutz et al., 2007; Alcantár-Escalera et al. 2013). We are taking advantage of that helminths release their eggs to the environment along with the host faeces and are easily collectable during keeping the frogs captivity for overnight in the laboratory. We have an ongoing project with already collected faecal samples from water frogs, in which we aim to test our formerly developed DNA barcodes (not yet published) for helminth parasites. Through the application of barcodes we can step into the monitoring phase of DAMA but without the need to kill any more host specimens. This potential makes this method highly valuable and also serviceable for monitoring hosts with vulnerable populations or strictly protected status. The monitoring of host population is also in progress in order to clarify the distribution of *P. lessonae* and gather more knowledge about the breeding system of water frogs in HNP. If we can collect more *P. lessonae* individuals it can answer our earlier assumption in **Paper II**, in which we hypothesized that, the missing parasite species may still occur in Hortobágy National Park, but recently became restricted to *P. lessonae*.

5. Összefoglaló

Ökológiai megközelítésben, úgy gondolunk a parazitizmus fogalmára, mint két faj szimbiotikus interakciójára, ahol hosszas intim kapcsolat áll fenn a parazita és gazdaszervezete között. Köztudott, hogy a paraziták tápanyagokat vonnak el a gazdaszervezettől és csökkenthetik annak túlélési, illetve szaporodási esélyeit, amely bizonyos esetekben a gazda pusztulásához is vezethet. Az élősködők diverzitása a Földön egyedülállónak mondható, hiszen a ma ismert élőlények megközelítőleg a fele parazita életmódot folytat. Korábban ezen élősködők sokféleségének felmérése kimerült az egyszerű fajleírásokban, illetve a faunisztikai jellegű vizsgálatokban. Az utóbbi évtizedekben, ezzel ellentétben a figyelem egyre inkább az olyan folyamatok feltárására irányult, amelyek a parazita közösségek összerendeződésének a mintázatait hivatottak minél jobban megismerni, továbbá az ezt irányító háttérmechanizmusokat vizsgálták. Ennek a diverzitásnak, továbbá a gazda-parazita kapcsolatok feltárásának elengedhetetlen eleme, hogy mind a gazda, mind pedig a parazita fajok pontos taxonómiai azonosításon essenek át. Továbbá bizonyító példányaik múzeumokban, vagy szabadon hozzáférhető gyűjteményekben is elérhetőek legyenek, így téve lehetővé a múltbéli és a jelenlegi mintázatok összehasonlítását.

Az endoparazita férgek (összefoglaló néven helminthek) világszerte a leginkább kutatott parazita csoportok közé tartoznak. Doktori értekezésemben ilyen helminth közösségek összetételét tanulmányoztam a *Pelophylax esculentus* komplexen. A Hortobágyi Nemzeti Parkban 40 évvel korábban végeztek alapkutatást a helyi gerincesek helminth élősködőinek feltárására, így összehasonlítható volt a helminth közösségek időbeli stabilitása is. Másrészről a gazda szervezeteknek az egymással történő hibridizációja és szaporodási rendszerük feltárása egy további szinten tette lehetővé ennek az összetett rendszernek a megismerését. Mindezek mellett, értekezésem során egy olyan nem-invazív parazitagyűjtési (lásd DAMA protokoll) módszer kezdeti fázisait is megvalósítottam, amely a DNS vonalkód eljárás segítségével egy gyors és költséghatékony módját teszi lehetővé a helminthek monitorozásának. Továbbá az invazív mintavételezést minimalizáló törekvésként, egyaránt hozzájárulhat a védett és fokozottan veszélyeztetett, illetve sérülékeny populációkkal rendelkező gazda szervezetek helminth faunájának nyomon követéséhez.

I. cikk *Parazita felmérések új típusú megközelítése* (Brooks et al. 2014)

Célkitűzés és módszerek

A parazita szervezetek, amelyek speciális transzmissziós stratégiákkal és komplex életciklussal bírnak, nem pusztán az emberek és haszonállataik, növények, illetve a vadállatok kórokozói, hanem igen fontos képviselői azon betegségek ökológiai és történeti hátterének, amelyek terjesztéséért felelősek. A hagyományos megközelítése az endoparazita gyűjtési módszereknek azon alapul, hogy minél több gazdaszervezetből igyekezzünk egyedeket gyűjteni és lehetőleg tegyük ezt korábban nem vizsgált élőhelyek és gazdafajok mintázásával. Ez az endoparazita helminthek esetében destruktív mintavétellel valósítható meg, amelyet az általunk bemutatott megközelítés csak a kezdeti, dokumentációs fázisában javasolja a felmérésnek. A célunk az volt, hogy:

(i) javasoljunk egy parazita felmérési protokollt, amely alapjául szolgálhat a különböző diszciplínák közötti együttműködés elősegítésének, továbbá kiváltson egy destruktív módszert, amelyet hosszú távú, nem-invazív parazitagyűjtéssel helyettesít, továbbá

(ii) egy olyan átfogó platformot biztosítson ezen diszciplínák számára, amely széleskörű információt szolgáltathat a parazita szervezetek evolúciójáról, epidemiológiájáról, továbbá a különböző gazdaszervezetek, földrajzi régiók, illetve ökoszisztémák közötti kapcsolatokról.

Eredmények

Kiemeljük, hogy a tradicionális parazita felméréseket újra köztudatba kell helyezni, de mindezt egy újfajta megközelítésben. Ezt mi DAMA protokollnak (dokumentáció, értékelés, monitoring és cselekvés) neveztük el, ami többek között azt javasolja, hogy nem szükséges a gazdaszervezetek nagy egyedszámban történő destruktív mintavételezése (endoparazita gyűjtés esetében az elpusztítása) ahhoz, hogy fontos információhoz juthassunk az élősködők sokféleségéről és ökológiájáról. A DNS vonalkód módszerben rejlő lehetőségek gyors és költséghatékony módjai lehetnek annak, hogy a parazitákat nyomon követhessük a gazdaszervezeteikben folytatott rejtett életmódjuk ellenére is, ahogy azt a DAMA protokoll *monitoring* fázisa is javasolja. Továbbá fontos, hogy az ilyen típusú felmérésekből származó összes releváns információt bárki számára hozzáférhető, valós időben frissíthető adatbázisokban (lásd például Encyclopedia of Life vagy a Global Biodiversity Information Facility) tegyük elérhetővé, ezáltal maximalizálva a különböző tudományterületek közötti együttműködés lehetőségét és annak hatékonyságát.

II. cikk *Helminth felmérés a DAMA protokoll alapján – kezdeti lépések: dokumentáció és értékelés* (Herczeg et al. 2016)

Célkitűzés és módszerek

Helminth felmérésünket a Hortobágyi Nemzeti Parkban (HNP) végeztük, ahol a 40 évvel korábban lefolytatott alapkutatás adott számunkra viszonyítási alapot a kecskebéka fajkomplex (*Pelophylax esculentus* komplex) endoparazita faunájának újravizsgálásához. A zöldbékák jó modellszervezeteknek bizonyulnak az ilyen típusú vizsgálatokra, hiszen fontos szerepet töltenek be az ökoszisztémában, gazdag endoparazita helminth faunával bírnak, illetve számos háttér információval rendelkezünk az élősködőik ökológiájáról is. A 2012-2013-as években 101 *Pelophylax* sp. egyedet gyűjtöttünk három különböző élőhelyi paraméterekkel rendelkező vizes élőhelyről. A gazdaszervezetek taxonómiai határozását molekuláris módszerek segítségével végeztük. Ebben a vizsgálatban eltértünk a hagyományos parazita felmérésektől és a DAMA protokollt követtük, amely szerint a gazdák destruktív mintavételezése csupán a felmérés kezdeti szakaszára korlátozódik, azt a későbbiekben a DNS vonalkódok alapján történő monitoring váltja majd fel, amely nem jár a gazdaszervezetek elpusztításával. Ennek reményében nem csak morfológia alapú taxonómiai azonosításra gyűjtöttünk parazitákat, hanem az egyedek nagy részét 96%-os etil-alkoholban konzerváltuk, hogy örökítő anyagukból DNS vonalkódokat készíthessünk. Ezek alapján a vizsgálat célja az volt, hogy:

(i) leírja a HNP-ban jelenleg élő zöldbékák helminth közösségeinek az összetételét,

(ii) összehasonlítsa azt a 40 évvel ezelőtti alapkutatás eredményeivel,

(iii) továbbá DNS mintákat gyűjtsön a parazitáktól annak érdekében, hogy a DNS vonalkódok segítségével nem-invazív alapú mintavételt tegyen lehetővé a jövőbeni monitoring számára.

Eredmények

A vizsgált gazda populációt *Pelophylax ridibundus* és a hibrid *Pelophylax esculentus* alkotta. A mindhárom populációra számított átfogó prevalencia értéke 76.2% volt, míg az egy gazdaegyedre vonatkoztatott átlagos intenzitás, pedig 2.44 parazita faj/gazdaegyed. Vizsgálatunk során legfeljebb 6 helminth faj fordult elő egy gazdaegyedben: 20 egyed (19.8%) a 101 *Pelophylax* sp-ből 1 fajt, 24 egyed (23.8%) 2 fajt, 19 egyed (18.8%) 3 fajt, 9 egyed (9%) 4 fajt, 3 egyed (3%) 5 fajt, míg 2 egyed (2%) összesen 6 helminth fajt hordozott.

Kutatásunk faunisztikai eredményei alapján három kategóriát állítottunk fel, eszerint: (i) állandó fajok (ii) hiányzó fajok és (iii) HNP-ra új fajok alkották a parazita közösségeket. A HNP-ban élő zöldbékák helminth közössége jelentős változáson esett keresztül az elmúlt évtizedekben, amely hat faj elvesztése mellett, két hortobágyi faunára új faj megjelenése jellemezte. Továbbá azt találtuk, hogy a lassú folyású vizes élőhelyhez kötődő *P. ridibundus* egyedekben a paraziták előfordulásának átlagos intenzitása szignifikánsan nagyobb volt (χ^2 = -4.782, df = 1, *P*= 0.002) mint az olyan élőhelyeken, amelyek állóvíz jellegűek. Ahogy azt a DAMA protokoll is javasolja, a parazita felmérésből származó információt online, valós időben frissíthető és mindenki számára ingyenesen hozzáférhető formában tettünk elérhetővé az *Encyclopedia of Life* (EOL) adatbázisban, továbbá a *flickr* fényképmegosztó portálon a helminth fajokról készült mikroszkópos felvételeket feltöltöttük, amelyeket az EOL adatbázisban lévő parazita profilokhoz társítottunk.

III. cikk *Pelophylax esculentus komplex faji összetétele és szaporodási rendszere Kelet-Magyarországon* (Herczeg et al. 2017)

Célkitűzés és módszerek

Disszertációm harmadik publikációja a *Pelophylax esculentus* komplexre fókuszál, amelyet a két szülői faj, a *Pelophylax ridibundus* és a *Pelophylax lessonae*, illetve ezek interspecifikus hibridje a *Pelophylax esculentus* alkot. A szülői fajok a hibridektől, kizárólag morfológiai karaktereket figyelembe véve nehezen különíthetőek el teljes bizonyossággal, továbbá a hibridek genotípusának (LR–diploid; LLR és LRR–triploid) meghatározása is molekuláris módszerek alkalmazását igényli. A Hortobágyi Nemzeti Park területén lévő három különböző típusú vizes élőhelyen vizsgáltuk a zöldbéka populációk fajösszetételét, amelynek meghatározását a szérum albumin intron-1 (SAI–1) gén fragmentumban található polimorfia alapján végeztük. Továbbá 15 mikroszatellita marker alkalmazásával megállapítottuk a hibrid egyedek genotípusát. Végül, populáció genetikai indexek számításával következtettünk a zöldbékák szaporodási rendszerére. Vizsgálatunkban tehát arra kerestük a választ, hogy:

(i) milyen fajok alkotják a Hortobágy zöldbéka populációit,

(ii) a hibrid egyedek milyen genotípussal (ploiditás) rendelkeznek,

(iii) és a vizsgált populáció szerkezete milyen hatással van a genetikai diverzitásukra és a klonális öröklődés menetére, amiből a szaporodási rendszerükre következtethetünk.

Eredmények

A molekuláris vizsgálatok eredményei alapján kijelenthetjük, hogy a fajkomplex mindhárom tagja jelen van a Hortobágyon. Két eltérő fajösszetételű populáció típust különböztettünk meg. Két élőhelyen csak *P. ridibundus* és *P. esculentus* (RE populáció) egyedeit gyűjtöttük, míg a harmadik élőhelyen mindhárom taxon előfordult (LER populáció). A mikroszatellit vizsgálat alapján kizárólag diploid genotípusú (LR) hibrid békákat találtunk, a triploid egyedek (LLR vagy LRR) jelenléte nem volt detektálható. Adataink alapján legalább két populáció esetén nagy bizonyossággal elmondható, hogy a hibridek az R genomjukat klonális módon adják tovább és a *P. lessonae*-val visszakereszteződve tudnak új hibrid nemzedéket létrehozni, így a Közép-Európában leginkább elterjedt LE szaporodási rendszerhez tartoznak.

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Part II

This part contains the original reprints, in which the methodology, results and their discussion are detailed.

List of papers

1. Brooks DR, Hoberg EP, Boeger WA, Gardner SL, Galbreath KE, Herczeg D, Mejía-Madrid HH, Rácz SE, Tsogsaikhan Dursahinhan A (2014) Finding them before they find us: informatics, parasites and environments in accelerating climate change. *Comparative Parasitology* 81:155–164 [$IF_{2014/2015}$: 0.7]
2. Herczeg D, Vörös J, Végvári Zs, Kuzmin Y, Brooks DR (2016). Helminth parasites of the *Pelophylax esculentus* complex (Anura: Ranidae) in Hortobágy National Park (Hungary). *Comparative Parasitology* 83:36–48 [IF_{2016}: 0.659]
3. Herczeg D, Vörös J, Christiansen DG, Benovics M, Mikulíček P (2017) Taxonomic composition and ploidy level among European water frogs (Anura: Ranidae: *Pelophylax*) in eastern Hungary. *Journal of Zoological Systematics and Evolutionary Research* 55:129–137 [IF_{2016}: 2.444]

Papers are referred through the text by their boldface roman numerals (**I-III**).

Own contribution to the papers included in the thesis

	I	II	III
Original idea	*	*	*
Study design and data collection		*	*
Data analysis		*	*
Manuscript preparation	*	*	*

Paper I

Finding them before they find us: informatics, parasites and environments in accelerating climate change

Brooks DR, Hoberg EP, Boeger WA, Gardner SL, Galbreath KE, Herczeg D, Mejía-Madrid HH, Rácz SE, Tsogsaikhan Dursahinhan A (2014) *Comparative Parasitology* 81:155–164 [$IF_{2014/2015}$: 0.7]

Summary

Parasites are agents of disease in humans, livestock, crops, and wildlife and are powerful representations of the ecological and historical context of the diseases they cause. Recognizing a nexus of professional opportunities and global public need, we gathered at the Cedar Point Biological Station of the University of Nebraska in September 2012 to formulate a cooperative and broad platform for providing essential information about the evolution, ecology, and epidemiology of parasites across host groups, parasite groups, geographical regions, and ecosystem types. A general protocol, documentation– assessment–monitoring–action (DAMA), suggests an integrated proposal to build a proactive capacity to understand, anticipate, and respond to the outcomes of accelerating environmental change. We seek to catalyze discussion and mobilize action within the parasitological community and, more widely, among zoologists and disease ecologists at a time of expanding environmental perturbation.

