Institute of Evolutionary Biology and Environmental Studies

Ecological and genetic structure of water frog populations: the role of habitat characteristics and invasive species

Master Thesis

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Zusammenfassung

Teil I: Invasive Seefrösche und triploide Teichfrösche als Bedrohung für die einheimischen Wasserfrösche (*Pelophylax* spp.)

Der Europäische Wasserfrosch-Komplex ist geeignet, um die Effekte eines invasiven Taxons auf einheimische Taxa zu untersuchen. Der invasive Seefrosch (*Pelophylax ridibundus)* beeinflusst einheimische Wasserfrosch-Populationen negativ durch Hybridisierung. Es wird angenommen, dass Hybridisierung mit dem Seefrosch eine Hauptursache für das Verschwinden der einheimischen Kleinen Wasserfrösche (*P. lessonae*) und Teichfrösche (*P. esculentus*) ist. Endpunkt der Invasion sind reine *P. ridibundus*-Populationen. Persistenz der einheimischen Taxa ist möglich, wenn die Taxa unterschiedliche Habitatpräferenzen zeigen. Um mögliche Habitatunterschiede aufzuzeigen, untersuchte ich, wie die Struktur von 16 Schweizer Wasserfroschpopulationen mit der Wassertemperatur zusammenhängt und ob es Unterschiede gibt zwischen temporären und permanenten Gewässern. Ich konnte zeigen, dass *P. ridibundus* fast ausschliesslich in permanenten und kalten Gewässern vorkommt. Im Gegensatz dazu bevorzugen beide einheimischen Taxa temporäre Gewässer. Deren unterschiedliche Präferenzen beeinflussen möglicherweise die Folgen der *P. ridibundus* Invasion. Allerdings ändern sich die Folgen für eine Population auch in hohem Masse, wenn triploide *P. esculentus* einwandern. Ich habe Triploide in 4 von 16 Gewässern gefunden. Die Invasion von triploiden *P. esculentus* kann im Gegensatz zur Invasion von *P. ridibundus* als kryptische Invasion bezeichnet werden, da die Triploiden nur unzureichend anhand äusserer morphologischen Merkmale bestimmt werden können. Ich nutzte Microsatelliten-Daten, Genotyp-Häufigkeiten, mtDNA-Sequenz Analysen und phänotypische Merkmale, um herauszufinden ob die Triploiden invasiv oder einheimisch sind. Ich fand heraus, dass vermutlich eine Invasion von Ungarn oder möglicherweise aus der Slowakei stattgefunden hat. Meine Resultate deuten darauf hin, dass die einheimischen Wasserfrösche trotz invasiver *P. ridibundus* persistieren können. Im Gegensatz zu *P. ridibundus* scheint es den invasiven Triploiden nicht möglich zu sein, die einheimischen Wasserfrösche zum Verschwinden zu bringen. Dennoch gefährden die Triploiden das einheimische LE-System.

Teil II: *Batrachochytrium dendrobatidis*-Infektionen bei Kaulquappen und Adulten aus dem hybridogenetischen *Pelophylax esculentus*-Komplex

Massensterben von Amphibien in den 90er Jahren wurden durch Chytridiomykose-Ausbrüche erklärt. Die Chytridiomykose ist eine Hautkrankheit, welche durch den Chytridpilz *Batrachochytrium dendrobatidis* (Bd) ausgelöst wird. Bd wurde als Hauptursache vieler Amphibienrückgänge auf allen Kontinenten mit Ausnahme der Antarktis identifiziert. Das Vorkommen von Bd ist begrenzt auf nicht austrockende aquatische Lebensräume. Daher untersuchte ich, ob die Bd-Infektionsrate von Amphibien in temporären Gewässern tiefer ist als permanenten Gewässern. Ich testete 528 Wasserfrosch-Kaulquappen auf Bd-Infektion in 16 Schweizer Gewässern, die Hälfte temporär und die Hälfte permanent. Ich konnte in keinem der Gewässer Kaulquappen mit Bd-Infektion finden. Das Fehlen der Infektion bei Kaulquappen im Gegensatz zu Adulten deutet darauf hin, dass unter natürlichen Bedingungen die Bd-Infektion von Wasserfröschen nach der Metamorphose stattfindet.

Part I: Invasive *Pelophylax ridibundus* and triploid *P. esculentus* threaten native water frog populations in Switzerland

The European water frog complex is suitable to study the effects of an invading taxon on a native population. The invading taxon *Pelophylax ridibundus* can disturb the local population systems through hybridization. Hybridization is supposed to be a proximate driver of replacement of the native water frogs *P. lessonae* and *P. esculentus*, ending in pure *P. ridibundus* populations. Limitation of *P. ridibundus* populations and persistence of native taxa is possible if taxa show different habitat preferences. To test for habitat differences, I analysed whether and how the structure of 16 swiss water frog populations depends on temperature and pond hydroperiod. I found *P. ridibundus* nearly exclusively in permanent and cold ponds, whereas both native taxa preferred temporary ponds. Those habitat preferences may influence the outcome of *P. ridibundus* invasions. However, the outcome for a population changes greatly if the invader is a triploid *P. esculentus*. I found triploids in 4 of 16 ponds. Invasive triploids may be described as a cryptic invasion because unlike *P. ridibundus*, triploids cannot easily be determined using external morphology. Using a combination of microsatellite data, genotype frequencies, mtDNA sequence analysis and phenotype determination I tested whether the triploids have invaded or whether they are native. The invasion took most likely place from Hungary or, with lower probability, perhaps from Slovakia or the Fontainebleau Forest in France. My results suggest that native water frogs may persist because the natives and the invading *P. ridibundus* differ in habitat preferences. In contrast to *P. ridibundus*, the found invasive triploids are not able to replace the native water frogs, but they threaten the LE-system.

1 Introduction

The introduction of non-native species may have several consequences for native species such as predation, competitive exclusion, introduction of diseases and habitat modification [1-3]. If the native species is closely related to the invader, the existence of the native specie may also be threatened genetically by hybridization [3, 4]. Hybridization and its consequences for native taxa can be well studied in members of the European water frog complex (*Pelophylax* spp.). The genetic mechanism of the unusual reproductive system makes them particularly prone to negative effects of invaders through hybridization [5].

The European water frog complex consists of a hybrid *Pelophylax esculentus* (genotype LR) and the parental species *Pelophylax lessonae* (genotype LL) and *Pelophylax ridibundus* (genotype RR). In Middle Europe populations consisting of *P. lessonae* and *P. esculentus* (LE-system) are the rule [6]. *P. ridibundus*, which was formerly absent [7] has invaded Middle Europe [8]. In England, France, Spain [9-11] and Switzerland [12] they were imported mainly for consumption of frog legs and for scientific purposes. Large numbers of *P. ridibundus* animals that escaped or were released into the wild in the middle of the twentieth century started to disturb the local population structure or even replace the native water frogs [13, 14].

A proximate driver of the ongoing establishment of *P. ridibundus* may be found in the unusual reproductive system of water frogs. The water frog hybrid *P. esculentus* with the genome LR reproduces by hybridogenesis [15]. During the gametogenesis the *lessonae* genome (L) is discarded and gametes containing only the *ridibundus* genome (R) are produced [16]. Such hybrids have an R genome that is clonally inherited (i.e. without recombination) and a Mendelian (L) inherited genome. *P. esculentus* frogs mate with *P. lessonae* to restore *P. esculentus* hybrids. In LE-systems, the *P. ridibundus* offspring resulting from matings between hybrids usually die because of the mutation load that the R genome carries [17]. The mortality of these *P. ridibundus* offspring is essential for the persistence of LE-systems [18, 19].

Vorburger & Reyer [5] showed how invasive *P. ridibundus* can disturb the system through hybridiza-

tion. Invasive *P. ridibundus* have normal sexually inherited genomes. If they mate with *P. esculentus*, then the clonally inherited R of the hybrid genome can recombine and therefore reduce mutation load. As a consequence, matings between *P. ridibundus* and *P. esculentus* and those between *P. esculentus* hybrids all produce viable *P. ridibundus* offspring. In the end, a pure *P. ridibundus* population results from the invasion.

There are several reasons why the outcome of the invasion by *P. ridibundus* may not be as predicted by Vorburger & Reyer [5]. First, Vorburger & Reyer [5] assumed that all taxa have the same ecological niche. Earlier studies showed, however, that the three taxa have different niches [20, 21]. If species performance depends on the habitat, then the native taxa may persists despite the severe consequences of hybridization with invasive *P. ridibundus*. Second, recent studies showed that there may be cryptic invasions within the *P. ridibundus* invasion [14, 22]. Invasions are called "cryptic" if some invasive taxa, including triploid *P. esculentus*, cannot easily be determined using external morphology. In particular, invasion by triploid water frogs, as they occur in some eastern and northern European countries, would greatly change the outcome of the invasion. If there are triploids, the *P. esculentus* may form all-hybrid populations without any parental species [23].

The goal of my master thesis was to test these two possibilities. I recorded the frequencies of the three taxa in ponds in northern Switzerland and tested whether taxon frequencies depend on pond characteristics. Based on previous studies on the ecology of water frogs [20, 24-26] I evaluated whether water frogs show differential preferences for pond water temperature and pond hydroperiod. Additionally, I used genetic methods to identify the origin and ploidy of the invasive water frogs.

2 Methods

2.1 Study design and water frog sampling

2.1.1 Study area

I selected 16 ponds in northern Switzerland (Table 1). The ponds are located in cantons Zürich and Thurgau in an area of about 195 square kilometers (N 47° 34.9 - 47° 41.2, E 8°36.1 - 8°49.5). Pond selection was based on the presence of water frogs, pond hydroperiod (temporary or permanent) and proximity to forests (M. Lippuner, personal communication).

Table 1 List of sampled ponds in northern Switzerland and their Swissgrid coordinates

Pond No.	Pond Name	Coordinates x	y
I	Heinrichsee	694610	273420
II	Tümpel bei Buck	704100	279860
III	Ober Weiher	694150	282600
IV	Länzigloch	687050	277160
V	Altlauf Inselen	691625	272425
VI	Pfaffensee	695000	273100
VII	Seewädeli	701600	279500
VIII	Grube Oberboden	689600	277650
IX	Cholgruebsee	693990	274230
X	Enteler-Weiher	692400	274700
XI	Bruggloch	687300	272800
XII	Kiesgrube südöstl. Feldhof	687500	270820
XIII	Barchetsee	698850	274750
XIV	Kiesgrube Rhinauer Feld	689300	277375
XV	Weiher Gütighausen	696550	271400
XVI	Retentionsbecken Breiten	693750	271400

2.1.2 Sampling

I caught 528 water frog tadpoles during daytime in July and August 2010 with a dip net. From each pond 28-36 tadpoles (one exception with 19 tadpoles) were captured haphazardly. Species identification was based on external morphology [27] and confirmed by DNA analysis [28]. To carry out the DNA analysis, I took a tissue sample from each tadpole. To get the tissue sample, a small piece of the tail was cut of with a scalpel and conserved in 95% EtOH. I released all tadpoles immediately after sampling. In addition, 40 and 12 adult and subadult frogs, respectively, were captured during two nights at the beginning of October 2010 in two ponds. During the following day I determined the sex of each frog phenotypically and cut from each individual a fingertip for tissue and a blood sampling. The tissue sample for DNA analysis was stored in 95% EtOH and the blood was smeared onto a microscope slide. Frogs were then released where they had been captured. Each tadpole and adult water frog was handled with unused, powder-free vinyl gloves.