Comp. Parasitol.
81(2), 2014, pp. 155–164

Finding Them Before They Find Us: Informatics, Parasites, and Environments in Accelerating Climate Change

Daniel R. Brooks,[1,7,8] Eric P. Hoberg,[2,7] Walter A. Boeger,[3,7] Scott L. Gardner,[1,7] Kurt E. Galbreath,[4] Dávid Herczeg,[5] Hugo H. Mejía-Madrid,[6] S. Elizabeth Rácz,[1] and Altangerel Tsogtsaikhan Dursahinhan[1]

[1] H. W. Manter Laboratory of Parasitology, University of Nebraska State Museum of Natural History, University of Nebraska, Lincoln, Nebraska 68588-0514, U.S.A.,
[2] U.S. National Parasite Collection, Agricultural Research Service, USDA, Beltsville Area Research Center, BARC East 1180 Beltsville, Maryland 20705, U.S.A.,
[3] Laboratório de Ecologia Molecular e Parasitologia Evolutiva, Universidade Federal do Paraná, Caixa Postal 19073, Curitiba, PR 81531-980, Brazil,
[4] Department of Biology, Northern Michigan University, 1401 Presque Isle Avenue, Marquette, Michigan 49855, U.S.A.,
[5] MTA-DE "Lendület" Behavioural Ecology Research Group, University of Debrecen 1 Egyetem-tér, Debrecen, Hungary,
[6] Departamento de Ecología y Recursos Naturales, Laboratorio de Ecología y Sistemática de Microartrópodos, Facultad de Ciencias, Universidad Nacional Autónoma de México, Mexico D.F., C.P. 04510, Mexico, and
[7] Organizers of the workshop: "Parascript in the 21st Century—Climate Change, Biodiversity and Emerging Disease," Cedar Point Biological Station, University of Nebraska, 5–6 September 2012

ABSTRACT: Parasites are agents of disease in humans, livestock, crops, and wildlife and are powerful representations of the ecological and historical context of the diseases they cause. Recognizing a nexus of professional opportunities and global public need, we gathered at the Cedar Point Biological Station of the University of Nebraska in September 2012 to formulate a cooperative and broad platform for providing essential information about the evolution, ecology, and epidemiology of parasites across host groups, parasite groups, geographical regions, and ecosystem types. A general protocol, documentation–assessment–monitoring–action (DAMA), suggests an integrated proposal to build a proactive capacity to understand, anticipate, and respond to the outcomes of accelerating environmental change. We seek to catalyze discussion and mobilize action within the parasitological community and, more widely, among zoologists and disease ecologists at a time of expanding environmental perturbation.

KEY WORDS: documentation–assessment–monitoring–action, climate change, biodiversity, emerging infectious disease, parasites, hosts, epidemiology, ecology, evolution.

STOCKHOLM PARADIGM

Parasitology finds itself in a time of exciting possibilities. During the past generation, parasites have become recognized as significant components of both biological diversity and as excellent model systems for general evolutionary (Brooks and McLennan, 1993) and ecological (Poulin, 1997; Poulin and Morand, 2004) studies. At this time, there is growing interest in parasites as we begin to understand more and more that there are direct connections among climate change, biodiversity dynamics, and emerging infectious disease (EID). Parasites occupy a central role in efforts to develop proactive protocols for monitoring changes in ecosystem structure and for detecting the potential for emerging disease in resident and colonizing host species, be they human, livestock, or wildlife (Daszak et al., 2000; Brooks and Hoberg, 2006, 2008, 2013; Patz et al., 2008; Agosta et al., 2010; Hoberg, 2010; Weaver et al., 2010; Hartigan et al., 2012; Altizer et al., 2013; Hoberg and Brooks, 2013). Parasites, especially those with specialized transmission dynamics, including complex life cycles, are not only agents of disease in humans, food-animal resources, crops, and wildlife, they are also powerful representations of the ecological and historical context of the diseases they cause (Dobson and Hudson, 1986; Dobson and May, 1986a, b; Dobson and Carper, 1992; Hoberg, 1997; Dobson and Foufopoulos, 2001; Marcogliese, 2001, 2005; Nieberding and Olivieri, 2007; Hoberg and Brooks, 2008; Hoberg et al., 2008; Rosenthal, 2008; Lafferty, 2009; Kilpatrick, 2011; Kuris, 2012). This is especially true for eukaryotic parasites.

Recognizing this nexus of professional opportunities and global public need, we gathered at the Cedar Point Biological Station of the University of Nebraska in September 2012 for a workshop to discuss the possibility of developing a cooperative

[8] Corresponding author.

platform for providing essential information about the evolution, ecology, and epidemiology of parasites broadly across host groups, parasite groups, geographical regions, and ecosystem types. Here we summarize our discussions and make some recommendations. We seek to catalyze discussion and mobilize action within the parasitological community and, more widely, among zoologists and disease ecologists, conservation biologists, and those in the policy arena at a time of expanding environmental perturbation.

Parasites are primary components of environmental change and, concurrently, contribute to developing a nuanced understanding of ecosystems in transition because they allow the incorporation of biological insights across considerable spatial and temporal scales. Parasites and parasitologists reside at the expanding nexus of interacting crises of biodiversity, climate stability and change, and emerging infectious diseases (Brooks and Hoberg, 2013; Mora and Zapata, 2013). Clearly, a substantial and potentially irreversible challenge to the distribution and continuity of biodiversity, ecosystem integrity and sustainability, and socioeconomic stability, through changing interfaces and ecotones, influencing patterns of disease, emerges directly from the footprint of accelerating climate warming and its attendant environmental perturbation (e.g., Parmesan and Yohe, 2003; Lovejoy and Hannah, 2005; Patz et al., 2005; Lawler et al., 2009; Post et al., 2009; Weaver et al., 2010; IPCC, 2007a, b, 2013; Meltofte et al., 2013). Equally clearly, the nature, scope, and scale of anthropogenic climate warming are pervasive, and anticipating unprecedented perturbation across the biosphere necessitates both the incorporation of historical and contemporary insights regarding the structure and distribution of biodiverse systems as well as the development of novel integrative approaches to serve as a framework in which to understand the impacts and effects of such change.

In this arena, we increasingly recognize that faunal assembly (structure and diversification) among hosts, parasites, and pathogens has often been associated with ecological perturbation as a driver of geographic and host colonization at varying spatial and temporal scales over Earth's history (e.g., reviewed in Hoberg and Brooks, 2008, 2010). In short, parasite diversification has unfolded (in part) through episodic shifts in climate and environmental settings in conjunction with both ecological mechanisms and host switching (e.g., Hoberg and Klassen, 2002; Nieberding et al., 2008; Hoberg et al., 2012; Hoberg and Brooks, 2013). Such an ecocentric view of parasite diversification, tied to considerable complexity in ecological processes, counters more than a century of coevolutionary thinking about the nature of the development of host–parasite assemblages (for comprehensive reviews, see Brooks and McLennan, 1993, 2002; Janz, 2011). Further, the apparent significance of host colonization in diversification poses a "parasite paradox" (Agosta et al., 2010) that stems from 2 observations: (1) Parasites demonstrate specificity (restricted and apparently specialized host ranges) and are resource specialists; and (2) such specialization occurs even though shifts onto relatively unrelated hosts are common in the phylogenetic diversification of parasite lineages and are even often directly observable in ecological time.

The articulation of what we herein refer to as the "Stockholm paradigm" serves as a conceptual foundation for resolution of the parasite paradox and provides a new integrative view of complex associations grounded in both a considerable body of experimental observations and in core principles emanating from 4 academic generations of researchers at Stockholm University (for a review, see Brooks and McLennan, 2002; Agosta et al., 2010; Janz, 2011, and references therein). In this paradigm, the resolution of the parasite paradox emerges through integration of 4 key ecological and historical concepts: ecological fitting; the oscillation hypothesis; the geographic mosaic theory of coevolution; and taxon pulses. Ecological fitting (Janzen, 1985) drives substantial opportunities for accelerated host colonization, prior to the evolution of a novel spectrum of capabilities for host exploitation, and is a function of both phenotypic flexibility and phylogenetic conservatism in traits related to the use of broad-based resources. Consequently, specialists may be involved in host-range expansion through shifts under a dynamic of ecological fitting. The oscillation hypothesis describes events downstream, setting the stage for alternating trends in the evolution of generalists and subsequent new specialists (Janz and Nylin, 2008; Nylin et al., 2014). More generally, and over time, novel combinations of interacting species emerge through processes defined within the geographic mosaic theory of coevolution (Thompson, 2005). Whether referring to helminths of vertebrates or phytophagous insects, such symbiotic assemblages originate, exist, and persist in a crucible of accelerating change that serves to demonstrate the equivalence of processes for faunal assembly, including host and geographic colonization across spatial scales and

through evolutionary and ecological time (Hoberg and Brooks, 2008, 2010; Hoberg, 2010). Thus, considerable complexity arising from taxon pulses (Erwin, 1985; Halas et al., 2005) driven by climate change and large-scale ecological perturbation lead to extensive biotic mixing (and mosaics) and further serve as the antecedents for episodes of rapid host switching, including outbreaks of emerging infectious diseases (Brooks and Hoberg, 2007, 2013; Hoberg and Brooks, 2008, 2013; Agosta et al., 2010).

A contrast of the Stockholm paradigm with the more traditional paradigm of coevolution in defining the nature of complex host–parasite associations is apparent. As noted above, classical coevolutionary models predict that host colonization becomes less likely as the intensity of co-adaptive responses (microevolutionary phenomena) increase across the time frame of an association (i.e., parasites become more specialized to their hosts). Thus, in this context, it is assumed that the process of coevolution itself should provide a high degree of protection against emerging diseases because it becomes more and more difficult for increasingly specialized parasites to jump hosts. Two logical conclusions from this classical view emerge: (1) Host switches should be rare at all scales; and (2) when events of host colonization occur, there must be an underlying genetic change in the parasite that is its precursor, and this change determines the capacity to be associated with a novel host (e.g., Kilpatrick, 2011). This immediately shifts the focus of discussions about climate change and emerging diseases to center on the possible mechanisms by which climate forcing can influence the origins of novel genetic variation (and the conditions or environmental regime to try them out in, where the latter, but not the former, is consistent with the Stockholm paradigm). As a consequence, the expectation remains that because novel genetic innovations must lead the way, emerging diseases will be rare under the classical paradigm.

Counter to this relatively simple scenario for coevolution, the Stockholm paradigm, by contrast, predicts that emerging diseases—in the form of parasites of humans, livestock, crops (we include novel phytophagous pest insects and insect parasitoids of beneficial insects), and wildlife—will be common rather than rare events during episodes of climate change. Colonization is based on those genetic capacities historically retained within a particular system that provide the potential for switching related to ecological fitting. An implicit feature is the assumption of a large sloppy fitness space (Agosta, 2006; Agosta and Klemens, 2008, 2009; Agosta et al., 2010) represented by an array of potential hosts from which most pathogens had been historically precluded by circumstances of time, space, and origin. Exposure of that space, and a concomitant increase in the rate and frequency of host colonization, cascades from accelerating climate change and associated events of biotic expansion. Concurrently, heightened rates of host switching are also predicted under this paradigm as habitats are disrupted and restricted and as patterns of sympatry among species are modified through range contraction and compression into increasingly reduced biogeographic areas.

The Stockholm paradigm also suggests an alternative pathway for addressing the implications of emerging disease. Over the past century, our expanding understanding of epidemiological processes has, for the most part, led to attempts to mitigate the damage posed by emergent diseases, with humanity tending to react to specific events as they occurred. Reactive management policies, however, are not economically sustainable, especially in the context of the Stockholm paradigm reflecting a fundamentally correct explanation of the evolution of interspecific associations. Thus, our largely reactionary mode for addressing outbreaks and ecological disruption could be supplanted by an additional strategy based on a proactive stance and tactics. In a mode defined as evolutionary risk assessment, we can use our knowledge of diversity, past environments, and biological processes in the context of the paradigm to aid in anticipating the future in a world of rapid change. While, like climate change, we cannot stop emerging diseases, we believe and suggest that a path to proactive risk management is less expensive, and thus more effective, than responding in the aftermath of an emerging crisis.

Parasitologists have 3 major contributions to offer in what we hope will become a more inclusive discussion of the relationships among climate change, emerging disease, and biodiversity dynamics. First, some of our organisms, much like the bacteria and viruses that occupy so much press attention and reporting on emerging diseases, cause acute and chronic diseases in humans, livestock, crops, and wildlife. Second, our organisms track broadly through ecosystems and, as such, reveal much about the trophic structural context of disease transmission. Finally, though there is still much to learn, we know a lot about our organisms, their evolution, and their

ecology. Clearly, this is a complex phenomenon, but a combination of technical advances, empirical experience, and a strong recognition of the need for, and importance of, baseline data to understand the structure and history of the biosphere has given us an integrative approach by which we as parasitologists can contribute in a proactive and adaptive manner towards a solution.

WHERE TO BEGIN

Proper valuation of biodiversity on scales from local to global depends on information derived from systematics. Biologists implicitly acknowledge that an understanding of systematics is the underpinning of all of the life sciences whenever they attach a species name to the organisms they are studying. Systematics is the branch of biology charged with the responsibility of making certain that every biologist who uses a particular name actually refers to "the same thing." Since Darwin, the assignment of a specific epithet to a group of organisms has been the proposal of a hypothesis that those organisms belong to what Darwin (1872) termed "communities of descent." That is, they are members of a diagnosably inclusive and mutually exclusive hereditary information system. As a result, the names we assign to the organisms we study are indices of information—not just about unique identity, but about an array of characteristics ranging from their reproductive biology to their development, ecology, and behavior, e.g., all the traits that, when combined, characterize their life and lifestyle. This gives rise to the adage "No name, no information; wrong name, wrong information." The seemingly inexhaustible potential of evolutionary diversification means that each species is marked by always amazing, often surprising, and sometimes extremely subtle, diagnostic differences requiring considerable taxonomic expertise to recognize and distinguish (e.g., Makarikov et al., 2013). Because no 2 species are the same, no matter how closely related, it is therefore essential to know with what you are dealing. The crucial nature of proper identification has been underscored for research as disparate as the study of parasites and sexual selection (e.g., McLennan and Brooks, 1991), of parasites as bio-indicators (e.g., Frank et al., 2013), and of parasites as biodiversity probes (Gardner and Campbell, 1992).

A second function of systematics is generating phylogenies, which are fundamental for all comparative evolutionary studies (Brooks and McLennan, 1991, 1993, 2002). Darwin's insight that all communities of descent are related to each other in a tree (in part a reticulating network) of life led to his dictum that the most likely explanation for similarity is inheritance from a common ancestor and not existence in common environments. This explains the massive evidence indicating that most aspects of the biology of parasites, including their ecology and behavior, are phylogenetically conservative, something anticipated by Harold Manter (1966) when he coined the term "parascript" (see Brooks and McLennan, 1993).

Parasitology, like most disciplines, suffers from the "taxonomic impediment," the global shortage of professional taxonomists and systematists (GTI, 1999), and it cannot be clearer that the need for expert taxonomists is now greater than ever in the past. In parallel, recognition of the taxonomic impediment emphasizes not just the need to develop and use our increasingly valuable existing archival collections of voucher specimens of hosts and parasites at all spatial scales, but also the need for a concomitant expansion of informatics resources to describe the biosphere. Although we encourage all countries and institutions to train and hire more taxonomists and to support museum infrastructure, we are realistic. In the short term, at least, we cannot assume that additional resources will be allocated for this important purpose. As a consequence, it becomes even more imperative that existing taxonomists cooperate both with each other and with other biodiversity specialists (for an extensive discussion of the benefits of such cooperation, see Brooks and McLennan, 2002).

In the following, we propose a general protocol, with the acronym DAMA, which we believe can be a blueprint for many different cooperative efforts. DAMA is our name and rationale for documentation–assessment–monitoring–action, an integrated proposal and rationale to build a proactive capacity to understand, anticipate, and respond to the outcomes of accelerating environmental change.

Document

For all biodiversity inventories, including those for parasites, the more we look, the more species we find. Moreover, the more we find, the more information about these species we discover. To make significant progress in understanding complex biological interactions globally, we need to know what parasites exist in as many different parts of the planet as possible (Hoberg, 1997; Brooks and Hoberg, 2000; Hoberg et al., 2013). This will require comprehensive taxonomic inventories. Importantly, and to maximize information content, each species name used in any

such inventory needs to be linked to voucher specimens available in properly maintained state-of-the-art archival collections with direct links to informatics resources describing specimen-associated ecological, phylogenetic, and population-level data (Frey et al., 1992; Haverkost et al., 2010; Cook et al., 2013). We cannot emphasize enough the need for a major sea change in the parasitological tradition of maintaining private collections, as these limit data information and sharing during a time of decreasing resource availability and change.