2.1.3 Water temperature measurement and hydroperiod determination

I measured the water temperature of each pond with temperature loggers (Maxim Thermochron iButtons DS1921G-F5, accuracy ±1°C from -30°C to +70°C). The loggers recorded the temperature every 60 min, starting at the day of getting the samples until August 27. The loggers were placed in waterproof tubes 18 cm below the water surface. To get a constant depth, the tubes were fixed at a buoy. All loggers where placed at the sunny site of the pond in open water. I calculated a mean temperature across all ponds. Each pond could be classified this way as warmer or colder (Δ°C) than the average.

The hydroperiod I determined from a survey of amphibian habitats [29]. Each pond classified as temporary dries at least one time a year.

2.2 Determination of taxa

2.2.1 Genetic determination by microsatellites

Laboratory Protocol ----------

Extracted DNA from all samples was used to determine the genotype by a microsatellite-based method [28]. For the extraction of DNA, a BioSprint 96 DNA Blood Kit (Qiagen, Valencia, CA) with magnetic-particle technology for DNA purification and the BioSprint 96 workstation were used in combination. The DNA extraction was following Qiagen's protocol for tissue extraction.

All samples I subjected to a PCR with the primer mix PM1-A consisting of 5 different primer pairs. Some samples were analysed afterwards additionally with three other Primer mixes consisting of four (PM1-B, PM2-B) and five (PM2-A) primer pairs, respectively (Table 2). PCRs were carried out with a final volume of 5.2 μl and contained 1 μl DNA extraction, 2.5 μl Qiagen Multiplex PCR Master mix, 1.13 μl RNase-free water and 0.57 μl primer mix. PCR condition for PM1 was 15 min initial denaturation at 95°C, 30 cycles of 30 s at 94°C, 90 s at 57°C, 60 s at 72°C and a final extension of 30 min at 60°C. The PCR conditions for PM2 were similar but with 31 cycles instead of 30 and 60°C instead of 57°C [30].

1.4 μl of the PCR products was mixed in a new 96-well plate with 20 μl LIZ-Hi-Di Formamide (Applied Biosystems). After denaturizing at 95° C for two minutes the samples were placed in the DNA analyzer (Applied Biosystems PRISM 3570 DNA analyzer). The alleles from each primer pair were scored using GeneMapper software (Applied Biosystems, vers. 3.7).

Table 2 Primer mixes and included primers used for the genetic determination of water frog genotypes. For some genomes (*) the specificity of according primer was unknown so far.

Primer mix	Primer pairs	Specificity	Dosage
PM1-A	Rrid064A	R/L*	-
	RICA5	L/R*	-
	RICA1b6	L/R	✓
	RICa1b5	L/R	✓
	Ga1a19	L/R	✓
PM1-B	Res16	L/R	✓
	Res20	L	-
	RICA2a34	L/R	-
	Re2CAGA3	R/L*	-
PM2-A	Res22	R	-
	ReGa1a23	L/R	-
	Rrid169A	R	-
	Rrid013A	L/R	-
	Rrid059A	L/R	-
PM2-B	Re1CAGA10	L/R	-
	RICA1a27	L/R*	-
	RICA18	L/R*	-
	Rrid135A	R	-

Genotyping ----------

I analyzed all samples with 5 primer pairs and some with additional 13 primer pairs [31-35]. Each primer pair is amplifying loci in either the L genome, the R genome, or both. The alleles were scored basing on a preliminary data set from Swiss, Swedish, Danish, Baltic and German samples. Due to the lack of previous extensive Swiss populations scoring, a number of alleles were so far unknown. By drawing conclusions from other primer pairs with L or R specific alleles, most unknown alleles could be allocate to either the L or R genome. Three primers out of the PM1-A primer mix amplify L and R specific alleles and show dosage effect. Dosage effect can be used to differentiate an LR genotype hybrid from an LLR or LRR genotype hybrid. The differentiation took place by comparing the relative intensities (peak heights) of the R and L alleles [28]. LR, LLR, LRR peak height ratios clustered into separate groups [36].

2.2.2 Taxon determination by erythrocyte size measurement

Erythrocytes of diploid water frog have a smaller size than the erythrocytes of triploid water frogs [37]. I measured erythrocyte size of adult and subadult water frogs from two ponds. I measured the width and length of 6 single erythrocytes per individual sample. Erythrocyte area was calculated using the formula for an ellipse.

2.2.3 Haplotype determination by sequencing mitochondrial DNA

Laboratory Protocol ----------

To score the mitochondrial DNA sequence, DNA was first extracted from tissue samples, amplified by PCRs and analyzed afterwards by electrophoresis and detection of fluorescent dye-labeled nucleotide fragments. For the DNA extraction, the BioSprint 96 DNA Blood Kit (Qiagen, Valencia, CA) in combination with the BioSprint 96 workstation was used. The extraction followed Qiagen's protocol for tissue extraction. Double-stranded amplification and cycle sequencing of mtDNA were achieved for the ND2 (1038 bp) and ND3 (340 bp) genes. PCRs were carried out with a final volume of 10 µl per sample, containing 1 µl DNA extraction (50-100 ng DNA), 5 µl SIGMA-Ready Mix Taq PCR –P4600, 2 µl RNase-free water and 1 µl of each primer (10 µMol). Two separate PCRs were carried out for ND2, containing primer pair L1/H1 or L2/H2 and ND3 containing primer pair L/H (Table 3) [38]. PCR condition for ND2 was 1 min initial denaturation at 94°C, 35 cycles of 30 s at 94°C, 60 s at 62°C, 60 s at 72°C and a final extension of 7 min at 72°C. The PCR conditions for ND3 were similar but with 45 s at 50°C instead of 60 s at 62°C. For purification of amplified DNA 0.5 µl ExoSAP-IT and 0.5 µl H_2O was added to each sample and incubated at 37°C for 45 min and 80°C for 15 min. To perform the sequencing reaction, 5 µl PCR product was mixed with 0.5 µl Big Dye Aliquot, 1.75 µl 5x sequence buffer, 2.75 µl ddH_2O and 1 µl L or H primer. PCR condition for ND2 was 45 s initial denaturation at 95°C, 30 cycles of 30 s at 95°C, 30 s at 62°C, 4 min at 62°C and a final extension of 1 min at 60°C, storing afterwards at 10°C. The PCR condition for ND3 was 30 s at 55°C instead of 62°C. To clean up the sequencing reactions, 75 µl of 0.6mM Sodium acetate $(ETOH)_4$ was added to each sample. Each sample was mixed by vortexing and stored for 15 min at room temperature before spinning for 18 min at 2000 rpm. The supernatant was removed and the samples were spinned again for 1 min at 1000 rpm upside-down to remove drops. Afterwards the remaining substance was resuspended in 13 µl of water. Electrophoresis and detection of fluorescent dye-labeled nucleotide fragments was carried out with an Applied Biosystems PRISM 3570 DNA analyzer. The DNA sequences were scored with MEGA 5 software [39].

Table 3 Sequence of mtDNA primers from genes ND2 and ND3.

Gene	Primer	Sequence (5'→3')
ND2	L1	AAGCTTTTGGGCCCATACCCC
	H1	GGGGCGATTTTTTGTCAGGTTG
	L2	GGACTCGCCCCYCTACACTTCTG
	H2	CTCCGCTTAAGGCTTTGAAGGC
ND3	L	AGTACACGTGACTTCCAATC
	H	TTGAGCCGAAATCAACTGTC

Sample selection----------

The mitochondrial DNA was analyzed for 95 samples. all from the 4 ponds where I found triploids. From pond VIII and XIV I analyzed 21 adult or subadult frogs each and 8 and 14 tadpoles, respectively. From pond V I analyzed 18 tadpoles and from pond XIII 13 tadpoles. The selection of individual samples within a pond was based on the genotype determination by microsatellites. From each pond with LLR triploids I analyzed at least 11 triploids. For diploid LR or RR I analysed 4-9 samples, except in cases where these genotypes were rare; then all samples from the pond were included. In the pond with LRR triploids the genotypes I analyzed were nearly equally distributed. None of the individuals sampled from all 4 ponds belonged to the genotype LL.