In addition to knowing what parasites occur in any given area, we also need to know how to find specimens of each particular parasite species when needed. As well, we need to know as much as possible about the natural history of each parasite species, especially with respect to its geographic origin, transmission dynamics, microhabitat preferences, and host range (e.g., what parasite species are present, when they are there, in what hosts, and in what parts of the hosts they are found in, and how they are transmitted).

It appears now that 1 of the most efficient ways to summarize this globally available species-specific data is through the construction of digital home pages for each species, as envisioned in the international initiative called the *Encyclopedia of Life* (www.eol.org). In order for inventory information to be maximally useful, all cooperating research groups must agree to share such information, and, ideally, all such inventory information should be stored in a form that can be updated in real time and that is freely and readily available on the internet, as embodied in the *Global Biodiversity Information Facility* (www.gbif.org).

Assess

Every species has a story to tell, and that story is fundamentally a story of descent with modification, meaning common ancestry, in the context of the selective crucible of a multivariate and changing set of environmental conditions through which each hereditary lineage passes. Species-specific information about ecology, behavior, and geographic distributions, examined in an evolutionary (i.e., phylogenetic) context, provides far more information than a simple list of species and their known properties (Brooks and McLennan, 2002), and this fundamentally Darwinian perspective is amplified in the case of parasites. Manter (1966) stated that parasites always tell not just the stories of their own ecology, but also that of their hosts and of the geographic distributions and complex ecosystems in which they live and evolve. He called for a research program integrating systematics, ecology, and biogeography, which he termed parascript. Parasite phylogenies began to appear in the late 1970s, and many of the initial studies integrating phylogeny, ecology, and biogeography used parasite systems as exemplars (Brooks, 1985; see summaries in Brooks and McLennan, 1991, 1993, 2002; Hoberg and Klassen, 2002). The coherent research program for Parascript that emerged in the early 1990s (Brooks and McLennan, 1993) has catalyzed, and continues to catalyze, significant basic research in this field.

In the past 15 yr, a case has been made that significantly greater information relevant to climate change, biodiversity dynamics, and emerging disease, that is, the critical information needed for making proactive, anticipatory policies, results when parasite biodiversity inventories are placed in an evolutionary context (Gardner and Campbell, 1992; Hoberg, 1997; Brooks and Hoberg, 2000, 2013; Hoberg et al., 2008). For this reason, we need a relevant and powerful paradigm—one that explains not only the dynamics of maintaining a pathogen in association with a particular host in a particular ecosystem, but one that also explains historical origins and how such associations can change rapidly in response to rapidly changing environments. The Stockholm paradigm provides such a foundation (see, e.g., Agosta et al., 2010, and references therein).

Monitor

We want to develop proactive and anticipatory policies for using basic information about parasites in climate change, biodiversity, and emerging disease. This approach will require that we monitor the parasite diversity documented in our inventories. Moreover, we must also be able to recognize distributional and ecological changes as soon as they occur, and we must know if those changes are unusual. This means we need to document not just parasite diversity in each area inventoried through time, but that inventories also need to be large scale across both time and space. Such an approach emphasizes that basic inventory work needs to be an ongoing process—it is not enough to collect parasites in 1 place at 1 time, as patterns can only be detected by sampling over wide geographic areas (Gardner and Campbell, 1992; Hoberg et al., 2008). Such an approach will allow us to compare findings within and among given places over time, and we envision a network of information growing in space and time that will be capable of alerting us to not just shifting spatial and ecological boundaries but also to

the critical changes in 1 place that will allow us to anticipate similar changes in other areas (e.g., Polley and Thompson, 2009).

Documentation, surveillance, and the monitoring of parasite biodiversity can encompass a continuum that collectively contributes to informatics resources of the highest quality (e.g., Hoberg et al., 2013). Across this spectrum are: (1) targeted taxonomic studies of single species of parasites; (2) limited surveys in single host species, perhaps at a restricted number of localities; (3) surveys and inventories at the ecosystem level based on standardized and comprehensive sampling protocols; and (4) fully integrated inventories of hosts and parasites and the application of population genetics/phylogeography to explore associations on both fine temporal and spatial scales. Ecosystem approaches and geographic coverage from landscape to regional scales feed into archival collections (parasites, hosts, and tissues) held in museum repositories and become the cornerstone for establishing baselines for parasite faunal diversity, abundance, epidemiological, populational, and spatial patterns, and disease emergence over time. Linked with phylogeny and biogeography on varying spatial and temporal scales, these provide a window into change in the biosphere across both evolutionary and ecological time (Hoberg, 1997; Brooks and Hoberg, 2000). Further, such essential inventory information can provide the data required for the development of modeling protocols to examine various scenarios for both environmental change and the distribution of disease (Waltari and Perkins, 2010) and can serve to validate predictions about biological outcomes, including events of ongoing geographic colonization and host switching (e.g., Hoberg et al., 2013; Kutz et al., 2013).

To maximize the efficient use of limited resources, parasite monitoring programs should be fully integrated with efforts to document and archive host diversity. Those of us who conduct parasite inventories have often had the frustrating experience of dealing with people who very much want information from us about the parasites of the hosts they are studying, and who are incredulous when we tell them such information, in most cases, does not exist. Those same investigators often refuse to allow destructive sampling of "their" organisms in order to provide the information they desire. Similarly, parasitologists who discard host carcasses after extracting the parasites waste critical data regarding the ecological and evolutionary context within which the parasites exist. They miss the opportunity to maximize the impact of their efforts and the value of their data. In short, increased cooperation between parasitologists and those who study host taxa has the potential to enhance the productivity of both realms of investigation, as well as to foster and open new paths of inquiry.

The appropriate application of molecular tools offers an important way in which to facilitate the description of complex parasite communities, though this approach does not stand alone. For example, the technique popularly known as genetic or DNA barcoding offers the possibility of performing nondestructive monitoring of hosts for parasites. This creates the potential to shift from logistically challenging field collections, necropsy, and morphological characterization based on assessments of a few hosts to more geographically extensive, site-intensive, and near-simultaneous sampling across ecosystems, thus linking landscape to regional scales for assemblages of host species and populations (e.g., Jenkins et al., 2005; Kutz et al., 2007). This means we now have or can develop the capacity to more readily assess parasite impacts on host species that are rare or endangered. As well, barcoding provides a quick and cost-effective means of establishing transmission patterns, since larvae and juveniles of any given species of parasite in a particular place will have the same barcode profile. Clearly, an understanding of transmission dynamics is a critical element of assessing the ecological context of parasites in their environments.

Barcoding alone, however, is inadequate for documenting and assessing parasite diversity; among some groups of flatworms, for example, barcoding applications remain challenging (Vanhove et al., 2013). There are 2 reasons for this inadequacy. First, barcodes by themselves do not provide a direct link to a species name, and it is only when a particular barcode, or set of barcodes, is validated relative to a physical morphospecies already linked to a name that it can be used to index the information about the species it represents. In short, it is only at that point that barcodes can become useful tools in assessment and monitoring. Importantly, there may also be significant issues associated with determining the precise number and identity of species represented by a set of barcodes that are obtained without reference to the specimens from which they have been derived.

While barcoding is thus an excellent alternative to destructive sampling for assessing transmission dynamics and for monitoring parasite diversity, it does not eliminate the need for some destructive sampling during the basic documentation phase of inventorying. This means that we as parasitologists

must continue to cooperate with host specialists in order to minimize destructive sampling—even in some cases foregoing any destructive sampling of particular host species. On a positive note, however, such destructive sampling can provide a means for making barcoding more time- and cost-effective. If, for example, 1,000 pinworms occur in the rectum of a *Chauna marina* (cane toad), a barcoder working alone would need to analyze all 1,000 worms to determine how many species were present. Working in concert with a parasite taxonomist, who can recognize that all 1,000 worms belonged to a single species, would enable the barcoder to save both considerable time and expense. Some of the most spectacular successes of barcoding have occurred when a systematist or ecologist had presorted a collection of specimens in this manner (e.g., Burns et al., 2008).

Looping back and reassessing: Monitoring is not just about redocumenting. It is also about reassessing. When ongoing documentation and monitoring produce new findings, we must ask important reassessment questions, such as: What is missing? What is new? What transmission dynamics need to be determined? Are new transmission dynamics implied by new host records? Does anything need to be redetermined? How has the environment changed or shifted over time? Significantly, those regions of the world where monitoring for EIDs is most badly needed are precisely where such reassessment should immediately take place. For example, high-latitude systems are under rapidly accelerating change and are among the most sensitive environments on the planet and thus require continued reassessment, and the ongoing work to survey and inventory complex host–parasite systems has already demonstrated substantial ecological perturbation in both marine and terrestrial habitats (e.g., Hoberg et al., 2013; Kutz et al., 2013; Meltofte et al., 2013). Thus, collaborative reassessment efforts across boundaries should be made in order to enable research groups to maintain already ongoing monitoring programs and in order to offer uninterrupted continuity during the development of basic research on the parasite diseases of both humans and wildlife. Decidedly, this will not happen if efforts are dispersed and not focused on precise aims.

Act

> "To be forewarned is to be forearmed." —Robert Greene, 1592 (or earlier)

Our call to action asks parasitologists to propose and implement policies for dealing with the intertwined crises of climate change, biodiversity, and emerging diseases based upon basic and sound biological principles. Those policies necessarily involve matters of socioeconomic development. More than most other biologists, parasitologists live in a research milieu in which basic and applied research programs are inextricably linked, so we should all have a deep understanding of this perspective and its importance. We believe that the most at-risk part of the biosphere is the source of our scientific infrastructure—technological humanity.

We also believe that the development of effective action plans for coping with the complexity of climate change, biodiversity, and emerging diseases begins with accepting that there is a critical need and that time is short. To the extent that we cannot stop or reverse climate change, we also cannot stop diseases from emerging. Clearly, the accumulation of pathogen pollution creates an increasing economic burden for humanity, and we know that preventing or anticipating problems is cheaper than crisis response. Therefore, if we do not want EIDs to become an unsustainable economic burden, we need to be proactive. We are not suggesting that humanity stop responding to crises as they occur, but we believe that there are economic reasons to attempt to anticipate problems, to mitigate them when possible, and to only respond rapidly to them when such mitigation fails. We cannot stop or reverse the climate change events that are occurring, but we can mitigate circumstances or adapt to them, at least in some situations (IPCC, 2007a, b, 2013).

Human knowledge is the basis of human adaptation. Phylogenetic conservatism—stored information about past evolutionary successes—is the primary source of evolutionary adaptability. This is the reason assessments need to tie inventory information to as much as is known about the evolutionary history of each parasite species and its closest relatives. The action plan implied in this proposal requires integrated knowledge of the past, present, and future. What *were* the drivers of emerging disease in the past, and how can we learn from them? What *is* happening now, and what factors are currently inhibiting or driving such changes? We simply must know more about the world in which we live. Finally, what future events *may* we anticipate that will be similar to what we know about the past and present?

We do not think our proposal is the only way to proceed, nor do we think that in all cases it will prove to be either feasible or the best pathway forward. We do, however, believe our proposal has merit,

especially in terms of linking human activities with basic evolutionary principles. As well, our proposal provides a framework for cooperation among many specialists and their institutions throughout the world, and it is based on the recognition of a common need. If we do not cooperate now, we will surely face far fewer options for mitigating or alleviating the impacts of global environmental change in the future.

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Paper II

Helminth parasites of the *Pelophylax esculentus* complex (Anura: Ranidae) in Hortobágy National Park (Hungary)

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Summary

The Document, Assess, Monitoring, Act (DAMA) protocol details an approach to integrating information about parasites into large-scale studies of biodiversity, climate change, and emerging diseases. This study represents an effort to put the DAMA protocol into practice. We collected 101 individuals of protected ranid frogs belonging to the *Pelophylax esculentus* complex during 2012 and 2013 in the Hortobágy National Park (HNP) in eastern Hungary in an area where an inventory of amphibian helminths had been conducted 40 yr previously. Collecting sites included flowing water, a fish pond system, and a wetland marsh system. We found the following helminth species: Digeneans: *Diplodiscus subclavatus*, *Haematoloechus variegatus*, *Opisthioglyphe ranae*, *Pleurogenes claviger*, *Pleurogenoides medians*; Nematodes: *Oswaldocruzia filiformis*, *Rhabdias esculentarum*; and Acanthocephala: *Acanthocephalus ranae*. *Rhabdais esculentarum* is a new species for the Hungarian fauna and *P. ridibundus* represents a new host record for *R. esculentarum* while *D. subclavatus*, *P. claviger*, and *P. medians* are new species for the helminthofauna of the HNP. Our findings showed a significant discrepancy from the results of baseline inventories carried out 40 yr ago, although the reasons for this discrepancy are not clear. We suspect that the previously reported helminth species that we did not encounter are restricted to *Pelophylax lessonae*, a host we have not yet collected at this location, but factors associated with climate change or anthropogenic impacts cannot be ruled out.

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Helminth Parasites of the *Pelophylax esculentus* Complex (Anura: Ranidae) in Hortobágy National Park (Hungary)

Dávid Herczeg,[1,6] Judit Vörös,[2] Zsolt Végvári,[3] Yuriy Kuzmin,[4] and Daniel R. Brooks[5]

[1] MTA-DE "Lendület" Behavioural Ecology Research Group, University of Debrecen, 1 Egyetem-tér, Debrecen, H-4032, Hungary (e-mail: herczegdavid88@gmail.com),
[2] Hungarian Natural History Museum, 13 Baross utca, Budapest, H-1088, Hungary (e-mail: jvoros@nhmus.hu),
[3] Department of Conservation Zoology, University of Debrecen – Hortobágy National Park Directorate, 2 Sumen Street, Debrecen, H-4024, Hungary (e-mail: vegvari@hnp.hu),
[4] Department of Parasitology, I. I. Schmalhausen Institute of Zoology, 15 Bogdan Khmelnitsky Street, Kyiv-30 01601 Ukraine (e-mail: rhabdias@gmail.com), and
[5] H. W. Manter Laboratory of Parasitology, University of Nebraska State Museum of Natural History, University of Nebraska, W 529 Nebraska Hall, Lincoln, Nebraska 68588-0514, U.S.A. (e-mail: dnlbrooks@gmail.com)

ABSTRACT: The Document, Assess, Monitoring, Act (DAMA) protocol details an approach to integrating information about parasites into large-scale studies of biodiversity, climate change, and emerging diseases. This study represents an effort to put the DAMA protocol into practice. We collected 101 individuals of protected ranid frogs belonging to the *Pelophylax esculentus* complex during 2012 and 2013 in the Hortobágy National Park (HNP) in eastern Hungary in an area where an inventory of amphibian helminths had been conducted 40 yr previously. Collecting sites included flowing water, a fish pond system, and a wetland marsh system. We found the following helminth species: Digeneans: *Diplodiscus subclavatus*, *Haematoloechus variegatus*, *Opisthioglyphe ranae*, *Pleurogenes claviger*, *Pleurogenoides medians*; Nematodes: *Oswaldocruzia filiformis*, *Rhabdias esculentarum*; and Acanthocephala: *Acanthocephalus ranae*. *Rhabdais esculentarum* is a new species for the Hungarian fauna and *P. ridibundus* represents a new host record for *R. esculentarum* while *D. subclavatus*, *P. claviger*, and *P. medians* are new species for the helminthofauna of the HNP. Our findings showed a significant discrepancy from the results of baseline inventories carried out 40 yr ago, although the reasons for this discrepancy are not clear. We suspect that the previously reported helminth species that we did not encounter are restricted to *Pelophylax lessonae*, a host we have not yet collected at this location, but factors associated with climate change or anthropogenic impacts cannot be ruled out.

KEY WORDS: Helminths of amphibians, *Pelophylax ridibundus*, *Pelophylax esculentus*, Digenea, Nematoda, Acanthocephala, *Rhabdias esculentarum*, Hungary.