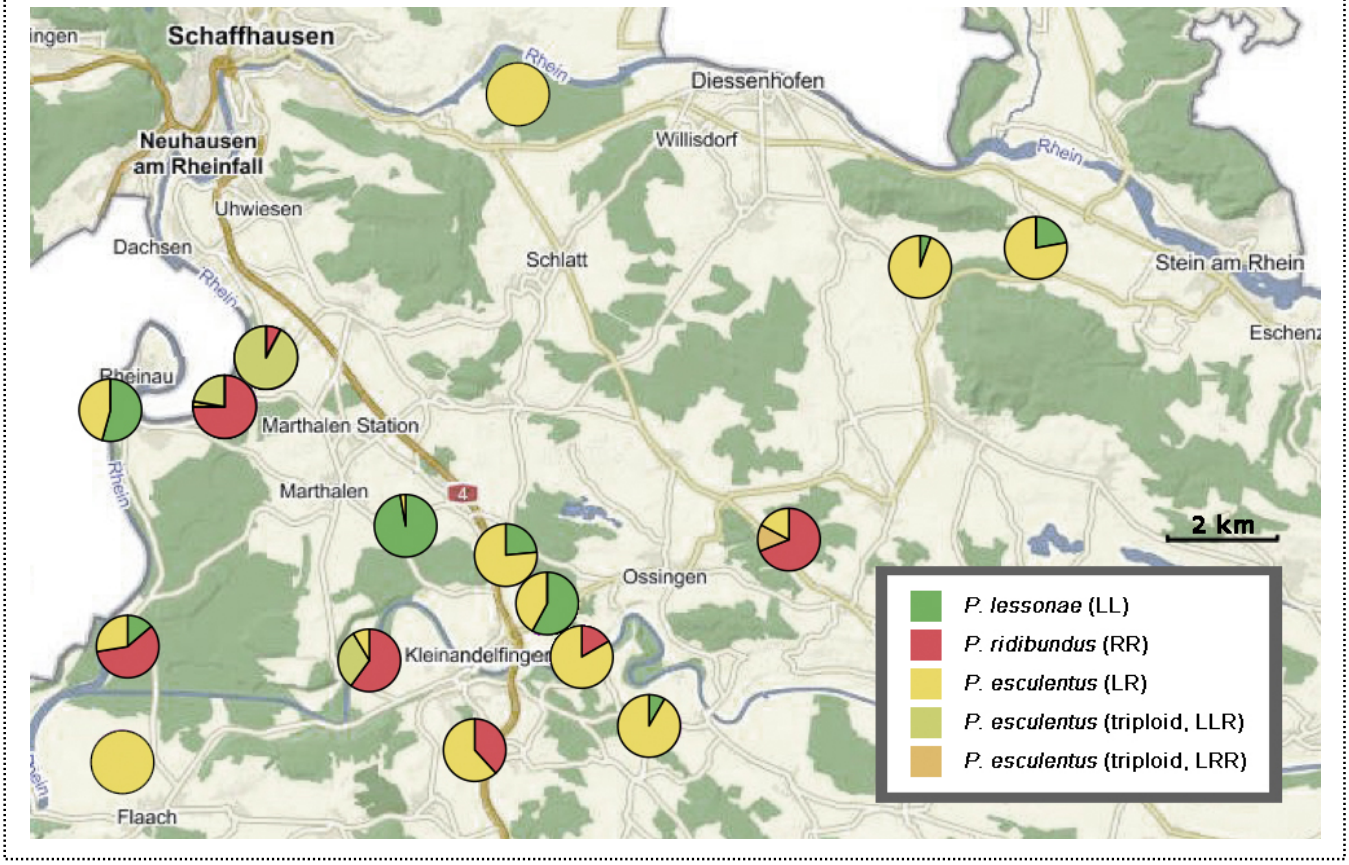

Figure 1 Water frog taxa composition of 528 tadpoles in 16 northern Swiss populations, including *P. lessonae*, *P. ridibundus* and *P. esculentus* with diploid and triploid genomes. The sample sizes were at least 28 tadpoles with one exception of 19 tadpoles.

Sample allocation to a haplotype----------
I allocated the mitochondrial DNA (mtDNA) sequence of my samples to reference sequences from the EMBL align database. The reference sequences were *ridibundus* mtDNA (AM900660 [38]), *lessonae* mtDNA (AM887976 [38]) and *Pelophylax bergeri* mtDNA (GU8121134 [40]). I used the gene ND2 and ND3 DNA to identify the haplotype according to Plötner [38].

2.3 Statistical analysis

I used logistic regression to test whether genotype proportions depended on pond temperature (deviation from the average pond temperature) and hydroperiod (temporary vs. permanent). The analysis was done with the statistic program R [41].

3 Results

3.1 Frequency of water frog taxa

3.1.1 Composition of genotypes in populations

I analyzed the genotypes of 528 tadpoles and 52 adult or subadult water frogs (Appendix 1). Genotype and taxon-composition varied strongly among ponds (Figure 1). 7 of 16 populations had only *P. lessonae* and *P. esculentus*, 6 included *P. ridibundus* and *P. esculentus*, and in one pond all three taxa were present. In two ponds, I found only diploid *P. esculentus*. In ponds with *P. lessonae* and *P. esculentus*, the relative frequencies of hybrids varied from 2.8% (pond X, n=36) to 94.4% (pond VII, n=36). In the *P. ridibundus* and *P. esculentus* composition, the frequency of hybrids varied from 31% (pond XIII, n=29) to 83.3% (pond VI, n=36). Pond XI was the only pond with all three taxa present

with the frequencies *P. ridibundus* 55.6%, *P. esculentus* 27.6% and *P. lessonae* 13.8% (n=29).

3.1.2 Temperature and hydroperiod dependence

Genotype composition depended on both temperature and hydroperiod. The parental taxa *P. lessonae* and *P. ridibundus* had higher frequencies in colder ponds ($p = 1.8 \cdot 10^{-14}$ and $p = 2.4 \cdot 10^{-2}$) whereas *P. esculentus* was more frequent in warmer ponds ($p = 2.0 \cdot 10^{-16}$) (Figure 2).

P. lessonae and *P. esculentus* were more frequent in temporary ponds than in permanent ponds ($p = 2.0 \cdot 10^{-4}$ and $p = 7.7 \cdot 10^{-5}$, respectively). *P. ridibundus* frogs have a higher prevalence in permanent ponds than in temporary ones ($p = 2.1 \cdot 10^{-9}$) (Figure 3).

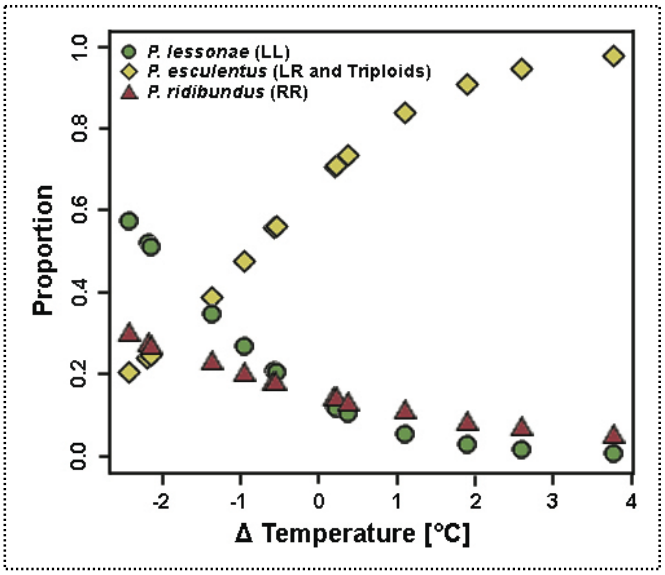

Figure 2 Logistic regression for the prevalence of *P. lessonae, P. esculentus and P. ridibundus* plotted against temperature. Frogs of the hybrid *P. esculentus* taxon have a higher prevalence in warmer ponds in contrast to the parental specie.

3.2 Triploid water frogs

3.2.1 Frequencies in populations

I found triploid water frogs with genotypes LLR and LRR. I found LLR triploids in 3 ponds with frequencies of 31.4% (pond V, $n_{total}=35$), 67.3% (pond VIII, $n_{total}=55$) and 32.3% (pond XIV, $n_{total}=62$), respectively. I found 4 triploids with an LRR genome in pond XIII , representing a frequency of 13.8% ($n_{total}=29$).

3.2.2 Determination of triploidy

Identification of triploid individuals was accomplished based on microsatellite data. If an individual showed at a microsatellite locus three different alleles it was classified as triploid. In 66 of 72 LLR inviduals three different alleles were found in three microsatellite loci (RlCA5b, Ga1a19 and Res16), in 5 individuals they existed in two microsatellite loci and in 1 individual they occurred in a single locus. The pair of L alleles was in all LLR triploids identical. The microsatellite loci showing two alleles were checked additionally for dosage. The triploidy was confirmed with dosage on RlCA6b in 67 of 72 LLR individuals, the remaining 5 individuals could not be checked due to failed allele identification. None of the 4 LRR triploids showed three alleles in any microsatellite loci. Dosage was checked for 4 microsatellite loci (RlCA6b, RlCA5b, Ga1a19, Res16) and all individuals clustered to a LRR group in all loci.

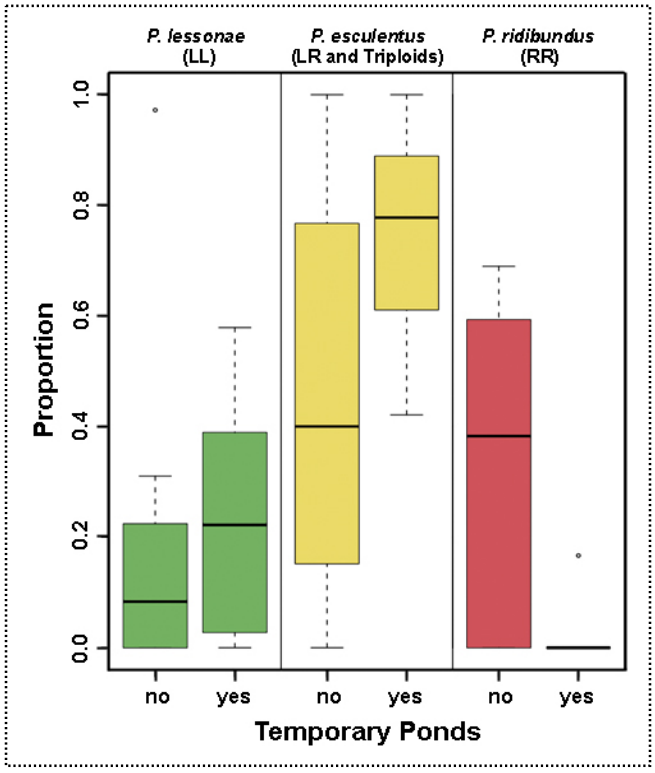

Figure 3 Frequencies of the taxa *P. lessonae, P. esculentus and P. ridibundus* plotted for temporary (yes) and non temporary (no) ponds. *P. ridibundus* is almost only present in permanent ponds whereas *P. lessonae* and *P. esculentus* are more frequent in temporary ponds.

In adult water frogs the triploidy was additionally confirmed by erythrocyte size measurement. Erythrocyte size clearly distinguished diploid and triploid frogs (Figure 4). The size of erythrocytes from diploid and triploid subadult individuals overlapped.

3.2.3 Similar triploid *P. esculentus* among European water frog populations

I caught 40 and 12 adult and subadult frogs, respectively, in two ponds containing LLR triploid tadpoles (pond VIII and pond VIX). All 40 adult water frogs could be allocated to a sex. I captured 20 females and 20 males. All LLR triploids (n=17) were exclusively males. Pond VIII (n_{total}=27) contained 48.2% LLR, 11.1% *P. ridibundus* and 40.7% *P. esculentus*. Pond XIV (n_{total}=26) contained 46.2% LLR, 27.9% *P. ridibundus* and 29.9% *P. esculentus*. I captured no *P. lessonae* or LRR individuals. The taxon suggests that the LE-population was invaded by both *P. ridibundus* and triploids that produce LL gametes (Figure 5). In the literature, four populations of males producing LL sperms were described. In all populations triploid hybrids consist exclusively of LLR males. One population is located in northwestern Hungary [42], two populations in western Slovakia [43] and one in the Fontainebleau Forest near Paris [17].

3.3 Haplotype of mtDNA

All 95 samples tested for their mitochondrial DNA could be allocated to either *ridibundus* or *bergeri* mtDNA (Table 4). 12 individuals from 2 ponds (VIII and XIV) had *ridibundus* mtDNA. The *ridibundus* mtDNA sequence of all individuals was identical, i.e. all had the same haplotype, but it could not be assigned to a single known haplotype. The 3 potential haplotypes (R17, R18, R19) can be found in populations in Russia, Serbia, Slovakia, Poland, Latvia, Romania, Bulgaria, France and Greece. 83 individuals had *bergeri* mtDNA; they occurred in all 4 ponds (V, VIII, XIII and XIV). The *bergeri* mtDNA sequence of all individuals was identical. Compared to the *bergeri* reference sequence, the *bergeri* mtDNA sequence of my samples had different bases at 3 positions (0.2%). Compared to the *lessonae* reference sequence different bases were at 54 positions (3.9%). For *bergeri* mtDNA no haplotypes are defined. The *bergeri* mtDNA is similar to the *lesso-*

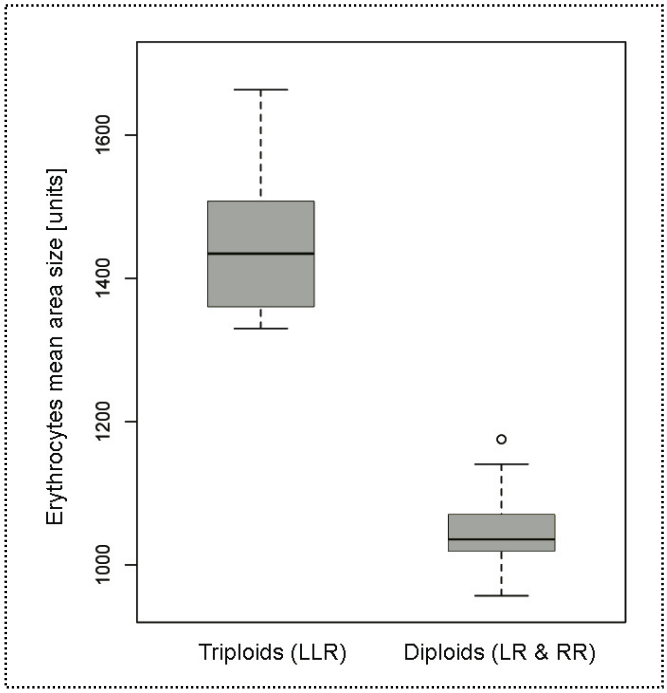

Figure 4 Triploid water frogs have larger erythrocyte mean area sizes than diploid individuals.

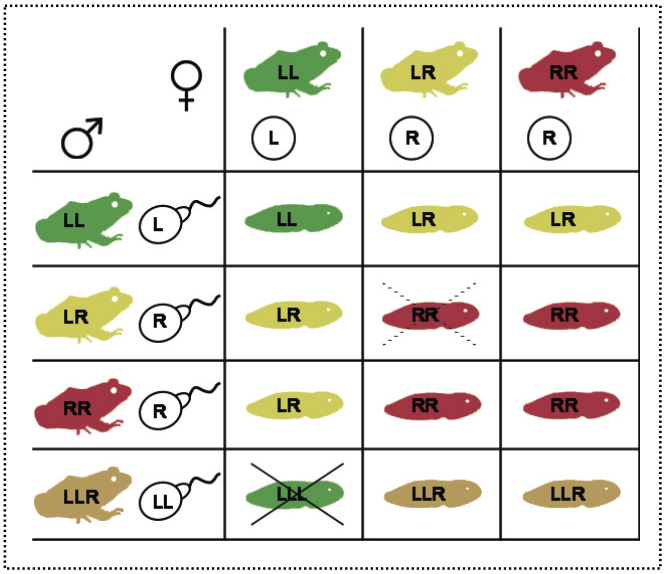

Figure 5 Crossing table of an LE-system invaded by *P. ridibundus* and LLR triploids. LLR triploids are exclusively males. Tadpoles resulting from mating between LLR males and LL females do not survive. Tadpoles resulting from LR matings are normally inviable, but they produce more viable offspring as more LR stem from primary hybridization. Parts of the illustration are taken from Christiansen 2009 [23].

nae mtDNA; a *lessonae* mtDNA haplotype very similar to the mtDNA sequences in my samples can be found in Italy.

Table 4 Amount of *bergeri* and *ridibundus* mtDNA found according to pond and genotype.

Pond	Genotype	Tested	Mitochondrial DNA	
			# *bergeri*	# *ridbundus*
V	LLR	11	11	-
	LR	3	3	-
	RR	4	4	-
VIII	LLR	17	17	-
	LR	9	9	-
	RR	3	-	3
XIII	LLR	4	4	-
	LR	5	5	-
	RR	4	4	-
XIV	LLR	20	19	1
	LR	6	6	-
	RR	13	2	7

4 Discussion

The results of my study supported the expectation that the native P. *lessonae* and *P. esculentus* and the invasive *P. ridibundus* have different niches according pond water temperature and hydroperiod. Such differences in habitat preference may allow the persistence of the native taxa in the presence of invasive *P. ridibundus*. In addition, I could demonstrate that triploid water frogs are present in Swiss ponds. Triploids invaded cryptically within the *P. ridibundus* invasion probably from a population located in Hungary, Slovakia or France and they may pose a new threat to the native LE-system.

I showed that *P. lessonae*, *P. esculentus* and *P. ridibundus* had differential preferences for pond water temperature and pond hydroperiod. The analysis of taxon frequencies and temperature showed that *P. ridibundus* and *P. lessonae* prefer colder ponds whereas *P. esculentus* prefers warmer ponds (Figure 2). The analysis of taxon frequencies and hydroperiod showed that *P. ridibundus* prefers permanent ponds whereas *P. esculentus* and *P. lessonae* prefer temporary ponds (Figure 3). The preference of *P. ridibundus* for colder water temperatures and permanent ponds is consistent with previous studies. Previous studies demonstrated, that temperature preference for *P. ridibundus* lies below that of *P. lessonae* [26] and that *P. ridibundus* tadpoles are less vulnerable to fish predators than other species [44]. The found preference of *P. lessonae* and *P. esculentus* for temporary ponds is also consistent with other studies. These showed that both species can perform well in temporary ponds [24, 45]. I found a preference of *P. lessonae* for colder ponds and a preference of *P. esculentus* for warmer ponds. This result contrasts with a previous study that found the opposite: performance of *P. lessonae* tadpoles is better at high temperature and of *P. esculentus* at low temperature [25]. However, other studies and my results suggest that the ecological niches of *P. ridibundus*, *P. esculentus* and *P. lessonae* are different. I could demonstrate that the taxa occur at different positions along the pond hydroperiod and temperature gradients (Figure 3/4). These differences may allow persistence of the native taxa. The native taxa may persist in temporary and warmer ponds. Hence, it seems unlikely that the invasive *P. ridibundus* completely replace the native taxa as the model of Vorburger & Reyer [5] predicts.

The model of Vorburger & Reyer [5] predicts, that invasive *P. ridibundus* primarily reduce the frequency of *P. lessonae* and since their parental species will become less common, *P. esculentus* declines as well, ending in a pure *P. ridibundus* population. Based on this model and the bad performance of *P. lessonae* in permanent ponds [24] I assume that invaded *P. ridibundus* reduce primarily the frequency of *P. lessonae*. Some tendency that invading *P. ridibundus* decrease primarily the frequency of *P. lessonae* was shown by my data. In 6 of 7 ponds where *P. ridibundus* was present I could find *P. esculentus* as well, but no *P. lessonae* (Figure 1). With my sample size of at least 29 tadpoles, the frequency of *P. lessonae* is expected to be lower than 10% in those ponds [46]. Due to the decreasing frequency of the parental species *P. lessonae*, it is expected that *P. esculentus* disappear as well. Three alternative scenarios could explain, however, that I observed *P. esculentus* beside *P. ridibundus*. First, *P. esculentus* is still produced by *P. lessonae* persisting in low frequencies in invaded ponds. Second, *P. esculentus* survived from the primary population but will be replaced by *P. ridibundus* in future. Third, *P. esculentus* immigrates or is produced by immigrating *P. lessonae*.

Besides different habitat preferences, the outcome of an invasion is likely to differ with the type of the invader. The invasion of *P. ridibundus* into an LE-system may lead to a replacement of both native taxa, whereas invasive triploid *P. esculentus* allow *P. esculentus* to persist and to form all-hybrid populations without any parental species [23]. I did not find an all-hybrid population but I found triploids in 4 out of 16 ponds. I wanted to determine the gametes produced by the triploid frogs and the origin of the triploid frogs. For the three ponds containing LLR triploids I had enough data for using a combination of microsatellite data, genotype frequencies, mtDNA sequence analysis and phenotype determination. The microsatellite analysis showed that all triploids carried the same allele combination on the two L genomes. This suggests that they produce diploid LL gametes where both LL genomes are transmitted without recombinations ("bihemiclones"; N. Pruvost, personal communication). LL gamete-producing LLR triploids would best explain the observed genotype frequencies (Figure 5). The mtDNA sequence found in triploids is consistent with the sequence from native water frogs (Table 4) and all triploids I found were males. Males as exclusive sex of LLR triploids and the likely pairwise inheritance of LL alleles are consistent with sex determination in *P. esculentus*. Sex determination in *P. esculentus* is a genetic XX-XY system [47] with a dominant male determining Y factor. Because the primary hybridization is usually the result of preferential mating between a *P. lessonae* male and a *P. ridibundus* female the Y factor is supposed to be present only in the L genome [17, 48]. Due to pairwise inheritance of the two L genomes, the Y factor is always transmitted and all offspring will be males. Crossing experiments would be needed to give certainty about the genome composition in the sperm of the LLR triploids.

There may be two reasons for the presence of triploids: the triploids may have invaded or they may be native. The following reasons lead me to argue that the LLR triploids are not native. First, the first individual had to be a male. Males producing diploid sperm are rare and no water frogs system of diploid males producing LL sperm have been observed until now [49]. Second, I found a second type of triploids (LRR) and two kinds of triploids in a water frog system were only observed in all-hybrid populations [49]. The following three reasons lead me to argue that the LLR triploids are invasive. First, Hungary, Slovakia and the Fontainebleau forest as potential origin populations are quite far away from my study site and no other similar population was found in between. Second, all found triploids have native mtDNA. This suggests that only males were introduced. Third, there was no evidence for triploid water frogs in Switzerland so far and I found in the same region beside LLR triploids also LRR triploids. The presence of two kinds of triploids suggests that triploids invaded Swiss ponds twice and cryptically.