For more than a decade, researchers have made the case that parasites should occupy a central role in our efforts to monitor changes in ecosystem structure emerging from global climate change, for detecting the potential for emerging diseases, and for changes in the colonization of human, livestock, or wildlife hosts (Brooks and Hoberg, 2000, 2006, 2013; Daszak et al., 2000; Agosta et al., 2010; Hoberg and Brooks, 2013; Brooks et al., 2014). To accomplish this it is thought that traditional faunal surveys of parasites must be brought back to center stage, but within a novel conceptual framework (Brooks et al., 2014). Traditional parasitological inventories of helminths aim to collect as many parasites from as many hosts as possible—especially hosts that have not been examined for parasites previously—gathering both taxonomic, ecologic, and population data from that extensive destructive sampling. In many cases today, however, the rationale for any such inventory encompasses host species of interest from the perspective of emerging diseases and biodiversity loss. Such hosts, however, often comprise vulnerable populations. Laws and regulations minimizing destructive sampling are aimed at maintaining populations of threatened or endangered animal and plant species in their natural habitats in the face of the current accelerated loss of biodiversity. However, collecting for such a scientific purpose is by no means the greatest threat to such populations (Minteer et al., 2014), and rapidly expanding urbanization, environmental pollution, habitat loss, fragmentation, and emerging infectious diseases pose greater threats (Blaunstein et al., 1994; Berill et al., 1997; Berger et al., 1998; Daszak et al., 1999; Corn, 2000; Cushman, 2006). Nonetheless, the paradoxical notion that parasitologists are "killing animals to save them" is deeply entrenched, leading to the paradox that many researchers desire information about the parasites of their hosts yet do not support inventory activities aimed at obtaining such data. If parasitologists believe that their inventory activities are justified, they need

[6] Corresponding author.

to advocate an approach that obviates such knee-jerk reactions by both the public and the scientific community in order to maximize and most efficiently obtain information pertaining to biodiversity.

Brooks et al. (2014) summarized a protocol (Document, Assess, Monitor, Act) with the acronym DAMA in reference to the above-noted need to sample threatened host species, suggesting that their integrated approach provides a proactive capacity to understand, anticipate, and respond to the outcomes of accelerating environmental change and its impact on parasitic and other diseases. While such an approach involves destructive sampling, the DAMA approach suggests that it is not necessary to kill large numbers of hosts to obtain important information about parasite biodiversity. In short, much more important is having and maximizing information available about each species collected (Brooks and Hoberg, 2000). For example, information about parasite transmission dynamics provides critical information about trophic structure in ecosystems (Marcogliese and Cone, 1997; Marcogliese, 2005), and much of that information can now be obtained using nondestructive sampling.

Within the DAMA approach Brooks (1998), and Brooks and Hoberg (2000), suggest that if a host is rare in the inventory site, then fewer hosts should be sampled. If a host is rare and endemic over it is entire geographic range, the option exists to not perform destructive sampling; in such cases, information can still be obtained based upon what is found in phylogenetically or ecologically (or both) related host species in the same area (Brooks and McLennan, 2002).

Such a destructively minimalist approach involves collecting voucher specimens of each species of parasite encountered, along with their deposition in properly curated museum collections to maximize biodiversity information (Davis, 1996; Global Taxonomy Initiative, 1999), in order to provide important historical data and predictive baselines for understanding the patterns and distribution of organisms. This implies that the infrastructure for collections must be regarded as an integral component of any developing program to survey, inventory, and document biodiversity resources, and such voucher specimens become essential references for future nondestructive sampling within the context of the assessment and monitoring elements of DAMA.

Much of the previous work on anuran helminths has focused on faunistic inventories based upon collections taken during a single season. In this study we compare our faunistical findings with historical baselines set up 4 decades ago (Edelényi, 1972; Murai et al., 1983). While information derived from such long-term study allows the determination of the persistence and stability of parasite diversity (Bursey et al., 2010), the DAMA protocol departs from traditional surveys by suggesting that destructive sampling ends with this initial documentation phase. In this light we collected parasite material not just for classical morphology-based taxonomic indentification, but the majority of the helminth specimens were preserved in ethanol for the development of DNA barcodes (unpublished data). Methods such as DNA barcoding have the potential to provide quick and cost-effective ways to monitor parasite species and their transmission dynamics (Moszczynska et al., 2009; Locke et al., 2010, 2011; Prosser et al., 2013) and thus can be used as a baseline for future nondestructive study within the monitoring phase of DAMA using, for example, host feces, urine, or both. While such an approach precludes the possibility of obtaining direct documentation of statistically robust population estimates (noting that such data are part of the assessment phase in the DAMA protocol), such data can be obtained using noninvasive methods—with a caveat. At present, nondestructive sampling of parasites allows only the determination that at least 1 specimen of a given species of parasite is present in a host; hence, there is a critical need to develop better quantitative assessments of parasite loads based on data obtained from noninvasive methods.

The Hortobágy National Park (HNP) was founded in 1973 and is the largest contiguous alkaline steppe in Europe, covering 80,000 ha, and is the largest and oldest national park in Hungary. Approximately 30% of HNP is covered by wetlands including alkaline marshes, wet grasslands, and 75 fish ponds that cover 6,000 ha. The importance of HNP in sustaining wetland wildlife is recognized by the Ramsar Convention, designating 27,000 ha of the national park as Ramsar sites (Ecsedi, 2004).

Inventory data for helminths inhabiting amphibians in the HNP had been reported earlier by Edelényi (1972) and Murai et al. (1983). As in earlier studies of helminths of European amphibians (e.g., Buchvarov, 1962; Kuc and Sulgostowska, 1988a; Düsen and Öz, 2006; Popiolek et al., 2011), the hosts for most of the helminths previously reported from the amphibians of HNP are water frogs of the *Pelophylax esculentus* complex. Therefore we focused on those hosts in this study.

Systematists have begun untangling the complex evolutionary history of the *P. esculentus* complex. In

the process, they have cast doubt on many host identifications in older studies for which no voucher specimens (of hosts or parasites) exist, thus making comparisons with recent studies difficult. For example, Dely (1981) mentioned 2 members of the *P. esculentus* complex as occurring in HNP, *Pelophylax ridibundus* (Pallas, 1771) and *Pelophylax esculentus* (Linnaeus, 1758), the latter of which originates from the hybridization of *P. ridibundus* and *Pelophylax lessonae* (Camerano, 1882) (see Graf and Polls-Pelaz, 1989). Mészáros and Bartos (1978) and Hilbers (2008) recently reported *P. lessonae* from HNP as well. No voucher specimens exist for the *Pelophylax* spp. listed as hosts by Edelényi (1972) and Murai et al. (1983).

In this report we follow the DAMA protocol and present findings documenting the helminth fauna of water frogs in the HNP in eastern Hungary while discussing initial efforts in assessing that fauna with respect to previous studies (Edelényi, 1972; Murai et al., 1983).

MATERIALS AND METHODS

Study site

We collected water frogs from habitats associated with flowing water (47°26′43′′N; 21°10′10′′E and 47°26′38′′N; 21°08′26′′E), the fish pond system (47°37′13′′N; 21°04′35′′E), and marshland sites (47°36′03′′N; 20°52′53′′E) in the HNP.

Data collection

In many countries, including Hungary, all amphibian species are protected—some of them highly protected—and the collection of these individuals is strictly connected to research permits issued by the local authorities. These permits allow destructive sampling of a restricted number of individuals, which in most cases is not sufficient for assessing and monitoring parasite species. Water frogs were manually collected between May 2012 and August of 2013 using dip nets and by collecting fresh road kills. We collected 52 *Pelophylax* spp. individuals from 2 localities associated with flowing water (canals) and 49 individuals from Hortobágy fish ponds and the Egyek-Pusztakócs marsh system. Due to the high rate of hybridization among water frogs, we used molecular taxonomic techniques following the methods of Hauswaldt et al. (2012) to distinguish between *P. ridibundus*, *P. lessonae*, and the hybrid *P. esculentus*. This method is based on the PCR product size differences from the amplification of the serum albumin intron-1 gene fragment.

Preparation and fixation methods

We examined hosts for helminth parasites within 48 hr of capture. The frogs were overanaesthetized in ether-filled glass containers and the body cavity opened via longitudinal ventral incision. The heart, lungs, liver, gall bladder, and urinary bladder were separated and examined under a stereomicroscope. The alimentary tract was opened by cutting from the anterior esophagus to the rectum and the contents placed into Petri dishes containing Ringer's solution and examined under a stereomicroscope. All helminths collected for DNA data were placed in 96% EtOH for storage. Voucher specimens of adult digeneans were fixed in hot 4% formalin and stored in 70% EtOH. Nematodes were fixed in glacial acetic acid, washed in 70% EtOH, and stored in 70% EtOH + 5% glycerine. Acanthocephalans were relaxed in distilled water for 24 hr followed by fixation in alcohol formalin-acetic acid (AFA) for 24 hr and then stored in 70% EtOH. Digeneans and acanthocephalans were stained with aceto-carmine, dehydrated, cleared in cedar oil, and mounted in Damar gum. Nematodes were cleared in glycerol and examined as temporary mounts. Voucher specimens of parasites were deposited in the Parasitological collection and hosts were deposited to the Herpetological collection of the Hungarian Natural History Museum. Additional voucher specimens of *Rhabdias esculentarum* were deposited in the Helminthological collection of the Department of Parasitology, I. I. Schmalhausen Institute of Zoology, Kyiv, Ukraine.

Statistical analysis

We used generalized linear models (GLM) with a binomial error distribution term to determine the overall prevalence differences between sexes, host species, and collection sites. We used GLM with a Poisson error distribution term to determine the overall mean intensity of parasite species per hosts and for comparison of the previously mentioned variables. Based on the characteristics of collection sites, we pooled the data derived from the fish ponds and the Egyek-Pusztakócs marsh system, yielding 2 categories: habitats associated with still water (fish ponds and Egyek-Pusztakócs marsh system) and canals. Statistical analysis was carried out with QP. 3.0 (Rózsa et al., 2000) and in the R statistical environment (R Core Team, 2013). Overall prevalence and overall mean intensity data are followed by 95% confidence intervals; measurement data is presented as mean followed by minimum and maximun values in parentheses.

RESULTS

Table 1 summarizes our helminth findings for the 101 individuals (48 males and 53 females) of *P. ridibundus* and *P. esculentus* we collected. The overall prevalence of endoparasitic helminths was 76.2% (95% CI = 66.89–83.78) in the examined water frog population. The overall mean intensity was 2.44 (95% CI = 2.17–2.71) helminth species per host. No host contained more than 6 species of parasites: 20 (19.8%) of 101 *Pelophylax* spp. harbored 1 species, 24 (23.8%) harbored 2 species, 19 (18.8%) harbored 3 species, 9 (9%) harbored 4 species, 3 (3%) harbored 5 species, and 2 (2%) harbored 6 species. We found that *P. ridibundus* individuals inhabiting canals had a significantly greater overall mean intensity of helminth species per host than did the same species in still water ($\chi^2 = -4.782$, df = 1, $P = 0.002$). No other significant differences were detected. Based upon these data we divided our faunistical findings

Table 1. Species of parasites collected in this study from the Hortobágy National Park, Hungary.*

Species	Family	Host record	Location	Collection number
Haematoloechus variegatus	Haematoloechidae Freitas & Lent, 1939	*P. ridibundus*, *P. esculentus*	lungs	NHMH-18465
Opisthioglyphe ranae	Telorchiidae Looss, 1899	*P. ridibundus*, *P. esculentus*	SI, LI	NHMH-18463
Diplodiscus subclavatus	Diplodiscidae Cohn, 1904	*P. ridibundus*, *P. esculentus*	rectum	NHMH-18464
Pleurogenes claviger	Pleurogenidae Looss, 1899	*P. ridibundus*	SI	NHMH-18461
Pleurogenoides medians	Pleurogenidae Looss, 1899	*P. ridibundus*, *P. esculentus*	SI, LI	NHMH-18462
Oswaldocruzia filiformis	Trichostrongylidae Leiper, 1912	*P. ridibundus*	SI	NHMH-18466
Rhabdias esculentarum	Rhabdiasidae Railliet, 1915	*P. ridibundus*, *P. esculentus*	lungs	NN 6.1, NN 6.2, NN 6.3, NN 6.4
Acanthocephalus ranae	Echinorhynchidae Cobbold, 1879	*P. ridibundus*, *P. esculentus*	SI	NHMH-18467

* SI = small intestine; LI = large intestine; NHMH = Parasitological collection, Hungarian Natural History Museum, Budapest, Hungary; NN = Helminthological collection, Department of Parasitology, I. I. Schmalhausen Institute of Zoology, Kyiv, Ukraine. All specimens were adults.

into 3 categories: Persistent core species; Missing species; and Species new to the area.

Persistent core species

Digenea: *Haematoloechus variegatus* (Rudolphi, 1819) Looss, 1899; *Opisthioglyphe ranae* (Frölich, 1791) Looss, 1899; Nematoda: *Oswaldocruzia filiformis* (Goeze, 1782); and Acanthocephala: *Acanthocephalus ranae* (Schrank, 1788) Lühe, 1911. These species were reported in HNP previously and are commonly reported species in European amphibians. Consequently, these species seem to form a strong and persistent core of parasite species in the anurans of HNP.

Missing species

These helminths were reported in previous studies, were confirmed by examination of deposited voucher specimens, and were absent from our inventory. Digenea: *Codonocephalus urnigerus* (Rudolphi, 1819) metacercaria; *Clinostomum complanatum* (Rudolphi, 1814) metacercaria; *Cephalogonimus retusus* (Dujardin, 1845) Odhner, 1910; *Gorgodera cygnoides* (Zeder, 1800) Looss 1899; *Haplometra cylindracea* (Zeder, 1800) Looss, 1899; *Haematoloechus asper* (Looss, 1899); *Pleurogenes loossi* (Looss, 1890) Africa, 1930; and Nematoda: *Cosmocerca ornata* (Dujardin, 1845).

Species new to the area

Diplodiscus subclavatus (Pallas, 1760) Diesing, 1836

Description ($n = 3$): Body pyramidal, 3.8 (2.7–5.7) mm long and 1.83 (1.1–2.6) mm wide. Oral sucker terminal 0.43 (0.3–0.6) mm wide with well developed oral diverticula. Oesophageal bulb 0.45 (0.35–0.6) mm long and 0.56 (0.4–0.9) mm in diameter. Acetabulum terminal and large with central accessory sucker. Acetabulum 1.93 (1.2–2.5) mm wide and one third of the total body length.

Site of infection: Rectum.

Type host and type locality: Rana spp., Germany (Goeze, 1782).

Other reported hosts and geography: European fire-bellied toad, *Bombina bombina* (Vojtková, 1961, former Czechoslovakia; Vojtková et al., 1963, former Czechoslovakia; Kozák, 1966, former Czechoslovakia; Vojtková and Krivanec, 1970, former Czechoslovakia); yellow-bellied toad, *Bombina variegata* (Kozák, 1966, former Czechoslovakia; Prokopic and Krivanec, 1975, former Czechoslovakia); common toad, *Bufo bufo* (Vojtková, 1961, former Czechoslovakia; Sey, 1964, 1968, Hungary; Kozák, 1973, former Czechoslovakia; Sey, 1991, Hungary; Cox, 1971, United Kingdom; Shimalov and Shimalov, 2001, Belarus); European green toad, *Bufo viridis* (Sey, 1964, 1968, 1991, Hungary; Vashetko and Siddikov, 1999, Uzbekistan; Murvanidze et al., 2008, Georgia); European tree-frog, *Hyla arborea* (Vojtková, 1961, former Czechoslovakia); lemon-yellow tree frog, *Hyla savignyi* (Yildirimhan et al., 2012, Turkey); edible frog, *Pelophylax esculentus* (Edelényi, 1942, 1960, Hungary; Sey, 1964, 1968, Hungary; Popovic and Mikes, 1989, Serbia; Sey, 1991, Hungary; Bjelic-Cabrilo et al., 2009, Serbia; Chikhlaev et al., 2009, Russia; Popiolek et al., 2011, Poland); pool frog, *Pelophylax lessonae* (Vojtková, 1961, former Czechoslovakia; Vojtková et al., 1963, former Czechoslovakia; Kuc and Sulgostowska, 1988b, Poland; Bakhoum et al., 2011, Belarus; Popiolek et al., 2011, Poland); marsh frog, *Pelophylax ridibundus* (Edelényi, 1960, Hungary; Buchvarov, 1962, Bulgaria; Sey, 1964, Hungary; Popovic and Mikes, 1989, Serbia; Sey, 1991, Hungary; Yildirimhan et al., 1996, Turkey; Düsen and Öz, 2006, Turkey; Murvanidze et al., 2008,

Georgia; Popiolek et al., 2011, Poland); *Pelophylax* spp. (Sey, 1983; Sey and Eöry, 1992, Hungary); moor frog, *Rana arvalis* (Kozák, 1973, former Czechoslovakia; Sey, 1991, Hungary); agile frog, *Rana dalmatina* (Kozák, 1973, former Czechoslovakia); Uludağ frog, *Rana macrocnemis* (Murvanidze et al., 2008, Georgia); common frog, *Rana temporaria* (Bovien, 1916, Denmark; Vojtková et al., 1963, former Czechoslovakia; Kozák, 1966, former Czechoslovakia; Prokopic and Krivanec, 1975, former Czechoslovakia; Kuc and Sulgostowska, 1988b, Poland; Sey, 1991, Hungary); alpine newt, *Ichthyosaura alpestris* (Barus et al., 1963, former Czechoslovakia); common newt, *Lissotriton vulgaris* (Prokopic and Krivanec, 1975, former Czechoslovakia; Cedhagen, 1988, Sweden; Bertman, 1994, Poland); northern crested newt, *Triturus cristatus* (Barus et al., 1963, former Czechoslovakia; Vojtková, 1963, former Czechoslovakia; Frandsen, 1974, Denmark; Bertman, 1994, Poland); sand lizard, *Lacerta agilis* (Lewin, 1992, Poland); European grass snake, *Natrix natrix* (Sey, 1991, Hungary; Bertman, 1993, Poland; Buchvarov et al., 2000, Bulgaria; Shimalov and Shimalov, 2000, Belarus; Murvanidze et al., 2008, Georgia); dice snake, *Natrix tessellata* (Buchvarov et al., 2000, Bulgaria); nose-horned viper, *Vipera berus* (Shimalov and Shimalov, 2000, Belarus).