With my data I could not determine the exact origin of the LLR triploids but my data suggest some tendencies. The data lead me to argue that the LLR triploids produce LL sperms. Triploids producing LL sperms are rare among the hybridogenetic water frog complex. According to literature, such LLR triploids producing LL sperms can be found in four different populations. One population is located in northwestern Hungary [42], two populations in western Slovakia [43] and one in the Fontainebleau Forest near Paris [17]. In all populations triploid hybrids consist exclusively of LLR males. The reproduction of the LLR males takes place by the fertilization of diploid LL sperm with haploid R eggs. In the populations from France and Slovakia the R eggs are produced by *P. esculentus* females [17, 43], whereas in Hungary they originate from *P. ridibundus* [42]. Although I cannot determine the exact origin of the triploids, Hungary is most likely while Slovakia is less likely. Hungary as potential origin of the found triploids is most likely, if the triploids invaded together with *P. ridibundus*. Only in the Hungarian population the LLR triploids live together with *P. ridibundus*. Additionally is noted in literature that *P. ridibundus* frogs from Hungary were introduced in Britain already in 1935 [11] and most introduced frogs to Switzerland originated from East or South-Eastern Europe [50]. Slovakia as potential origin of the found triploids is not supported by microsatellite data. Microsatellite data from my triploids and triploids from Slovakian population showed very different allele combinations (N. Pruvost, unpublished data). Comparison of allele combinations found in Swiss triploids with allele combinations found in triploids of populations in Hungary would give additional information about the origin. In conclusion, the invasive triploids most likely

originated from Hungary or, with lower probability, from Slovakia or the Fontainebleau Forest in France.

My results are evidence for a second and cryptic invasion [22] beside *P. ridibundus*: this is the first detection of triploid water frogs in Switzerland. The LLR and LRR triploids I found need other taxa as genome donors to persist. The LLR triploids I found produce LL gametes and need therefore an R-genome donor to persist. The R-genome donor may be *P. esculentus* or *P. ridibundus*. If only *P. esculentus* as donor is present, then the LLR triploids depend on *P. esculentus*. *P. esculentus* depends on their parental specie *P. lessonae* and therefore the triploids cannot replace both of them. I suggest that the LLR triploids are only in combination with *P. ridibundus* able to replace the native water frogs completely. In the three ponds where I found the LLR triploids, they live together with *P. esculentus* and *P. ridibundus*. I assume that *P. ridibundus* will prevail there in future or build a population with LLR triploids. It is hard to predict how the native populations structure will develop in presence of triploids. The found triploids exclude the R genome during gametogenesis and make exclusively LL gametes, whereas *P. ridibundus* and *P. esculentus* make R gametes [16]. Resulting LLR triploids from mating between the two taxa have therefore a conflict which genome is excluded during gametogenesis. If R gametes are produced by the triploids, the triploids are not able to persist. Hence I suggest, that LL gametes are produced, because I found high frequencies of triploids in my populations and in the populations in Hungary, Slovakia and France they live together with *P. esculentus* or *P. ridibundus* [17, 43].

The gametes produced by the LRR triploids are unknown. According to literature [49], haploid and diploid L and R gametes are possible. In Swiss populations they are only able to persist, if they produce diploid gametes, because all potential mating partners produce haploid gametes. Depending if the LRR triploids produce RR or LR gametes, the triploids depends on other genome donors. If they produce RR gametes, they depend on *P. lessonae* as L-genome donor and if they produce LR gametes they depend on a R-genome donor as *P. esculentus* or *P. ridibundus*. In any case, they are, similar to LLR triploids, only able to replace the native water frogs completely in combination with *P. ridibundus*. Triploids, however, destroy the LE-system but do not replace the native taxa. Therefore *P. ridibundus* is still the proximate driver of replacement because it can replace the native water frogs completely by themselves or in combination with triploids. Nevertheless also triploids threaten the Swiss water frog populations. Even if they do not replace the native water frogs completely, they decrease their frequencies. Additionally the triploids may threaten the native taxa in different habitats than *P. ridibundus* if they have different niches than *P. ridibundus*. I could demonstrate that *P. ridibundus* is mainly limited on cold and permanent ponds, but for the triploids the habitat limitations may be different. Different habitat limitations of triploids may also affect LE-systems in warm and temporary ponds.

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References

[1] Lodge DM. Biological Invasions: Lessons for Ecology. Trends in Ecology and Evolution. 1993;8(4):133-137.

[2] Manchester SJ, Bullock JM. The impacts of non-native species on UK biodiversity and the effectiveness of control. Journal of Applied Ecology. 2000;37(5):845-864.

[3] Sakai AK, Allendorf FW, Holt JS, Lodge DM, Molofsky J, With KA, Baughman S, Cabin RJ, Cohen JE, Ellstrand NC, McCauley DE, O'Neil P, Parker IM, Thompson JN, Weller SG. The population biology of invasive species. Annual Review of Ecology and Systematics. 2001;32:305-332.

[4] Rhymer JM, Simberloff D. Extinction by hybridization and introgression. Annual Review of Ecology and Systematics. 1996;27:83-109.

[5] Vorburger C, Reyer H-U. A genetic mechanism of species replacement in European waterfrogs? Conservation Genetics. 2003;4(2):141-155.

[6] Berger L. On the origin of genetic systems in European water frog hybrids. Zoologica Poloniae. 1990;35:5-27.

[7] Plötner J. Die westpaläarktischen Wasserfrösche. Bielefeld (Laurenti-Verlag). 2005.

[8] Pagano A, Dubois A, Lesbarrères D, Lodé T. Frog alien species: a way for genetic invasion? Comptes Rendus Biologies. 2003;326:85-92.

[9] Arano B, Llorente G, Garcia-Paris M, Herrero P. Species Translocation Menaces Iberian Waterfrogs. Conservation Biology. 1995;9(1):196-198.

[10] Schmeller DS, Pagano A, Plenet A, Veith M. Introducing water frogs - Is there a risk for indigenous species in France? Comptes Rendus Biologies. 2007;330(9):684-690.

[11] Zeisset I, Beebee TJC. Population genetics of a successful invader: the marsh frog *Rana ridibunda* in Britain. Molecular Ecology. 2003;12(3):639-646.

[12] Grossenbacher K. Verbreitungsatlas der Amphibien in der Schweiz. Documenta Faunistica Helvetica. 1988;7:1-207.

[13] Hofer-Polit D. Aussterben von *Rana lessonae* und *Rana esculenta* durch die Ausbreitung von *Rana ridibunda*. Elaphe. 1998;6:79-80.

[14] Holsbeek G, Jooris R. Potential impact of genome exclusion by alien species in the hybridogenetic water frogs (*Pelophylax esculentus complex*). Biological Invasions. 2010;12(1):1-13.

[15] Schultz RJ. Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. American Naturalist. 1969;103:605-619.

[16] Tunner HG, Heppich S. Premeiotic Genome exclusion during oogenesis in the common edible frog, *Rana-esculenta*. Naturwissenschaften. 1981;68(4):207-208.

[17] Graf JD, Polls Pelaz M. Evolutionary genetics of the *Rana esculenta* complex. Evolution and Ecology of Unisexual Vertebrates (editors Dawley, RM & Bogart, JP); New York State Museum Bulletin 466, Albany, USA. 1989:289-302.

[18] Hellriegel B, Reyer H-U. Factors influencing the composition of mixed populations of a hemiclonal hybrid and its sexual host. Journal of Evolutionary Biology. 2000;13(6):906-918.

[19] Som C, Anholt BR, Reyer H-U. The effect of assortative mating on the coexistence of a hybridogenetic waterfrog and its sexual host. American Naturalist, The. 2000;156(1):34-46.

[20] Pagano A, Crochet PA, Graf J-D, Joly P, Lode T. Distribution and habitat use of water frog hybrid complexes in France. Global Ecology and Biogeography Letters. 2001;10(4):433-441.

[21] Pagano A, Joly P, Plenet S, Lehman A, Grolet O. Breeding habitat partitioning in the *Rana esculenta* complex: The intermediate niche hypothesis supported. Ecoscience. 2001;8(3):294-300.

[22] Holsbeek G, Mergeay J, Hotz H, Plötner J, Volckaert FAM, De Meester L. A cryptic invasion within an invasion and widespread introgression in the European water frog complex: consequences of uncontrolled commercial trade and weak international legislation. Molecular Ecology. 2008;17(23):5023-5035.

[23] Christiansen DG. Gamete types, sex determination and stable equilibria of all-hybrid populations of diploid and triploid edible frogs (*Pelophylax esculentus*). BMC Evolutionary Biology. 2009;9(1):135.

[24] Anholt BR, Negovetic S, Rauter C, Som C. Predator complement determines the relative success of tadpoles of the Rana esculenta complex. Evolutionary Ecology Research. 2005;7(5):733-741.

[25] Negovetic S, Anholt BR, Semlitsch RD, Reyer H-U. Specific responses of sexual and hybridogenetic European waterfrog tadpoles to temperature. Ecology. 2001;82(3):766-774.

[26] Sinsch U. Thermal influence on the habitat preference and the diurnal activity in three European *Rana* species. Oecologia. 1984;64:125-131.

[27] Bühler C, Cigler H, Lippuner M. Amphibienlarven - Bestimmung. Fauna Helvetica 17, 2007.

[28] Christiansen DG. A microsatellite-based method for genotyping diploid and triploid water frogs of the *Rana esculenta* hybrid complex. Molecular Ecology Notes. 2005;5(1):190-193.

[29] Lippuner M, Rohrbach T. Ökologie des Springfrosches (*Rana dalmatina*) im westlichen

Bodenseeraum. Zeitschrift für Feldherpetologie. 2009;16:11-14.
[30] Christiansen DG, Reyer H-U. From clonal to sexual hybrids: Genetic recombination via triploids in all-hybrid populations of water frogs. Evolution. 2009;63(7):1754-1768.
[31] Arioli M. Reproductive patterns and population genetics in pure hybridogenetic water frog populations of *Rana esculenta*. Ecology department: University of Zurich; 2007.
[32] Garner TWJ, Gautschi B, Röthlisberger S, Reyer H-U. A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. Molecular Ecology. 2000;9(12):2173-2175.
[33] Hotz H, Uzzell T, Guex G-D, Alpers D, Semlitsch RD, Beerli P. Microsatellites: a tool for evolutionary genetic studies of western Palearctic water frogs. Mitteilung aus dem Museum für Naturkunde Berlin, Zoologische Reihe. 2001;77:43-50.
[34] Jakob C. Structure and Dynamics of Pure Hybridogenetic Water Frog Populations. PhD Thesis, University of Zurich, Switzerland. 2007.
[35] Zeisset I, Rowe G, Beebee TJC. Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R-lessonae*. Molecular Ecology. 2000;9(8):1173-1174.
[36] Christiansen DG, Fog K, Pedersen BV, Boomsma JJ. Reproduction and hybrid load in all-hybrid populations of *Rana esculenta* water frogs in Denmark. Evolution. 2005;59(6):1348-1361.
[37] Günther R. Die Erythrozytengrösse als Kriterium zur Unterscheidung diploider und triploider Teichfrösche, *Rana "esculenta"* L. (Anura). Biologisches Zentralblatt. 1977;96:457-466.
[38] Plötner J, Uzzell T, Beerli P, Spolsky C, Ohst T, Litvinchuk SN, Guex G-D, Reyer H-U, Hotz H. Widespread unidirectional transfer of mitochondrial DNA: a case in western Palaearctic water frogs. Journal of Evolutionary Biology. 2008;21(3):668-681.
[39] Kumar S, Tamura K, Nei M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics. 2004;5(2):150-163.
[40] Akin C, Bilgin CC, Beerli P, Westaway R, Ohst T, Litvinchuk SN, Uzzell T, Bilgin M, Hotz H, Guex G-D, Plötner J. Phylogeographic patterns of genetic diversity in eastern Mediterranean water frogs were determined by geological processes and climate change in the Late Cenozoic. Journal of Biogeography. 2010;37(11):2111-2124.
[41] R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2008. http://www.R-project.org.
[42] Tunner HG, Heppich-Tunner S. A new population of water frogs discovered in Hungary. Proc Sixth Ord Gen Meet Societas Europaea Herpetologica. 1992;Budapest 1991:453-460.
[43] Mikulicek P, Kotlik P. Two water frog populations from western Slovakia consisting of diploid females and diploid and triploid males of the hybridogenetic hybrid *Rana esculenta* (Anura Ranidae). Mitteilung aus dem Museum für Naturkunde Berlin, Zoologische Reihe. 2001;77:59-64.
[44] Teplitsky C, Plenet S, Joly P. Tadpoles' responses to risk of fish introduction. Oecologia. 2003;134(2):270-277.
[45] Semlitsch RD, Reyer H-U. Performance of tadpoles from the hybridogenetic *Rana esculenta* complex: Interactions with pond drying and interspecific competition. Evolution. 1992;46(3):665-676.
[46] DiGiacomo RF, Koepsell TD. Sampling for detection of infection or disease in animal populations. Journal of the American Veterinary Medical Association. 1986;189(1):22-23.
[47] Schempp W, Schmid M. Chromosome-banding in Amphibia. VI. BrdU-replication patterns in Anura and demonstration of XX-XY sex chromosome in *Rana esculenta*. Chromosoma. 1981;83(5):697-710.
[48] Blankenhorn HJ. Reproduction and mating behavior. *Rana lessona-Rana esculenta* mixed populations. The Reproductive Biology of Amphibians (editors Taylor DH & Guttmann SI); Plenum Press New York. 1977:389-410.
[49] Reyer H-U. From hybridization trough polyploidy to specification? The evolutionary potential of hybridogenetic water frogs (*Rana esculenta*). Institute of Evolutionary Biology and Environmental Studies: University of Zurich; 2010.
[50] Pagano A, Joly P, Hotz H. Taxon composition and genetic variation of water frogs in the mid-Rhone floodplain. Comptes rendus de l'Académie des sciences Série 3, Sciences de la vie. 1997;320(9):759-766.

Part II: *Batrachochytrium dendrobatidis* infection in tadpoles and adults of the hybridogenetic *Pelophylax esculentus* complex

Mass mortalities of amphibians in the 1990ties were related to cutaneous chytridiomycosis, a disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd). Bd has been recognized as a proximate driver of many amphibian declines on all continents except Antarctica. Bd occurrence is highly dependent on aquatic environments since the fungus cannot survive desiccation. Therefore I tested whether the infection rate of amphibians is lower in temporary ponds than in permanent ponds. I tested 528 water frog tadpoles for Bd infection in 16 ponds, half of them temporary and half permanent. I could not detect Bd infection in any tadpole in any of the ponds although a few adults and subadults in some ponds were infected. This indicates that under natural conditions the Bd infection of water frogs takes place primary after metamorphosis.

1 Introduction

The major threats to biodiversity are habitat destruction and degradation [1, 2] but other threats have been recognized in recent years: Over-exploitation, invasive species, pollution and diseases are additional threats to species [3, 4]. One of the most imperiled classes of vertebrates are the amphibians with 32% of over 6200 species threatened [4].

Since the mid-1970s, worldwide amphibian population declines were observed and extinctions are documented [4]. During the 1990ties dramatic amphibian population declines and mass mortalities were reported from both Australia and Central America [5-7]. Diagnostic investigations on the dead amphibians at these sites showed strong evidence that cutaneous chitridiomycosis, a disease caused by a chytrid fungus, was the reason for these mortalities [8]. The chytrid fungus *Batrachochytrium dendrobatidis* (Bd) [9] was recognized as the etiological agent of the disease and therefore as the proximate driver of many declines worldwide [8, 10]. On a global scale the pathogen has been identified on all continents except Antarctica with different severity of impact (http://www.bd-maps.net/).

Knowledge of distribution patterns and requirements of Bd is important for a better understanding of the ecology of the disease and for mitigation strategies against Bd. With specific strategies, the distribution of Bd can be curtailed and susceptible species be protected. On a large geographical scale, different studies have analyzed distribution patterns of Bd. There is a tendency that Bd prevalence is associated with cooler temperatures [11]. On a smaller geographical scale, other habitat factors play an important role. The fungus is highly dependent on an aquatic environment [11, 12] because it cannot survive desiccation [13]. The aquatic requirement of Bd seems to be a main candidate for habitat limitation [13]. That non-aquatic habitats may be a limitation for Bd was shown in a field study from southeast Queensland, Australia [12]. Kriger & Hero [12] compared the infection level of frog species breeding in ponds of different hydroperiods. They demonstrated that frog species breeding in permanent ponds were significantly more likely to be infected with Bd than were frogs breeding in temporary ponds. Another study [14] did not confirm a significant variation in chytrid levels of amphibians between temporary and permanent breeding habitats, but the authors assume that Bd in temporary ponds was re-introduced each year. Bd was most likely re-introduced by infected immigrating amphibians.

I analyzed if the hydroperiod of ponds has an influence on the Bd occurrence and prevalence. To do so, I compared Bd prevalence and occurrence in temporary and permanent ponds. I expected that the occurrence of infected amphibians is lower in temporary ponds than in permanent ponds. I chose for my study a single species that occurs in both temporary and permanent ponds. By choosing a single species I can avoid the influence of species differences in contrast to the study of Kriger & Hero [12]. The results of my study may help to decide whether the construction of temporary ponds could be a mitigation strategy against Bd.

The investigated ponds are located in northern Switzerland, a region where Bd was detected in approximately half of the water bodies (U. Tobler & B. R. Schmidt, unpublished data). The region shows a patchy distribution of Bd occurrence and neighboring ponds can differ in Bd presence/absence [15].

For the determination of the Bd occurrence and prevalence I chose frogs from the *Pelophylax esculentus* complex, because they fulfill three conditions necessary for this study: First, they breed in both permanent and temporary ponds [16]. Second, they are present in a region, where Bd infected and uninfected ponds occur in a patchy distribution [15]. Third, a Bd survey in ~70 ponds in northern Switzerland showed that 11.3% of 212 frogs of the *Pelophylax esculentus* complex tested were positive for Bd (U. Tobler, unpublished data).

Samples to determine Bd infections were taken from tadpoles because tadpoles can only be infected within the pond, whereas adults might have been infected on land. Due to the hydric requirement of Bd, the zoospores present in the water of temporary ponds likely have to be re-introduced by infected amphibians every season. The probability of a re-introduction increases with higher amphibian migration between ponds. Thus, I expect that the effect of hydroperiod is only apparent in more isolated ponds.

2 Methods

2.1 Study design and water frog sampling

2.1.1 Study area

I selected 16 ponds in northern Switzerland (Table 1). The ponds are located in cantons Zürich and Thurgau in an area of about 195 square kilometers (N 47° 34.9 - 47° 41.2, E 8°36.1 - 8°49.5). The selection for the ponds was based on the presence of water frogs (M. Lippuner, personal communication), pond hydroperiod (temporary or permanent) and proximity to forests.

Table 1 List of sampled ponds in northern Switzerland and their Swissgrid coordinates

Pond No.	Pond Name	Coordinates x	y
I	Heinrichsee	694610	273420
II	Tümpel bei Buck	704100	279860
III	Ober Weiher	694150	282600
IV	Länzigloch	687050	277160
V	Altlauf Inselen	691625	272425
VI	Pfaffensee	695000	273100
VII	Seewädeli	701600	279500
VIII	Grube Oberboden	689600	277650
IX	Cholgruebsee	693990	274230
X	Enteler-Weiher	692400	274700
XI	Bruggloch	687300	272800
XII	Kiesgrube südöstl. Feldhof	687500	270820
XIII	Barchetsee	698850	274750
XIV	Kiesgrube Rhinauer Feld	689300	277375
XV	Weiher Gütighausen	696550	271400
XVI	Retentionsbecken Breiten	693750	271400

2.1.2 Sampling

I caught 528 water frog tadpoles during daytime in July and August 2010 with a dip net. From each pond 28-36 tadpoles (only 19 tadpoles at one pond) were captured haphazardly. Species identification was based on external morphology [17] and confirmed by DNA analysis [18]. To carry out the DNA analysis, from each tadpole a tissue sample was taken. To test the tadpoles for Bd occurrence, each tadpole was swabbed with a sterile cotton swab (Copan Italia, Italy) over the mouthpart for about 10 seconds. The swabs were then put back in their original plastic tubes and frozen at –20°C after return from the field. After sampling, I immediately released all tadpoles into their pond of origin. In addition, 40 and 12 adult and subadult frogs, respectively, were captured at 2 nights at the beginning of October 2010 in two ponds. The water frogs were captured by hand. After capture, they were swabbed 20 times across the ventral surface, 10 times at each forehand and femoral. Each tadpole and adult water frog was handled with unused, powder-free vinyl gloves and then released where they were captured.