Specimens deposited and collection numbers: One, deposited in the Parasitological Collection of the Hungarian Natural History Museum (NHMH-18464).

Remarks

Previous reports from HNP did not mention the presence of this digenean species even though *D. subclavatus* is a common and widely distributed endoparasite of amphibians throughout Europe. Members of *Diplodiscus* in Eurasia and closely related North American (*Megalodiscus*) and Neotropical (*Catadiscus*) representatives of the Paramphistomidae are among the most-commonly reported anuran digeneans.

Pleurogenes claviger (Rudolphi, 1819) Looss, 1896

Site of infection: Small intestine.

Type host and type locality: Bufo viridis (Laurenti, 1768), Europe (Rudolphi, 1819).

Other reported hosts and geography: Bufo bufo (André, 1912, Switzerland; Cox, 1971, United Kingdom; Frandsen, 1974, Denmark; Cedhagen, 1988, Sweden); *Bufo viridis* (Shimalov and Shimalov, 2001, Belarus); *Natrix natrix* (Mihalca et al., 2007, Romania); *Pelophylax esculentus* (André, 1913, Switzerland; Edelényi, 1942, Hungary; Sey, 1964, 1968, Hungary; Cox, 1971, United Kingdom; Frandsen, 1974, Denmark; Kuc and Sulgostowska, 1988b, Poland; Chikhlaev et al., 2009, Russia; Bjelic-Cabrilo et al., 2009, Serbia); *Pelophylax ridibundus* (Sey, 1964, Hungary; Combes and Gerbeaux, 1970, Spain; Kuc and Sulgostowska, 1988a, Poland; Sey and Eöry, 1992, Hungary; Oguz et al., 1994, Turkey; Yildirimhan et al., 1996, Turkey); *Pelophylax* spp. (Sey and Eöry, 1992, Hungary); *Rana arvalis* (Sey, 1964, Hungary); *Rana macrocnemis* (Yildirimhan et al., 1997; Yildirimhan, Bursey et al., 2006, Turkey); *Rana temporaria* (André, 1913, Switzerland; Bovien, 1916, Denmark; Cox, 1971, United Kingdom; Frandsen, 1974, Denmark; Cedhagen, 1988, Sweden; Tkach et al., 2000, Ukraine); *Lacerta agilis* (Sharpilo et al., 2001, Ukraine).

Specimens deposited and collection numbers: One, deposited in the Parasitological Collection of the Hungarian Natural History Museum (NHMH-18461).

Remarks

Previous reports from HNP did not mention the presence of this digenean species. A few reports from Hungary exist but their occurence is not frequent. We found only 1 specimen in the small intestine of *P. ridibundus* collected from canals and could not generate measurements because of the poor condition of the only available voucher specimen.

Pleurogenoides medians (Olsson, 1876) Travassos, 1921

Description ($n = 5$): Body elongate, 81.2 (65–94) µm long and 50 (35–65) µm wide. Oral sucker is subterminal and round, 15 (10–20) µm in diameter. The ventral sucker is round, 12.6 (12–13) µm in diameter. The testes are symmetrical, posterior to the cecum, 10 (9–11) µm in diameter. The ovary is elongate, lateral to the cecum in the left side, 11.5 (9–14) µm long and 7 (6–8) µm wide.

Site of infection: Small and large intestines.

Type host and type locality: B. viridis, Denmark (Olsson, 1876).

Other reported hosts and geography: Bombina bombina (Vojtková, 1961, former Czechoslovakia; Vojtková et al., 1963, former Czechoslovakia; Vojtková and Vojtek, 1975, former Czechoslovakia); *Bombina variegata* (Vojtková and Vojtek, 1975, former Czechoslovakia); *Bufo bufo* (Cox, 1971, United Kingdom; Kozák, 1973, former Czechoslovakia;

Vojtková and Vojtek, 1975, former Czechoslovakia; Fernandez et al., 1986, Spain; Shimalov and Shimalov, 2001, Belarus); *Bufo viridis* (Kolendo, 1959, Poland; Vojtková and Vojtek, 1975, former Czechoslovakia); natterjack toad, *Epidalea calamita* (Vojtková and Vojtek, 1975, former Czechoslovakia); *Hyla arborea* (Kozák, 1973, former Czechoslovakia; Vojtková and Vojtek, 1975, former Czechoslovakia; Düsen and Öz, 2004, Turkey); *Pelophylax esculentus* (Edelényi, 1942, 1960, Hungary; Sey, 1964, Hungary; Rodrigues et al., 1973 Portugal; Popovic and Mikes, 1989, Serbia; Chikhlaev et al., 2009, Russia); *Pelophylax lessonae* (André, 1913, Switzerland; Bovien, 1916, Denmark; Kopriva, 1957, former Czechoslovakia; Vojtková, 1961, former Czechoslovakia; Vojtková et al., 1963, former Czechoslovakia; Kozák, 1966, 1968, former Czechoslovakia; Sey, 1968, Hungary; Kozák, 1973, former Czechoslovakia; Vojtková and Vojtek, 1975, former Czechoslovakia; Buchvarov, 1977, Bulgaria; Kuc and Sulgostowska, 1988b, Poland; Tkach et al., 2003, Ukraine; Bjelic-Cabrilo et al., 2009, Serbia); *Pelophylax ridibundus* (Edelényi, 1960, Hungary; Buchvarov, 1962, 1977, 1983, Bulgaria; Combes and Gerbeaux, 1970, Spain; Cox, 1971, United Kingdom; Kozák, 1973, former Czechoslovakia; Kuc and Sulgostowska, 1988a, Poland; Popovic and Mikes, 1989, Serbia; Oguz et al., 1994, Turkey; Yildirimhan et al., 1996, Turkey; Hassan and Saeed, 2001, Iraq; Düsen and Öz, 2006, Turkey; Saglam and Arikan, 2006, Turkey; Murvanidze et al., 2008, Georgia); *Pelophylax* spp. (Sey and Eöry, 1992, Hungary); *Rana arvalis* (Kozák, 1973, former Czechoslovakia; Vojtková and Vojtek, 1975, former Czechoslovakia); *Rana dalmatina* (Kozák, 1973, former Czechoslovakia; Prokopic and Krivanec, 1975, former Czechoslovakia; Buchvarov, 1977, Bulgaria; Düsen et al., 2009, Turkey); *Rana macrocnemis* (Yildirimhan, Bursey et al., 2006, Turkey; Düsen, 2007, Turkey; Murvanidze et al., 2008, Georgia); *Rana temporaria* (Bovien, 1916, Denmark; Kopriva, 1957, former Czechoslovakia; Vojtková and Krivanec, 1970, former Czechoslovakia; Prokopic and Krivanec, 1975, former Czechoslovakia; Vojtková and Vojtek, 1975, former Czechoslovakia; Cedhagen, 1988, Sweden); *Lissotriton vulgaris* (Vojtková, 1963, former Czechoslovakia); *Triturus cristatus* (Vojtková, 1963, former Czechoslovakia); *Lacerta agilis* (Lewin, 1992, Poland; Sharpilo et al., 2001, Ukraine); *Natrix tessellata* (Buchvarov et al., 2000, Bulgaria).

Specimens deposited and collection numbers: One, deposited in the Parasitological Collection of the Hungarian Natural History Museum (NHMH-18462).

Remarks

This is a new record for HNP. It has been reported previously in water frogs from Hungary (Edelényi, 1942, 1960; Sey, 1964, 1968; Sey and Eöry, 1992).

Rhabdias esculentarum (Cipriani, Mattiucci, Paoletti, Santoro and Nascetti, 2012)

Description ($n = 4$): Body small, 4.99 (4.34–6.41) mm long, 283 (268–304) μm wide (maximum width at vulval region). Body cuticle partly inflated, especially at the anterior and posterior ends. Buccal capsule wide, 9 (8–10) μm depth, 12.25 (8–14) μm outer diameter, 10 (9–13) μm inner diameter. Oesophageal length 389.5 (362–433) μm (7.9 % of total body length), width 27.5 (26–30) μm at anterior end, 61.75 (57–65) μm at oesophageal bulb. Nerve-ring near middle of oesophagus. Vulva postequatorial, distance from anterior end to vulva 2,759.5 (2,209–3,581) μm. Tail simple and pointed, length 205.5 (183–240) μm (4.16% total body length).

Site of infection: Lungs.

Type host and type locality: Pelophylax lessonae, Lake Vico, Latium, central Italy (Cipriani et al., 2012).

Other reported hosts and geography: Pelophylax esculentus (Cipriani et al., 2012, Italy).

Specimens deposited and collection numbers: Four, deposited in the Helminthological collection of the Department of Parasitology, I. I. Schmalhausen Institute of Zoology (NN 6.1; NN 6.2; NN 6.3; NN 6.4).

Remarks

The shape of the anterior end, shape of the buccal capsule and oesophagus, and the presence of ventral inflation of the body wall posterior to the anus are the characters allowing the specimen to be assigned to *R. esculentarum*. Using these characters, these worms differ from *Rhabdias bufonis* (Schrank, 1788) Stiles and Hassall, 1905; from *Pelophylax* spp.; from *Rhabdias rubrovenosa* (Schneider, 1866) Semenov, 1929; and from *Rhabdias sphaerocephala* (Goodey, 1924), all of which are all known from European bufonids. *Pelophylax ridibundus* is a new host record for *R. esculentarum* and Hungary is a new geographic distribution record.

DISCUSSION

As suggested by Brooks et al. (2014), assessment in the DAMA protocol is a 2-part process. The first of these is comparison of data with baselines (i.e., what

is already known about the parasites, hosts, and geographic location of an inventory) to address the question of how current findings relate to previous findings. The latter requires the understanding and acceptance that new inventories of places and hosts assessed previously are just as important, and possibly even more so, than are inventories of new hosts and new places. This study provides an excellent example of this assertion.

The current amphibian helminth fauna in the HNP appears to differ significantly from that reported previously by Edelényi (1972) and Murai et al. (1983). First, the original study of amphibian helminths in the HNP listed *Pleurogenes loossi* as present. We did not find this species but did find *Pleurogenoides medians* and *Pleurogenes claviger*.

Second, Murai et al. (1983) listed 3 species of digeneans inhabiting the lungs of water frogs: 2 species of *Haematoloechus* (*H. variegatus* and *H. asper*) and *Haplometra cylindracea*. In contrast, we found only *H. variegatus* in this study. All 3 species are historically common: *H. variegatus* has been found in Bulgaria (Buchvarov, 1977), Georgia (Murvanidze et al., 2008), Hungary (Edelényi, 1942, 1960; Sey, 1964, 1968; Murai et al., 1983; Sey and Eöry, 1992), Ukraine (Tkach et al., 2000), Poland (Kuc and Sulgostowska, 1988a, b; Popiolek et al., 2011), Portugal (Rodrigues et al., 1973), Russia (Chikhlaev et al., 2009), Serbia (Popovic and Mikes, 1989; Bjelic-Cabrilo et al., 2009), Turkey (Yildirimhan et al., 1996; Saglam and Arikan, 2006; Yildirimhan, Goldberg et al., 2006), and the United Kingdom (Cox, 1971). *Haematoloechus asper* has been reported from Georgia (Murvanidze et al., 2008), Hungary (Sey, 1964, 1968; Murai et al., 1983), Ukraine (Tkach et al., 2000), and Russia (Chikhlaev et al., 2009); and *H. cylindracea* has been reported from Belarus (Ryzhikov et al., 1980), Bulgaria (Buchvarov, 1977), former Czechoslovakia (Vojtková and Vojtek, 1975), Hungary (Murai et al., 1983), Iran (Mashaii, 2005), Latvia (Ryzhikov et al., 1980), Lithuania (Ryzhikov et al., 1980), Russia (Ryzhikov et al., 1980), Sweden (Cedhagen, 1988), Turkey (Yildirimhan, Bursey et al., 2006; Düsen, 2007, 2012), Ukraine (Ryzhikov et al., 1980; Tkach et al., 2000), and the United Kingdom (Cox, 1971).

Murai et al. (1983) also reported *Cephalogonimus retusus* and *Cosmocerca ornata* in the small intestine and *Gorgodera cygnoides* in the urinary bladder of water frogs in the HNP. Neither species was found in our study. All 3 species are common parasites of amphibians: *C. retusus* has been reported from Armenia (Ryzhikov et al., 1980), Azerbaijan (Ryzhikov et al., 1980), Bulgaria (Buchvarov, 1977; Buchvarov et al., 2000), Georgia (Ryzhikov et al., 1980; Murvanidze et al., 2008), Hungary (Edelényi, 1942, 1960; Sey, 1968; Murai et al., 1983), Portugal (Rodrigues et al., 1973), Russia (Ryzhikov et al., 1980), Serbia (Popovic and Mikes, 1989), and the United Kingdom (Cox, 1971); and *C. ornata* has been reported from Belarus (Shimalov and Shimalov, 2001; Shimalov et al., 2000, 2001), Bulgaria (Buchvarov, 1977), Georgia (Murvanidze et al., 2008), Hungary (Murai et al., 1983), Iran (Mashaii, 2005), Italy (Galli et al., 2000, 2001), Poland (Kuc and Sulgostowska, 1988a, b; Grabda-Kazubska and Lewin, 1989; Popiolek et al., 2011), Russia (Chikhlaev et al., 2009), Sweden (Cedhagen, 1988), Turkey (Düsen and Öz, 2004, 2006; Yildirimhan, Bursey et al., 2006; Düsen, 2007), United Kingdom (Walton, 1933; Cox, 1971), and Uzbekistan (Vashetko and Siddikov, 1999). *Gorgodera cygnoides* has been reported from Azerbaijan (Ryzhikov et al., 1980), Belarus (Ryzhikov et al., 1980; Shimalov and Shimalov, 2001), Bulgaria (Buchvarov, 1977, 1983; Kirin, 2002), former Czechoslovakia (Vojtková and Vojtek, 1975), Georgia (Ryzhikov et al., 1980; Murvanidze et al., 2008), Greece (Hristovski et al., 2006), Hungary (Edelényi, 1942, 1960; Sey, 1964, 1968; Murai et al., 1983), Kyrgyzstan (Ryzhikov et al., 1980), Macedonia (Hristovski et al., 2006), Poland (Kuc and Sulgostowska, 1988a, b; Popiolek et al., 2011), Portugal (Rodrigues et al., 1973), Russia (Ryzhikov et al., 1980), Serbia (Popovic and Mikes, 1989), Tadzhikistan (Ryzhikov et al., 1980), Turkey (Yildirimhan et al., 1996; Düsen and Öz, 2006; Yildirimhan, Goldberg et al., 2006; Yildirimhan et al., 2009; Düsen and Oguz, 2010), Ukraine (Ryzhikov et al., 1980), United Kingdom (Cox, 1971), and Uzbekistan (Ryzhikov et al., 1980). The absence of *C. retusus*, *C. ornata*, *G. cygnoides*, *H. asper*, *H. cylindracea*, and *P. loossi* were confirmed by sampling in 2013.