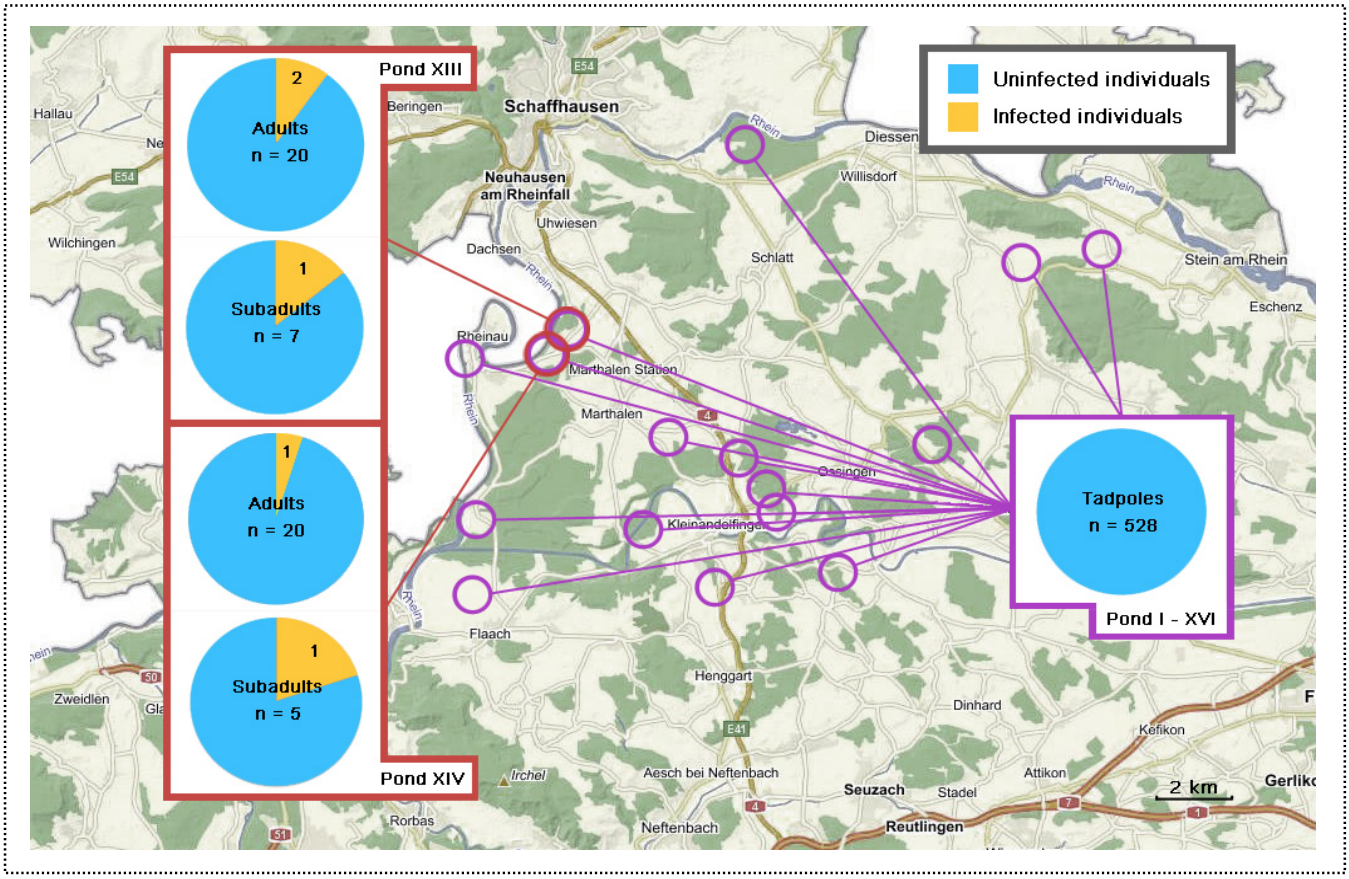

Figure 1 Number of *Batrachochytrium dendrobatidis* (Bd) infected water frog individuals. No water frog tadpole in any of the 16 populations was infected with Bd, visualized by the pie chart on the right. In pond VIII (left, top) 2 of 20 adults and 1 of 7 subadults were infected. In pond XIV (left, bottom) 1 of 20 adults and 1 of 5 subadults were infected.

2.2 Analysis of Bd infection status

2.2.1 Extraction of the chytrid fungus and detection by real-time PCR

To determine the Bd infection status of individuals, each individual was swabbed separately and checked afterwards for the presence of Bd with rt-PCR. The procedure of DNA extraction from the swab samples and rt-PCR assay corresponds to the protocol by Boyle et al. (2004) [19]. Instead of triplicates used by Boyle et al. [19] each sample was run in duplicates and the rt-PCR analysis was only repeated, if the duplicates showed inconsistent results. Zoospore equivalents (100,10,1,0.1) were run at each PCR-plate as controls. Because the extraction reagent is inhibitory to the rtPCR, samples were diluted 1:10 prior to the rtPCR analyses. As in Boyle et al. [19] untransformed zoospore equivalents are reported. Samples showing genomic equivalents (GE) above 0.1 were determined as Bd-positive and the individuals therefore classified as infected [20]. PCR assays were conducted using the Applied Biosystems Fast7500 and Bd-specific primers (ITS-1, 5.8s). The score for Bd-infection was done with Fast7500 software (Applied Biosystems).

3 Results

3.1 Bd infection rate in water frogs

3.1.1 Bd prevalence among tadpole

I checked 528 water frog tadpoles from 16 different ponds. The sample size per pond varied between 19 and 36 tadpoles. I could not detect any *Batrachochytrium dendrobatidis* infection in any water frog tadpoles (Figure 1).

3.1.2 Adult and subadult infection rate

In pond VIII 2 of 20 adults and 1 of 7 subadults frogs showed Bd infection resulting in a total prevalence of 11.1%. In pond XIV 1 of 20 adults and 1 of 5 subadults showed an infection with Bd, resulting in a prevalence of 8% (Figure 1). The mean quantity of zoospore load (genomic equivalents) per individual was between 0.003 and 0.477 (Table 2). The PCR indicated the presence of Bd on four additional adult frogs (Pond XIV) but the Bd zoospore loads were below the threshold of 0.1. Hence, they were classified as not Bd-infected.

Table 2 *Batrachochytrium dendrobatidis* (Bd) zoospore load (genomic equivalent) of infected adult and subadult water frogs from pond VIII and XIV. Individuals yielding the threshold of 0.1 are classified as Bd infected.

Pond	Stage	Zoospore load Mean ± SD	Status Bd
VIII	Adult	0.153 ± 0.021	Infected
	Adult	0.168 ± 0.010	Infected
	Sub adult	0.218 ± 0.045	Infected
XIV	Adult	0.477 ± 0.116	Infected
	Adult	0.023 ± 0.018	Not infected
	Adult	0.003 ± 0.004	Not infected
	Adult	0.014 ± 0.017	Not infected
	Adult	0.032 ± 0.024	Not infected
	Sub adult	0.162 ± 0.021	Infected

4 Discussion

I found no *Batrachochytrium dendrobatidis* infection in 528 water frog tadpoles. Thus, the hypothesis described in the Introduction could not be tested. There may be two reasons for this result. First, Bd may be present in such a low prevalence that I could not detect it. Second, Bd does not infect water frog tadpoles under natural conditions.

Low Bd prevalence and small samples sizes decrease the probability to prove Bd presence at a site. With my sample sizes of 29-36 tadpoles per pond I can detect Bd with 95% certainty with a prevalence of 8-10% and more in a population [21]. Bd prevalence of water frog adults varies among infected Swiss ponds from 0.0% to 83.3% (average 29.7%), based on 13 ponds and samples sizes between 4 and 46 individuals (U. Tobler, unpublished data). According to this data, I assume that Bd prevalence is lower among tadpoles than among adults. For each study site separately, Bd prevalence has to be lower than 10%. Across all ponds, sample size is much larger and prevalence must be very low. I conclude that under natural conditions water frog tadpoles do not get infected with Bd even though adults in the pond may be infected. The laboratory infection experiment of Baumgartner [22] showed that tadpoles of the *P. esculentus* complex can get infected with Bd. In this experiment, the exposure to Bd zoospores resulted in an infection of 7.8% of the tadpoles. Whereas water frog tadpoles get infected with Bd under laboratory conditions I could not detect Bd infection under natural conditions. Under natural conditions, the tadpoles are either not infected at all, or the Bd zoospore loads are below the detection threshold.

Kilpatrick et al. [23] showed that the susceptibility to Bd infection varies among amphibian species and within species among different developmental stages. The tadpole stage of water frogs seems to be rarely infected in the field, whereas 24.2 % of adults (n=182) were infected at Bd positive sites (U. Tobler, unpublished data). In amphibians as *Rana temporaria* infected tadpoles are rarely detected (Trenton W.J. Garner, personal communication). In contrast, up to 100% of *Alytes obstetricans* tadpoles can be infected at Bd positive sites and an infection prevalence of 44.8% among adults (n=1607) was demonstrated (U. Tobler, unpublished data). However, at least for the water frogs my findings suggest that under natural conditions infection takes place primarily after metamorphosis.

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References

[1] Pimm SL, Raven P. Biodiversity - Extinction by numbers. Nature. 2000;403(6772):843-845.

[2] Wilcove DS, Rothstein D, Dubow J, Phillips A, Losos E. Quantifying threats to imperiled species in the United States. Bioscience. 1998;48(8):607-615.

[3] Collins JP, Storfer A. Global amphibian declines: sorting the hypotheses. Diversity and Distributions. 2003;9(2):89-98.

[4] Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW. Status and trends of amphibian declines and extinctions worldwide. Science. 2004;306(5702):1783-1786.

[5] Laurance WF, McDonald KR, Speare R. Epidemic disease and the catastrophic decline of Australian rain forest frogs. Conserv Biol. 1996;10(2):406-413.

[6] Lips KR. Decline of a tropical montane amphibian fauna. Conservation Biology. 1998;12(1):106-117.

[7] Lips KR. Mass mortality and population declines of anurans at an upland site in western Panama. Conservation Biology. 1999;13(1):117-125.

[8] Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, Hines HB, Lips KR, Marantelli G, Parkes H. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(15):9031-9036.

[9] Longcore JE, Pessier AP, Nichols DK. *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. Mycologia. 1999;91(2):219-227.

[10] Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD, Hines HB, Kenyon N. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth. 2007;4(2):125-134.

[11] Johnson ML, Speare R. Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. Diseases of Aquatic Organisms. 2005;65(3):181-186.

[12] Kriger KM, Hero J-M. The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. Diversity and Distributions. 2007;13(6):781-788.

[13] Johnson ML, Berger L, Philips L, Speare R. Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms. 2003;57(3):255-260.

[14] Padgett-Flohr GE, Hopkins RL. Landscape epidemiology of *Batrachochytrium dendrobatidis* in central California. Ecography. 2010:688-697.

[15] Schmidt BR, Furrer S, Kwet A, Lötters S, Rödder D, Sztatecsny M, Tobler U, Zumbach S. Desinfektion als Maßnahme gegen die Verbreitung der Chytridiomykose bei Amphibien. Zeitschrift für Feldherpetologie. 2009;15:229-241.

[16] Van Buskirk J. Habitat partitioning in European and North American pond-breeding frogs and toads. Diversity and Distributions. 2003;9(5):399-410.

[17] Bühler C, Cigler H, Lippuner M. Amphibienlarven - Bestimmung. Fauna Helvetica 17, 2007.

[18] Christiansen DG. A microsatellite-based method for genotyping diploid and triploid water frogs of the *Rana esculenta* hybrid complex. Molecular Ecology Notes. 2005;5(1):190-193.

[19] Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms. 2004;60(2):141-148.

[20] Tobler U, Schmidt BR. Within- and Among-Population Variation in Chytridiomycosis-Induced Mortality in the *Toad Alytes obstetricans*. PloS ONE. 2010;5(6):e10927.

[21] DiGiacomo RF, Koepsell TD. Sampling for detection of infection or disease in animal populations. Journal of the American Veterinary Medical Association. 1986;189(1):22-23.