Murai et al. (1983) did not report members of 2 common amphibian helminth taxa that we detected, including nematodes belonging to the genus *Rhabdias*, which includes species living in the lungs of both amphibians and some lizards (Cipriani et al., 2012; Kuzmin, 2013). Most studies of *Rhabdias* in European amphibians have reported *R. bufonis*, and Edelényi (1960) reported this worm from *P. esculentus*, *P. ridibundus*, and *Bufo viridis* near the HNP. However, Murai et al. (1983) did not list any species of *Rhabdias*. Recent research suggests that specimens identified as *R. bufonis* represent a species complex

(Tkach et al., 2014). Cipriani et al. (2012) recently described *R. esculentarum* from both *P. lessonae* and *P. esculentus* in Italy, and our specimens in water frogs from HNP correspond to that species. We have not collected parasites from *Bufo bufo* (Linnaeus, 1758) or *B. viridis*, so we do not know if both *R. bufonis* and *R. esculentarum* do, or did, co-occur in the HNP. If the report of *R. bufonis* in *Pelophylax* spp. by Edelényi (1960) actually referred to *R. esculentarum*, then *R. esculentarum* must be assigned to the group of persistent core species of helminths in the water frogs of HNP.

Interestingly, *D. subclavatus* occurs in the rectum of frogs throughout Europe. We are therefore surprised that neither Edelényi (1972) nor Murai et al. (1983) listed *D. subclavatus*. Given its widespread geographic and host range, we would expect this species to be 1 of the persistent core species as well.

If we provisionally assign *R. esculentarum* to the category of persistent core species, the changes in the helminth fauna of water frogs in HNP consists of the loss of 5 species of digenean, 1 species of nematode, and the addition of *D. subclavatus*. These species seem to have little in common with respect to their basic ecology: as a group they live in the rectum, small intestine, urinary bladder, and lungs. Their first intermediate hosts include both gastropods and bivalves, and their second intermediate hosts range from the surface of plants and the exoskeleton of crustaceans to odonate larvae and tadpoles.

One thing the missing species may have in common is their anuran host. Of the 3 members of the *P. esculentus* complex occurring in HNP, Edelényi (1972) and Murai et al. (1983) listed only *P. ridibundus* and *P. esculentus* as hosts, and all the missing helminth species were reported from these 2 species. Unfortunately, no vouchers of collected hosts were deposited, and it is possible that some of the hosts identified previously as *P. ridibundus* were actually *P. esculentus* or *P. lessonae*. In this study, we collected only *P. ridibundus* and *P. esculentus*, so it is tempting to suggest that the missing parasites still occur in the HNP but that they are restricted to *P. lessonae*, which we have not yet collected. Such an explanation leads to the question: Has this species experienced a decline, or even extirpation, in HNP?

The answer to the above question may be due to localized anthropogenic phenomena, incuding the effects of urbanization, agriculture, or both, which may have reduced or changed the composition of the invertebrate fauna necessary to maintain the transmission dynamics of the missing species of helminths and which may have allowed *D. subclavatus*, if it is truly new to HNP, to become established. Given the protected status of the study site, we do not believe that there have been such significant anthropogenic changes inside HNP, implying that regional effects, including climate change, may play an important role in this regard. At this higher level of assessment we would expect to see a geographic pattern distinguishing the species that have been lost from those that persist. However, at this level no such obvious patterns exist that might explain the differences in results between our study and the previous inventories.

Previous reports did not list information about the prevalence and intensity of infection, and all water frogs were identified as either *Rana ridibunda* (=*P. ridibundus*) or *Rana esculenta* (=*P. esculentus*). It is possible that the missing parasites were quite rare in previous studies, and that they still exist as rare species today, which is supported by our finding of only a single specimen of *P. claviger* during 2 yr of sampling. Alternatively, it is also possible that *P. lessonae* and the missing parasites were previously more common and that they have become rare in the HNP. We suggest that additional efforts be made to find and assess the status of *P. lessonae*, and their parasites, in the HNP.

A second class of baseline comparisons is evolutionary, via asking "Parascript" (Manter, 1966) questions about the phylogenetic context of the ecology and distribution of the parasites found in an inventory. Such data are rare, and not often applied to inventory studies in parasitology, despite a generation of calls for such action (e.g., Brooks and McLennan, 1993; Brooks, 1998; Brooks and Hoberg, 2000, 2006, 2007, 2013; Hoberg, 1997, 2010; Hoberg and Brooks, 2008, 2010, 2013; Hoberg et al., 2012, 2013; Brooks et al., 2014). Because the documentation phase of the DAMA protocol precludes the possibility of obtaining such direct documentation of statistically robust population estimates, our results must be considered highly preliminary, at best, given the small sample size necessitated by the constraints of collecting permits. Nevertheless, such population level data are a critical part of the assessment phase in the DAMA protocol and they can be obtained in the future using noninvasive methods that can provide both qualitative (presence–absence) and quantitative (relative abundance) data (e.g., Endo et al., 2009). As such, our data serve as a starting point and marker for the monitoring element of the HNP within the context of

the DAMA protocol. Thus, while it may seem somewhat clumsy to undertake an inventory and monitoring project in separate phases, we believe that this approach maximizes the amount of information obtained while minimizing the amount of destructive sampling needed over the long term. In short, we believe that the DAMA protocol initiated herein provides an excellent means of obtaining useful information from parasite inventories (both within the HNP and elsewhere), even though limited destructive sampling occurs. The addition of molecular genetic data derived from parasites collected in this study will allow additional critical information to be gathered using nondestructive techniques in the future.

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Paper III

Taxonomic composition and ploidy level among European water frogs (Anura: Ranidae: *Pelophylax*) in eastern Hungary

Herczeg D, Vörös J, Christiansen DG, Benovics M, Mikulíček P (2017) *Journal of Zoological Systematics and Evolutionary Research* 55:129–137 [IF_{2016}: 2.444]

Summary

The western Palaearctic water frogs in the genus *Pelophylax* comprise several distinct species and three hybridogenetic hybrid forms. In this study, we focus on the *Pelophylax esculentus* complex, which consists of two sexual species, *Pelophylax ridibundus* and *Pelophylax lessonae* and their hybridogenetic hybrid, *Pelophylax esculentus*. Specifically, we investigated taxonomic composition and ploidy level of water frogs sampled in three different types of wetland habitats in the Hortobágy National Park (HNP), eastern Hungary. Using variation in serum albumin intron 1 (SAI–1) and 15 microsatellite loci we detected the presence of all members of the *P. esculentus* complex in the studied localities. In one locality all three taxa occurred syntopically, while in others water frog populations consisted of *P. ridibundus* and *P. esculentus* exclusively. The genomic composition of the 63 examined hybrid specimens analysed with microsatellites showed the occurrence of diploid genotypes only. We used a population genetics approach (allelic richness, gene diversity, multilocus genotypes and multilocus disequilibrium) to infer the breeding system of water frogs at HNP. Our data indicate that at least in two populations hybrids form gametes with clonally transmitted *P. ridibundus* genome and produce a new hybrid generation by mating with *P. lessonae*.

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[1]*MTA–DE 'Lendület' Behavioural Ecology Research Group, Department of Evolutionary Zoology and Human Biology, University of Debrecen, Debrecen, Hungary;* [2]*Hungarian Natural History Museum, Budapest, Hungary;* [3]*Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland;* [4]*Department of Botany and Zoology, Faculty of Science, Masaryk University in Brno, Brno, Czech Republic;* [5]*Department of Zoology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovak Republic*

Taxonomic composition and ploidy level among European water frogs (Anura: Ranidae: *Pelophylax*) in eastern Hungary

Dávid Herczeg[1], Judit Vörös[2], Ditte G. Christiansen[3], Michal Benovics[4] and Peter Mikulíček[5]

Abstract

The western Palaearctic water frogs in the genus *Pelophylax* comprise several distinct species and three hybridogenetic hybrid forms. In this study, we focus on the *Pelophylax esculentus* complex, which consists of two sexual species, *Pelophylax ridibundus* and *Pelophylax lessonae*, and their hybridogenetic hybrid, *Pelophylax esculentus*. Specifically, we investigated taxonomic composition and ploidy level of water frogs sampled in three different types of wetland habitats in the Hortobágy National Park (HNP), eastern Hungary. Using variation in serum albumin intron 1 (SAI-1) and 15 microsatellite loci, we detected the presence of all members of the *P. esculentus* complex in the studied localities. In one locality, all three taxa occurred syntopically, while in others water frog populations consisted of *P. ridibundus* and *P. esculentus* exclusively. The genomic composition of the 63 examined hybrid specimens analysed with microsatellites showed the occurrence of diploid genotypes only. We used a population genetic approach (allelic richness, gene diversity, multilocus genotypes and multilocus disequilibrium) to infer the breeding system of water frogs at HNP. Our data indicate that at least in two populations, hybrids form gametes with clonally transmitted *P. ridibundus* genome and produce a new hybrid generation by mating with *P. lessonae*.

Key words: serum albumin intron-1 (SAI-1) – clonal reproduction – hybridogenesis – microsatellites – breeding system - asexual vertebrates

Introduction

Western Palaearctic water frogs of the genus *Pelophylax* include fourteen distinct species and three hybrid forms reproducing by hybridogenesis (Graf and Polls-Pelaz 1989; Günther 1990; Plötner 2005; Lymberakis et al. 2007; Plötner et al. 2012). This special mode of reproduction resembles parthenogenesis in many aspects. While parthenogenetic females clonally produce diploid eggs, which give rise to a new generation of daughters, hybridogenetic individuals (hybrids) transmit clonally only one chromosome set; the other set of chromosomes is eliminated during gametogenesis and is restored in each generation by mating. Sexual and hybridogenetic taxa of water frogs form three different complexes in Europe. In central Europe, the *Pelophylax esculentus* complex is formed by two sexual species, the marsh frog, *Pelophylax ridibundus* (Pallas, 1771; genotype RR) and the pool frog, *Pelophylax lessonae* (Camerano, 1882; genotype LL). The interspecific mating of the two species produces the hybridogenetic edible frog, *Pelophylax esculentus* (Linnaeus, 1758; genotype LR). The mode of hybridogenesis in *P. esculentus* includes clonal transfer of either the genome of *P. ridibundus* (R genome) or the genome of *P. lessonae* (L genome), rarely simultaneous transfer of both genomes to gametes (Uzzell and Berger 1975; Berger et al. 1978; Polls-Pelaz 1994). Most *P. esculentus* in central Europe eliminate the L genome during gametogenesis, clonally transmit the R genome and backcross with syntopically living *P. lessonae* (LE system). Hybrid forms that eliminate the R genome form *lessonae* gametes and backcross with *P. ridibundus* (RE system) are less common and have been found only in eastern Germany, Poland and eastern Ukraine (Uzzell and Berger 1975; Berger 1977; Uzzell et al. 1980; Borkin et al. 2004; Biriuk et al. 2016). Besides hybrid forms living and mating with one or the other parental species, there are *P. esculentus* populations that are reproductively independent of the sexual species. Such all-hybrid populations (EE system) are found mostly in northwestern, less in central and eastern Europe (Günther 1973; Ebendal 1979; Berger 1988; Fog 1994; Hoffmann et al. 2015). Persistence of all-hybrid populations is achieved by mating of individuals, which produce different types of gametes (e.g. Christiansen et al. 2005; Christiansen and Reyer 2009). In addition to diploid hybrids, these populations typically comprise a fraction of triploid individuals possessing either two L and one R genome (LLR) or two R and one L genome (LRR). Finally, syntopic occurrence of both parental species and hybrids (LER population) is recorded from central and eastern Europe (Günther et al. 1991; Gubányi 1992; Kotlík and Šůlová 1994; Mikulíček et al. 2015). However, these populations are rare and were not studied in details. In Slovak LER populations, hybrids form R gametes (as in the LE system) and *P. ridibundus* individuals likely do not contribute to perpetuation of the hybridogenetic lineages (Mikulíček et al. 2015).

Due to the high rate of hybridization and overlapping morphological characters, there is no easy way to distinguish between hybrids of different ploidy levels and parental species. Various methods give reasonable results including erythrocyte size measurements (Günther 1977; Polls-Pelaz and Graf 1988; Ogielska et al. 2001; Mezhzherin et al. 2010) combined with morphometry (Hotz and Uzzell 1982; Gubányi and Korsós 1992; Günther and Plötner 1994; Tognarelli et al. 2014), electrophoresis of allozymes (Plötner and Klinkhardt 1992; Hotz et al. 2001; Mikulíček and Kotlík 2001; Mezhzherin et al. 2010) or albumin (Lőw et al. 1989), DNA flow cytometry (Vinogradov et al. 1990; Borkin et al. 2004; Arioli et al. 2010; Mikulíček et al. 2015) and microsatellites (Christiansen 2005; Arioli 2007; Christiansen and Reyer 2009; Pruvost et al. 2013).

Corresponding author: Dávid Herczeg (herczegdavid88@gmail.com)
Contributing authors: Judit Vörös (jvoros@nhmus.hu), Ditte G. Christiansen (dittegc@gmail.com), Michal Benovics (michal.benovics@gmail.com), Peter Mikulíček (pmikulicek@fns.uniba.sk)

Our understanding of the taxonomic and genotypic composition in Hungarian water frog populations is limited: few studies have been published and many of them are not based on genetic data. Several publications from the early and mid-20th century did not distinguish between *P. lessonae* and *P. esculentus* individuals (Bolkay 1909; Fejérváry 1921; Fejérváry-Lángh 1943; Dely 1953). In Hungary, studies based on both morphology and genetic data include those conducted in western (Gubányi 1988, 1990; Lőw et al. 1989; Gubányi and Pekli 1991; Tunner and Heppich-Tunner 1992), north-eastern (Berger et al. 1988) and eastern parts of the country (Mészáros 1973; Mészáros and Bartos 1978; Gubányi and Korsós 1992). Eastern Hungarian populations have not been well represented, and only the papers published by Mészáros and Bartos (1978) and Gubányi and Korsós (1992) examined the genetic structure of water frogs collected in Hortobágy National Park (hereafter HNP). Except from one population in western Hungary (Tunner and Heppich-Tunner 1992), we have no information about the type of breeding system of water frogs. Furthermore, our study is the first which attempts to assess breeding system of water frogs without the implementation of crossing experiments. Our intention was to apply population genetic approach to determine the type of gametes produced by hybrids and to infer the breeding system of water frogs.

The aims of the present study were to (1) determine the taxonomic composition of the *P. esculentus* complex in HNP using molecular markers, (2) specify the ploidy levels of hybrid individuals and (3) examine the effect of population structure on genetic diversity and the rate of clonal inheritance in water frogs.

Materials and Methods

Study area

This study was conducted in HNP, the largest continuous alkaline steppe in Europe covering 80 000 hectares. Founded in 1973, the area is the largest and oldest National Park in Hungary, abundant in wetland habitats like alkaline marshes, fishponds, wet grasslands and wet meadows. The expanded protected area also includes 75 fishponds covering a total of 6000 ha and ranging between 1 and 790 ha each (Ecsedi 2004).

Sampling

We collected 164 water frogs during the years 2012–2014 from three different types of wetland habitats. Fieldwork was carried out between April and October each year, respectively. Each site was visited at least three times during the years (in total, thirteen times). Habitats comprise slowly flowing water at the Nádudvar-Kösély canal (hereafter NKC), a fish pond system at Hortobágy fish ponds (HFP) and a marshland system at Egyek-Pusztakócs (EPMS). There are no direct connections between these wetland habitats which can provide easy passage for water frog migration. The straight geographic distance between the localities EPMS and NKC is more than 27 km, 15 km between EPMS and HFP and 21 km between HFP and NKC (Fig. 1). Sampling sites were visualized with Quantum GIS 2.8 (QGIS Development Team, 2015). A brief description of localities, sample sizes and GPS coordinates is given in Table 1. Frogs were captured at night manually and with dip nets. Additional individuals were collected from fresh road kills. DNA from water frogs was extracted from buccal swabs (Tubed Sterile Dryswab™, MWE) using DNeasy Blood and Tissue Kit spin columns (QIAGEN Inc., Valencia, CA, USA) and from toe clips of road-killed individuals. Voucher specimens of water

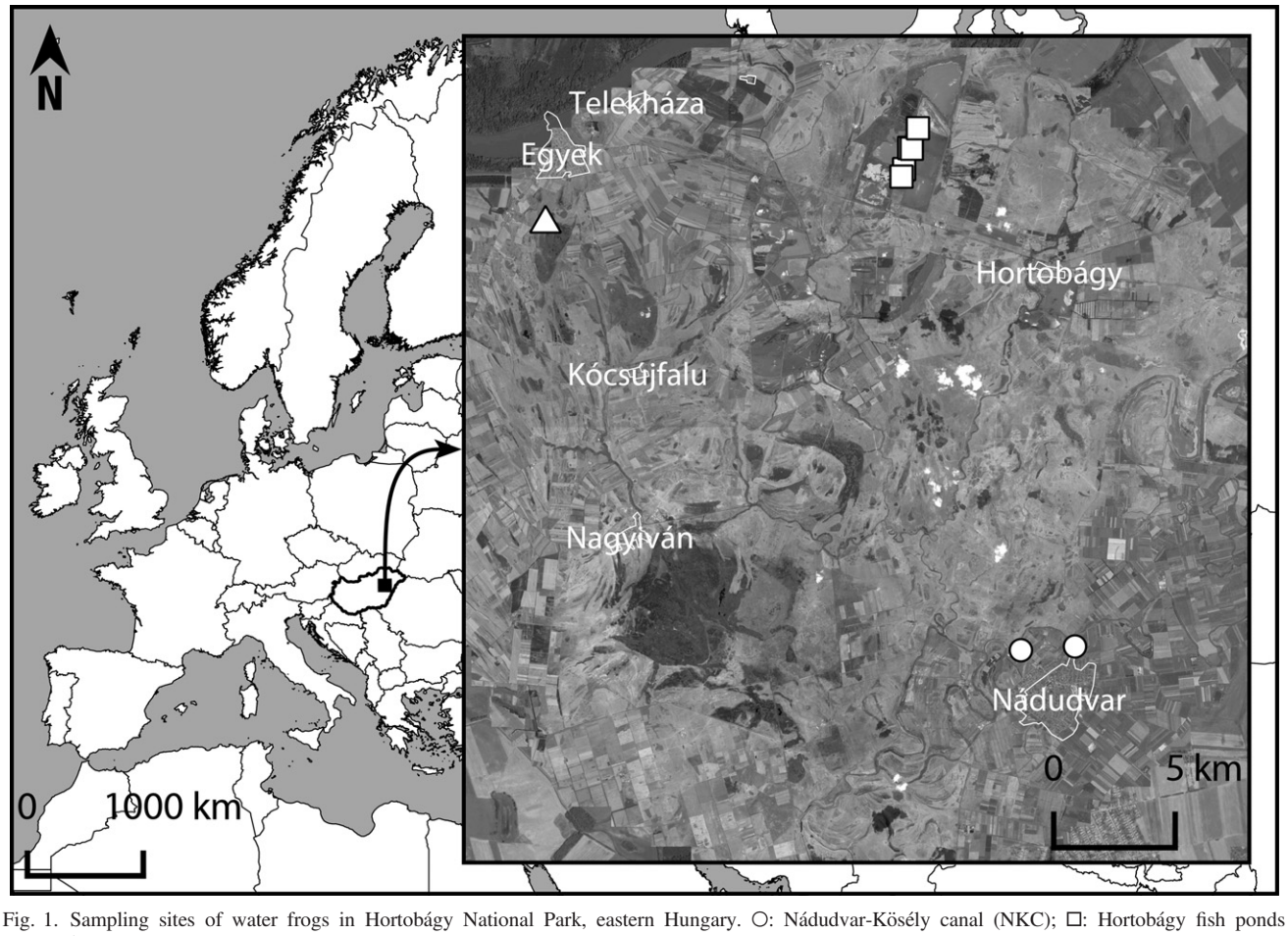

Fig. 1. Sampling sites of water frogs in Hortobágy National Park, eastern Hungary. ○: Nádudvar-Kösély canal (NKC); □: Hortobágy fish ponds (HFP); Δ: Egyek-Pusztakócs marsh system (EPMS)

Table 1. Geographic coordinates, sample sizes and brief description of sample localities at HNP

Locality	Abbreviation	Longitude	Latitude	Sample size	Habitat
Nádudvar-Kösély canal	NKC	47°26′43	21°10′11	30	Irrigation canal with moderate reed belt
		47°26′38	21°8′25	32	Irrigation canal with moderate reed belt
Hortobágy fish ponds	HFP	47°37′15	21°4′36	13	Small 'pool' near bigger fishpond
		47°37′43	21°4′46	12	Bank of bigger fishpond with moderate reed belt
		47°37′42	21°4′51	12	Bank of bigger fishpond with no reed belt
		47°38′9	21°5′3	20	Bank of bigger fishpond with moderate reed belt
		47°37′5	21°4′31	24	Canal alongside the railway with poor vegetation
Egyek-Pusztakócs marsh system	EPMS	47°36′3	20°52′53	21	Marshland with temporary water level

frogs were deposited in the Herpetological Collection of the Hungarian Natural History Museum (Table S1).

Species and ploidy level identification

We used molecular markers to distinguish, first, among the taxa *P. ridibundus*, *P. lessonae* and *P. esculentus*, and second, between the diploid and triploid individuals of hybrids. Taxon identification was determined using the technique described by Hauswaldt et al. (2012) and is based on allele size polymorphism in intron 1 of the serum albumin gene (SAI-1; Plötner et al. 2009), with a slight modification in PCR protocol. Specifically, we used a PCR mix of a total volume of 10 μl containing 1 μl 10X Taq buffer with $(NH_4)_2SO_4$ (ThermoScientific, Waltham, MA, USA), 0.48 μl of both forward (5′-TCCATACAAATGTGCTAAGTAGGTT-3′) and reverse (5′-GACGGTAAGGGGACATAATTCA-3′) primers (10 μM), 1 μl dNTPs (10 μM), 0.6 μl $MgCl_2$ (25 mM), 0.08 μl Taq DNA polymerase (5 U μl^{-1}) (ThermoScientific, Waltham, MA, USA), 5.56 μl nuclease free water and 0.8 μl of DNA. The PCR profile comprised an initial denaturation at 94°C for 90 sec, followed by 35 cycles of denaturation (30 sec at 94°C), annealing (40 sec at 59°C) and elongation (100 sec at 72°C), and a final elongation step at 72°C for 10 min. Three μL of PCR product was run on 1.8% of agarose gels. To verify SAI-1 fragments, we sequenced representative alleles on a Hitachi 3130 Genetic Analyzer (Applied Biosystems, UK). Consensus sequences were compiled using BioEdit version 7.0.9.0 (Hall 1999) and aligned manually. The sequences were then compared with those deposited in GenBank.

Ploidy determination and genotype composition of hybrids (diploid RL, triploid LLR and LRR) was based on analysis of species-specific polymorphism in selected microsatellite loci (Table 2). Besides Hungarian samples, we added three triploid LLR frogs from Kozí Chrbát, Slovakia, whose ploidy and genotypic composition was confirmed in the study of Pruvost et al. (2015). Two multiplex PCR amplification sets with forward primers fluorescently labelled with FAM, VIC, NED and PET were performed as follows. Reactions for Multiplex 1 were 6 μl total volume and contained 1 μl template DNA, 3 μl 2× QIAGEN Multiplex PCR kit (QIAGEN Inc., Valencia, CA, USA), 1.58 μl nuclease free water and 0.06 μl (10 μM) of both forward and reverse primers. Reactions for Multiplex 2 were the same except that we used 0.12 μl from Res14 and 0.18 μl from Re2Caga3 forward and reverse primers. In order to achieve equivalent allele intensity (i.e. those concentration of primers to all alleles amplify in similar amount), different primer volume was used. Multiplex PCR was performed using the following conditions: 15 min at 95°C, followed by 30 cycles of 30 sec at 94°C, 90 sec at 60°C and 60 sec at 72°C, followed by the final step 30 min at 60°C. PCR products were run on an ABI 3130 Genetic Analyzer (Applied Biosystems, UK) with a LIZ-500 size standard. The alleles (relative peak heights) were scored with GeneMarker software (Softgenetics, State College, PA, USA) manually by a single observer.

Estimation of null alleles, genetic diversity and clonality

Null alleles in microsatellite loci refer to alleles present in an individual that are not amplified because of mutation(s) in priming sites. An individual heterozygous in a specific locus, but having a null allele, is thus scored as a homozygote, because the null allele is not amplified during PCR. In hybridogenetic water frogs, which behave genetically as F1 hybrids, all individuals should be heterozygous in loci that amplify in both parental genomes. A homozygous genotype must be due to either a null allele or an allele, which is not species-specific, but occurs in both genomes. For loci that amplify only in one parental genome, all null alleles are 'visible' as non-amplification. We estimated frequency of null alleles in microsatellite loci as follows: first, we assigned all alleles to specific parental genomes (R or L) according to microsatellite data from the nearest genetically investigated populations in Slovakia (Mikulíček and Pišút 2012; Pruvost et al. 2013, 2015; Hoffmann et al. 2015). In loci amplifying in both genomes, alleles were either genome specific (e.g.

Table 2. Microsatellite loci used in this study to determine the ploidy level, the genetic diversity and clonality of *Pelophylax esculentus*. Loci were amplified either in one of the parental genomes (R or L) or in both parental genomes. Alleles found in Hungarian samples are given in parentheses.

Locus	Genome specificity (alleles)	References	Tag
Multiplex 1			
RICA1b6	L (73, 79, 83, 85) R (73, 85, 90, 97, 99)	Arioli (2007)	PET
RICA1b5	L (120, 122) R (135)	Garner et al. (2000)	NED
Ga1a19	L (99) R (105, 108, 118, 120, 134, 148)	Arioli (2007)	FAM
Rrid064A	R (223, 225, 227, 228, 230, 232, 236)	Christiansen and Reyer (2009)	NED
RICA5	L (248, 258, 260, 264)	Garner et al. (2000)	VIC
RICA2a34	L (110, 131, 137, 142, 144)	Christiansen and Reyer (2009)	VIC
Rrid013A	L (288) R (289, 292, 295, 298, 301, 304)	Hotz et al. (2001)	PET
Multiplex 2			
Res14	L (139) R (139, 145, 150)	Zeisset et al. (2000)	FAM
RICA18	L (178, 180, 182, 184, 206)	Garner et al. (2000)	NED
Res22	R (84, 86, 110, 112)	Zeisset et al. (2000)	NED
Rrid059A	L (101) R (113, 115, 133, 136, 138)	Hotz et al. (2001)	VIC
Rrid169A	R (184, 188, 192, 194, 196, 210, 212, 215)	Christiansen and Reyer (2009)	VIC
Re1Caga10	L (96, 98) R (102, 106, 108, 110, 114, 116, 118)	Arioli (2007)	FAM
Res20	*	Zeisset et al. (2000)	PET
Re2Caga3	R (173, 189, 204, 209, 213, 218, 231, 239, 244, 248)	Arioli (2007)	PET

*Neither L nor R genome was amplified by the primer.

allele 135 was present in R genome and alleles 120 and 122 in L genome in the locus RICA1b5; Table 2) or revealed significant differences in frequency between the genomes (e.g. allele 139 in the locus Res14 was fixed in L genome, but occurred in low frequency also in R genome). When an allele was not species-specific, it was assigned to the R or L genome according to its frequency observed in parental gene pools. After assigning the alleles to specific parental genomes, we counted the number of missing alleles and expected they were not amplified because they were null alleles. Finally, we calculated the frequency of null alleles locus by locus.

Because R and L genomes rarely or never recombine, all population genetic parameters were calculated for each genome separately. To estimate genetic diversity within the *P. esculentus* populations, we used allelic richness (AR) and gene diversity (*He*). AR and *He* were calculated in the program SPAGeDi 1.5, allowing simultaneous analysis of haploid and diploid data (Hardy and Vekemans 2002). The level of genome clonality was determined, based on the number of multilocus genotypes (MLG) and multilocus disequilibrium ($\bar{r}_d$). The number of MLGs was calculated using the program GenAlEx 6.4 (Peakall and Smouse 2006). MLG is the number of identical combinations of alleles found in microsatellite datasets; few combinations and high frequencies of these combinations may indicate clonal reproduction. In the absence of recombination, frequent MLGs here defined as four or more encounters (in agreement with Pruvost et al. 2015) would represent hemiclones (i.e. clonally transmitted haploid genomes *sensu* Vrijenhoek 1979). Multilocus disequilibrium is a score of non-random association of alleles at microsatellite loci and varies on a scale from ca. zero to one. Low $\bar{r}_d$ values are related to high recombination rate and thus would indicate low levels of clonality. Contrarily, high $\bar{r}_d$ values might be associated with clonal inheritance, as clonal genomes are inherited *en bloc*, without recombination. Multilocus disequilibrium was calculated in the program Multilocus 1.3 (Agapow and Burt 2001).

Results

Taxonomic composition

SAI-1 gene fragment and microsatellites data identified all members of the *P. esculentus* complex in the studied localities. The 164 frogs examined comprised 100 *P. ridibundus*, 1 *P. lessonae* and 63 *P. esculentus* individuals. In *P. ridibundus,* SAI-1 amplification resulted in a single band on agarose gels at approximately 850 bp, while in several individuals there was a second band at approximately 700 bp. In *P. lessonae,* the PCR produced one band at approximately 300 bp. In hybrids, a 300-bp-long fragment was always in combination with either 850-bp or 700-bp fragment. We compared our sequences with GenBank entries and found that the 300-bp PCR band generated from *P. lessonae* and *P. esculentus* (GenBank accession number KX838289) individuals was identical to *P. lessonae* GenBank entries FN432385 from Germany and FN432383 from *P. lessonae* in Italy. The sequence obtained from the 850-bp band of *P. ridibundus* (GenBank accession number KX838291) and *P. esculentus* matched Genbank entry JQ965524 from *P. ridibundus* in Latvia. The 700-bp band derived from both *P. ridibundus* and *P. esculentus* (GenBank accession number KX838290) matched GenBank entries JQ965529 and JQ965528 sampled in Poland. Both GenBank sequences are assigned to *Pelophylax kurtmuelleri* (Gayda, 1940), a disputable taxon distributed in the Balkans, whose allele in the SAI-1 gene was found in central European *P. ridibundus* (Hauswaldt et al. 2012). Table 3 summarizes the species composition ratio between different habitats and populations. *Pelophylax ridibundus* was the most frequent species (10–69% per each locality, 61% of all individuals), whereas *P. esculentus* occurred in moderate frequencies (31–86% per each locality, 38% of all individuals), and *P. lessonae* was represented by a single individual (0–5% per each locality, 0.6% of all individuals).

We recorded two types of populations occurring in HNP. In NKC and HFP, we found *P. ridibundus* and *P. esculentus* individuals (RE population) and a population with all three taxa, but dominated by *P. esculentus* occurred at EPMS (LER population; Table 3). In NKC and HFP, both sexes of *P. ridibundus* co-occurred with females and males of *P. esculentus*. In NKC, 95% of the hybrids were females, while in HFP, the proportion was 61% compared to males.

Ploidy level of hybridogenetic hybrids

We screened a total of 63 hybrid individuals from all three populations and diagnosed their ploidy level with a set of microsatellite loci (Table 2). The number of different alleles per locus ranged from 1 to 10, with a mean of 5.8. All individuals were indicated to be LR genotype. Three primers (Ga1a19, RICA1b5 and Rrid013A) amplified L and R loci with dosage effect (Fig. 2) demonstrating low levels of polymorphism, often a single L and one R allele were amplified in hybrids. In LLR frogs from a Slovak population, the L peak was clearly higher than the R peak, while in LR frogs, they were of similar height. For each locus, the dosage values clustered into two distinct groups. The three LLR individuals always fell in the left group (Fig. 2) confirming that triploid frogs are detectable by this method. The right group with dosage values around zero are classified as diploid LR frogs. It thus appears that no LRR frogs were present in the sample. The three reference LLR individuals had consistent LLR dosage values at all three loci and were heterozygous for several L-specific alleles at other loci, but the few individuals with LLR dosage values in our study differed for Ga1a19 (N = 2), RlCA1b5 (N = 4) and Rrid013A (N = 1) and did not show heterozygosity for L-specific alleles at other loci. Therefore, those individuals were considered to be diploids.

Null alleles, genetic diversity and clonality in *P. esculentus*

The highest frequency of null alleles was found in the locus Re1Caga10 (0.313 and 0.217 in the L and R genome, respectively). This locus was subsequently expelled from the analyses

Table 3. Summary table of water frogs collected during the years 2012–2014 at HNP. The table also includes juvenile individuals (N = 46).

	Taxon														
	P. ridibundus					*P. lessonae*					*P. esculentus*				
Locality	N	%	♀	♂	ASR	N	%	♀	♂	ASR	N	%	♀	♂	ASR
NKC	42	68	17	18	0.51	0	0	0	0	–	20	32	18	1	0.05
HFP	56	69	12	24	0.66	0	0	0	0	–	25	31	11	7	0.38
EPMS	2	10	1	0	0	1	5	0	0	–	18	86	5	0	0
Total	100	61	30	42	0.58	1	0.6	0	0	–	63	38	34	8	0.19

ASR, adult sex ratio is expressed by convention as the proportion of males in the adult population.

of genetic diversity and clonality. Frequency of null alleles in other loci was low, ranging from 0 to 0.116.

Genetic diversity within *P. esculentus* was slightly higher in L than in R genome. Allelic richness and gene diversity ranged between 1.310–3.680 and 0.051–0.550 in L genome, and 1.900–3.610 and 0.287–0.447 in R genome, respectively (Table 4). Very low values of allelic richness (AR = 1.310) and gene diversity (*He* = 0.051) were observed in the L genome in the population HFP. Multilocus genotype (MLG) analysis revealed overall four frequent (four or more times encountered) MLGs in R and one in L genome, respectively, although frequency of MLGs varied among the localities (Table 5). In all localities, most hybrids had frequent R MLGs and rare L MLGs, although the frequency of a single L MLGs was high in HFP.

Multilocus disequilibrium ($\bar{r}_d$), a measure of clonality, varied in both genomes (L genome 0.017–1.000; R genome 0.051–0.375) (Table 6). The lowest $\bar{r}_d$ value in the L genome was found at EPMS and co-occurred with the presence of a *P. lessonae* frog, with exclusively rare L MLGs and with the highest gene diversity (*He*) in the L genome. Thus, all available information agrees on high levels of recombination in the L genome at EPMS. Similarly, the lowest $\bar{r}_d$ in the R genome was found at HFP, where the highest percentage of *P. ridibundus*, the highest number of rare R MLGs and the highest genetic diversity in the R genome (both AR and *He*) were also encountered. Again, the results obtained independently from population composition, genetic diversity and clonality agree with each other indicating high levels of recombination in the R genome at HFP.

Discussion

Taxonomic composition of water frogs in Hortobágy National Park

At the three sites investigated in the Hortobágy National Park (HNP), the water frog population comprised all species of the *P. esculentus* complex. The collected material was dominated by *P. ridibundus* (61% of all sampled individuals) and *P. esculentus* (38% of all sampled individuals). In both taxa, males and females were detected. Our results correspond to previous studies

Table 4. Genetic diversity calculated separately for L and R genomes in hybrid individuals. Sample size at each locality is given in parentheses.

	L genome		R genome	
Locality	AR	*He*	AR	*He*
NKC (20)	3.680	0.496	2.730	0.371
HFP (25)	1.310	0.051	3.610	0.447
EPMS (18)	3.330	0.550	1.900	0.287
Mean	3.140	0.469	3.010	0.389

AR, allelic richness; *He*, gene diversity

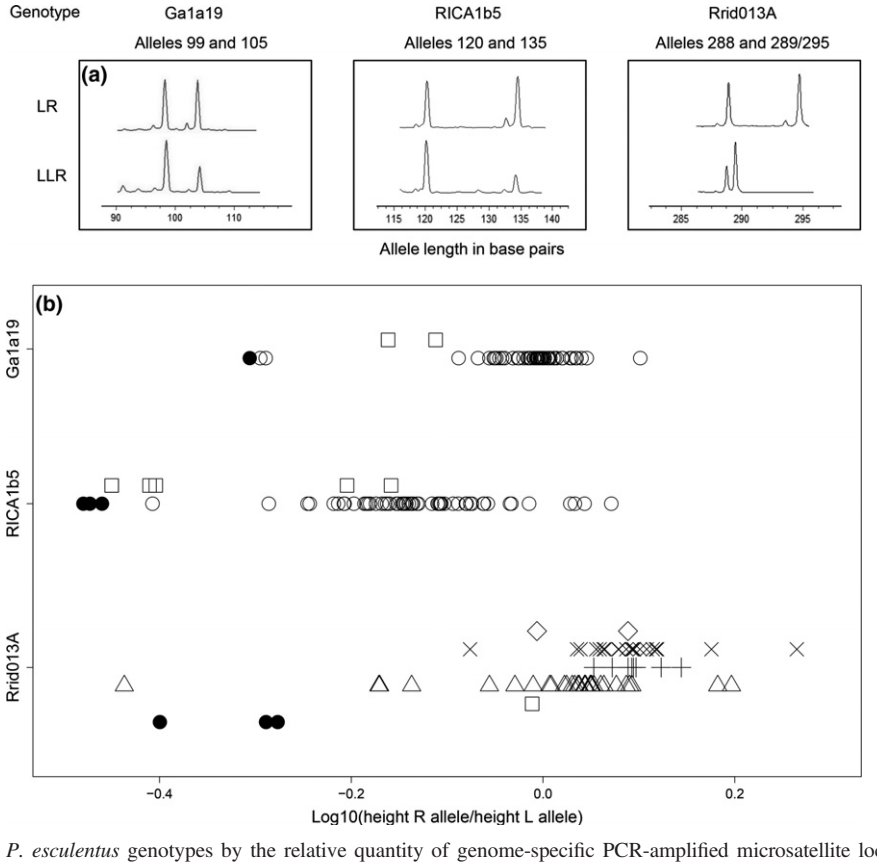

Fig. 2. Differentiation of *P. esculentus* genotypes by the relative quantity of genome-specific PCR-amplified microsatellite loci. (a) Genotype-specific amplification patterns at three microsatellite loci. At locus Ga1a19, the allele of 99 base pairs is L specific; allele 105 is R specific. At locus RICA1b5, allele 120 is L specific; allele 135 is R specific. At locus Rrid013A, allele 288 is R specific and alleles 289 and 295 are L specific. The relative peak heights differ between hybrid genotypes. (b) Allele height ratios were log10-transformed and ranged between −0.305 and 0.101 in Ga1a19; −0.479 and 0.071 in RICA1b5; −0.436 and 0.264 in Rrid013A. Legend: Ga1a19: □ – L99/R108, ○ – L99/R105; RICA1b5: □ – L122/R135, ○ – L120/R135; Rrid013A: ◇ – R288/L304, X – R288/L301, + – R288/L298, Δ – R288/L295, □ – R288/L292, ● – filled symbols are reference LLR individuals.

Table 5. Multilocus genotype (MLG) analysis in three localities of the *P. esculentus* complex. R-A to R-D and L-A represent different multilocus genotypes detected in R and L genome of *P. esculentus* with a frequency of four or more. MLGs presented only one to three times were summarized as R others and L others.

Multilocus genotypes	NKC	HFP	EPMS
R-A	3	0	6
R-B	1	4	0
R-C	4	5	5
R-D	4	3	3
R others	7	12	4
L-A	5	20	0
L others	15	5	18

Table 6. Multilocus disequilibrium ($\bar{r}_d$), a measure of clonality, calculated separately for L and R genomes in hybrid individuals. Sample size at each locality are given in parentheses.

Locality	L genome	R genome
NKC (20)	0.107	0.221
HFP (25)	1.000	0.051
EPMS (18)	0.017	0.375
Mean	0.375	0.216

reporting *P. ridibundus* and *P. esculentus* from the region (Marián 1963; Dely 1967; Murai et al. 1983). At EPMS, we collected a single juvenile *P. lessonae* individual. *Pelophylax lessonae* may be more abundant in EPMS or our results were affected by small sample size ($N = 21$). It is also possible that *P. lessonae* has suffered population loss and is particularly rare in HNP. Mészáros and Bartos (1978) confirmed the presence of *P. lessonae* in HFP, but they did not provide sample size data of collected specimens, so we cannot make any conclusions about the past abundance. Given the protected status of the study site, we do not hypothesize that there have been such significant anthropogenic changes inside HNP, implying that regional effects and climate change may play an important role. On the other hand, Mester et al. (2015) reported higher frequencies of *P. lessonae* at EPMS based on morphological and call monitoring data. This may support our first explanation about low sample size.

At two permanently flooded sites, we found RE populations dominated by *P. ridibundus* and living sympatrically with *P. esculentus*, while the site with temporary water level proved to be an LER population, on which both parental species and the hybrid co-occurred. The characteristics of the habitats in HFP and NKC seem to be optimal for *P. ridibundus* which prefers large and deep water bodies where the water is continuously renewed with oxygen (Morand and Joly 1995; Holenweg and Reyer 2000; Pagano et al. 2001; Mikulíček et al. 2015). This matches our findings, in which 98% ($N = 98$) of the *P. ridibundus* individuals were collected from sites with these habitat characteristics. *Pelophylax esculentus* shows no strict characteristics in habitat preference and it can be found practically in every type of water bodies (Berger 1973; Ildos and Ancona 1994). In the examined populations, hybrids occurred in all types of wetland habitats in HNP, while the only one collected *P. lessonae* individual was found in the shallow water body with temporary water level in EPMS as previously reported by Mester et al. (2015). After the regulation of the river Tisza in the 19th century at EPMS, the extensive floods have disappeared and erosion intensified, which all led to an accelerated alkalization of the region. Today, the area characterized by extensive Pannonic salt steppes and marshes (Aradi et al. 2003). In the past few decades, the water supply systems of the marshes were reconstructed by the result of a LIFE-Nature programme (Déri et al. 2009). This human-mediated environmental changes may provide possibly new habitats for *P. lessonae,* which populations show declining trend in Central Europe (Holsbeek and Jooris 2009; Mayer et al. 2013).

Ploidy level

In this study, we examined the genotypic composition of 63 *P. esculentus* individuals using microsatellite loci. Nine frogs showed contradictions in their genotype determination. One locus indicated LLR genotype (Fig. 2) either by dosage effect (seven individuals) or by heterozygosity (two individuals) but all the remaining loci in these frogs suggested LR genotype. Thus, we considered all examined hybrids to be LR diploids. In contrast, the three LLR reference frogs from Slovakia were easily recognizable as triploids as they showed dosage effect and/or heterozygosity at 4–5 loci. Although our dataset did not include any LRR individuals, the principle would have been the same. We therefore conclude that any triploid frogs would reliably have been distinguished from LR frogs with the current set of microsatellites as previously demonstrated in several other studies (Christiansen 2005; Hoffmann et al. 2015; Pruvost et al. 2015).

The presence of triploids in Hungarian water frog population was reported by Tunner and Heppich-Tunner (1992) in northwestern Hungary. They described a population system comprising *P. ridibundus* of both sexes, diploid *P. esculentus* of both sexes and exclusively male triploid (LLR) *P. esculentus*. The ploidy level of hybrids was confirmed with electrophoresis of the albumin locus and chromosome examination. The observation of Tunner and Heppich-Tunner (1992) was not confirmed recently by the study of Pruvost et al. (2015) and by our field research (J. Vörös, D. Herczeg, L. Choleva, P. Mikulíček; unpublished data) in the same area. Therefore, that was the only report of a triploid population in Hungary; all others (Mészáros and Bartos 1978; Berger et al. 1988; Gubányi and Korsós 1992; this study) encountered only diploid hybrids.

Using population genetics to infer breeding system

The RE-system with syntopic occurence of hybrids with P. ridubundus without P. lessonae tends to be uncommon in Europe; isolated populations observed in eastern Ukraine (Borkin et al. 2004; Biriuk et al. 2016), western and eastern Poland (Rybacki and Berger 2001), Germany (Günther 1990), France (Pagano et al. 1997) and Slovakia (Mikulíček et al. 2015). Whether hybrids in Hungarian RE populations eliminate the R genome and form L gametes, as in some German and Polish populations (Uzzell and Berger 1975; Berger 1977; Uzzell et al. 1980) or the simultaneous production of the L and R gametes, as was observed in populations in Ukraine (Vinogradov et al. 1990, 1991; Biriuk et al. 2016) remains an open question that can only be fully answered by crossing experiments. Alternatively, Hungarian hybridogenetic lineages can produce R gametes (as in the vastly distributed LE system), be reproductively dependent on *P. lessonae* individuals, which, however, should reach extremely low densities in studied populations. In this case, *P. ridibundus* would not serve as a host species in hybridogenetic reproduction, but just coexists with hybrids, as was observed in the mid-Rhône drainage in France (Pagano et al. 1997) or in western Slovakia (Mikulíček et al. 2015).

In the absence of crossing experiments, we aimed to settle the question by assessing the level of clonality in the L and the R

genome. If all L genomes in hybrids come from sexually reproducing *P. lessonae*, and the R genomes come partly from clonal gametes of *P. esculentus* and partly but less frequently from sexually reproducing *P. ridibundus*, then the L genome should show no clonality (i.e. high number of MLGs and a value of $\bar{r}_d$ around zero), while the R genome should show some evidence of clonality. Genetic diversity (both AR and *He*) decreases by clonal reproduction and should therefore be higher in the L than in the R genome.

The three localities analysed produced different results. In both, EPMS and NKC, as expected, the L genome was indeed less clonal (lower score of $\bar{r}_d$, higher number of MLGs) and had the higher level of genetic diversity than the R genome. This suggests that at EPMS and NKC, populations belong to the LE system typical in central Europe, in which hybrids make R gametes and only can produce new hybrids by mating with *P. lessonae*. This effect was especially apparent in EPMS. This makes sense, as EPMS was the locality with typical *P. lessonae*-specific habitat, where *P. ridibundus* was rare, where the only *P. lessonae* in the present study was found and where Mester et al. (2015) suggested a higher proportion of *P. lessonae*. In contrast, at NKC, the habitat was more similar to *P. ridibundus* preferences, *P. ridibundus* dominated in numbers and no *P. lessonae* was found there. If only few individuals of *P. lessonae* are present and have parented all the hybrids, genetic diversity in the L genome of the hybrids should indeed be low, as was observed at NKC. An even lower number of *P. lessonae* parents should produce a genetic signal in the hybrid offspring that is indistinguishable from clonal reproduction (low number of multilocus genotypes and high value of $\bar{r}_d$). This could explain why HFP could also belong to the LE system, although the L genome had a lower number of multilocus genotypes, a higher score of $\bar{r}_d$, and a lower level of genetic diversity than the R genome.

The application of $\bar{r}_d$ to infer breeding system of water frogs in the present study is to our knowledge novel. Previously, $\bar{r}_d$ was used to demonstrate genetic recombination in triploid hybrids in all-hybrid populations, but not to infer breeding system (Christiansen and Reyer 2009). We emphasize that the most accurate way to assess the type of gametes produced by hybrids and to determine the breeding system of water frogs is by crossing experiments; however, this is very time consuming. Therefore, our novel way of applying $\bar{r}_d$ in relation to breeding system seems to have great potential for use in future genetic water frog studies and might thus help extend our understanding of breeding system and their distribution. This method clearly has its limitations related to the number and polymorphism of selected genetic markers which might explain that it did not provide the expected result for all populations tested, but these first results represented a valuable first contribution to gathering experience with the method for the benefit of future studies.

In conclusion, additional efforts need to be carried out (e.g. sampling during mating activity) to clarify the distribution of *P. lessonae* at HNP and this may strengthen our assumption about the breeding system of water frogs inhabited in this nature reserve.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary table of water frog specimens used in this study.