[22] Baumgartner S. Experimental infection of three native European amphibian larvae by the pathogen *Batrachochytrium dendrobatidis*. Institute of Evolutionary Biology and Environmental Studies: University of Zurich; 2009.

[23] Kilpatrick AM, Briggs CJ, Daszak P. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. Trends in Ecology and Evolution. 2010;25(2):109-118.

Appendix

Appendix 1 Genotypes of water frog tadpoles, adults (*) and subadults (") and according mitochondrial DNA

Pond	Sample	Genotype	mtDNA	Pond	Sample	Genotype	mtDNA	Pond	Sample	Genotype	mtDNA
I	M.L.-1-01	LL	-	II	M.L.-1-42	LR	-	III	M.L.-1-83	LR	-
I	M.L.-1-02	LL	-	II	M.L.-1-43	LL	-	III	M.L.-1-84	LR	-
I	M.L.-1-03	LR	-	II	M.L.-1-44	LR	-	III	M.L.-1-85	LR	-
I	M.L.-1-04	LL	-	II	M.L.-1-45	LL	-	IV	M.L.-1-86	LL	-
I	M.L.-1-05	LL	-	II	M.L.-1-46	LR	-	IV	M.L.-1-87	LL	-
I	M.L.-1-06	LL	-	II	M.L.-1-47	LR	-	IV	M.L.-1-88	LL	-
I	M.L.-1-07	LR	-	II	M.L.-1-48	LR	-	IV	M.L.-1-89	LR	-
I	M.L.-1-08	LL	-	II	M.L.-1-49	LL	-	IV	M.L.-1-90	LL	-
I	M.L.-1-09	LR	-	II	M.L.-1-50	LR	-	IV	M.L.-1-91	LR	-
I	M.L.-1-10	LR	-	II	M.L.-1-51	LR	-	IV	M.L.-1-92	LR	-
I	M.L.-1-11	LR	-	II	M.L.-1-52	LR	-	IV	M.L.-1-93	LL	-
I	M.L.-1-12	LL	-	II	M.L.-1-53	LR	-	IV	M.L.-1-94	LL	-
I	M.L.-1-13	LL	-	II	M.L.-1-54	LL	-	IV	M.L.-1-95	LL	-
I	M.L.-1-14	LR	-	II	M.L.-1-55	LR	-	IV	M.L.-2-01	LR	-
I	M.L.-1-15	LR	-	III	M.L.-1-56	LR	-	IV	M.L.-2-02	LL	-
I	M.L.-1-16	LL	-	III	M.L.-1-57	LR	-	IV	M.L.-2-03	LL	-
I	M.L.-1-17	LL	-	III	M.L.-1-58	LR	-	IV	M.L.-2-04	LR	-
I	M.L.-1-18	LL	-	III	M.L.-1-59	LR	-	IV	M.L.-2-05	LL	-
I	M.L.-1-19	LR	-	III	M.L.-1-60	LR	-	IV	M.L.-2-06	LL	-
II	M.L.-1-20	LR	-	III	M.L.-1-61	LR	-	IV	M.L.-2-07	LL	-
II	M.L.-1-21	LR	-	III	M.L.-1-62	LR	-	IV	M.L.-2-08	LL	-
II	M.L.-1-22	LR	-	III	M.L.-1-63	LR	-	IV	M.L.-2-09	LL	-
II	M.L.-1-23	LR	-	III	M.L.-1-64	LR	-	IV	M.L.-2-10	LR	-
II	M.L.-1-24	LL	-	III	M.L.-1-65	LR	-	IV	M.L.-2-11	LR	-
II	M.L.-1-25	LR	-	III	M.L.-1-66	LR	-	IV	M.L.-2-12	LR	-
II	M.L.-1-26	LL	-	III	M.L.-1-67	LR	-	IV	M.L.-2-13	LL	-
II	M.L.-1-27	LR	-	III	M.L.-1-68	LR	-	IV	M.L.-2-14	LR	-
II	M.L.-1-28	LR	-	III	M.L.-1-69	LR	-	IV	M.L.-2-15	LL	-
II	M.L.-1-29	LR	-	III	M.L.-1-70	LR	-	IV	M.L.-2-16	LR	-
II	M.L.-1-30	LR	-	III	M.L.-1-71	LR	-	IV	M.L.-2-17	LL	-
II	M.L.-1-31	LR	-	III	M.L.-1-72	LR	-	IV	M.L.-2-18	LR	-
II	M.L.-1-32	LR	-	III	M.L.-1-73	LR	-	IV	M.L.-2-19	LR	-
II	M.L.-1-33	LR	-	III	M.L.-1-74	LR	-	IV	M.L.-2-20	LL	-
II	M.L.-1-34	LR	-	III	M.L.-1-75	LR	-	IV	M.L.-2-21	LL	-
II	M.L.-1-35	LR	-	III	M.L.-1-76	LR	-	IV	M.L.-2-22	LR	-
II	M.L.-1-36	LR	-	III	M.L.-1-77	LR	-	IV	M.L.-2-23	LR	-
II	M.L.-1-37	LR	-	III	M.L.-1-78	LR	-	IV	M.L.-2-24	LR	-
II	M.L.-1-38	LL	-	III	M.L.-1-79	LR	-	IV	M.L.-2-25	LR	-
II	M.L.-1-39	LL	-	III	M.L.-1-80	LR	-	V	M.L.-2-26	LLR	*bergeri*
II	M.L.-1-40	LR	-	III	M.L.-1-81	LR	-	V	M.L.-2-27	LLR	*bergeri*
II	M.L.-1-41	LR	-	III	M.L.-1-82	LR	-	V	M.L.-2-28	LLR	*bergeri*

Pond	Sample	Genotype	mtDNA	Pond	Sample	Genotype	mtDNA	Pond	Sample	Genotype	mtDNA
V	M.L.-2-29	LR	*bergeri*	VI	M.L.-2-73	LR	-	VII	M.L.-3-22	LR	-
V	M.L.-2-30	RR	-	VI	M.L.-2-74	LR	-	VII	M.L.-3-23	LR	-
V	M.L.-2-31	LR	*bergeri*	VI	M.L.-2-75	LR	-	VII	M.L.-3-24	LR	-
V	M.L.-2-32	RR	*bergeri*	VI	M.L.-2-76	LR	-	VII	M.L.-3-25	LR	-
V	M.L.-2-33	RR	*bergeri*	VI	M.L.-2-77	RR	-	VII	M.L.-3-26	LR	-
V	M.L.-2-34	RR	*bergeri*	VI	M.L.-2-78	LR	-	VII	M.L.-3-27	LR	-
V	M.L.-2-35	LLR	*bergeri*	VI	M.L.-2-79	RR	-	VII	M.L.-3-28	LR	-
V	M.L.-2-36	RR	*bergeri*	VI	M.L.-2-80	LR	-	VII	M.L.-3-29	LR	-
V	M.L.-2-37	RR	-	VI	M.L.-2-81	RR	-	VII	M.L.-3-30	LL	-
V	M.L.-2-38	RR	-	VI	M.L.-2-82	LR	-	VII	M.L.-3-31	LR	-
V	M.L.-2-39	RR	-	VI	M.L.-2-83	LR	-	VII	M.L.-3-32	LR	-
V	M.L.-2-40	RR	-	VI	M.L.-2-84	RR	-	VII	M.L.-3-33	LR	-
V	M.L.-2-41	RR	-	VI	M.L.-2-85	LR	-	VII	M.L.-3-34	LR	-
V	M.L.-2-42	RR	-	VI	M.L.-2-86	RR	-	VII	M.L.-3-35	LR	-
V	M.L.-2-43	LLR	*bergeri*	VI	M.L.-2-87	LR	-	VII	M.L.-3-36	LR	-
V	M.L.-2-44	RR	-	VI	M.L.-2-88	LR	-	VII	M.L.-3-37	LR	-
V	M.L.-2-45	LLR	*bergeri*	VI	M.L.-2-89	LR	-	VII	M.L.-3-38	LR	-
V	M.L.-2-46	RR	-	VI	M.L.-2-90	LR	-	VIII	M.L.-3-39	LR	*bergeri*
V	M.L.-2-47	RR	-	VI	M.L.-2-91	LR	-	VIII	M.L.-3-40	LLR	-
V	M.L.-2-48	LLR	*bergeri*	VI	M.L.-2-92	LR	-	VIII	M.L.-3-41	LLR	-
V	M.L.-2-49	LLR	*bergeri*	VI	M.L.-2-93	LR	-	VIII	M.L.-3-42	LLR	*bergeri*
V	M.L.-2-50	LLR	*bergeri*	VI	M.L.-2-94	LR	-	VIII	M.L.-3-43	LLR	*bergeri*
V	M.L.-2-51	RR	-	VI	M.L.-2-95	RR	-	VIII	M.L.-3-44	LLR	*bergeri*
V	M.L.-2-52	RR	-	VI	M.L.-3-01	LR	-	VIII	M.L.-3-45	LLR	*bergeri*
V	M.L.-2-53	RR	-	VI	M.L.-3-02	LR	-	VIII	M.L.-3-46	LLR	-
V	M.L.-2-54	RR	-	VII	M.L.-3-03	LR	-	VIII	M.L.-3-47	LLR	-
V	M.L.-2-55	LR	*bergeri*	VII	M.L.-3-04	LR	-	VIII	M.L.-3-48	LLR	*bergeri*
V	M.L.-2-56	LLR	*bergeri*	VII	M.L.-3-05	LR	-	VIII	M.L.-3-49	LLR	-
V	M.L.-2-57	RR	-	VII	M.L.-3-06	LR	-	VIII	M.L.-3-50	LLR	-
V	M.L.-2-58	LLR	*bergeri*	VII	M.L.-3-07	LL	-	VIII	M.L.-3-51	LLR	-
V	M.L.-2-59	RR	-	VII	M.L.-3-08	LR	-	VIII	M.L.-3-52	LLR	-
V	M.L.-2-60		-	VII	M.L.-3-09	LR	-	VIII	M.L.-3-53	LLR	-
V	M.L.-2-61	RR	-	VII	M.L.-3-10	LR	-	VIII	M.L.-3-54	LR	*bergeri*
VI	M.L.-2-62	LR	-	VII	M.L.-3-11	LR	-	VIII	M.L.-3-55	LLR	-
VI	M.L.-2-63	LR	-	VII	M.L.-3-12	LR	-	VIII	M.L.-3-56	LLR	-
VI	M.L.-2-64	LR	-	VII	M.L.-3-13	LR	-	VIII	M.L.-3-57	LLR	-
VI	M.L.-2-65	LR	-	VII	M.L.-3-14	LR	-	VIII	M.L.-3-58	LLR	-
VI	M.L.-2-66	LR	-	VII	M.L.-3-15	LR	-	VIII	M.L.-3-59	LLR	-
VI	M.L.-2-67	LR	-	VII	M.L.-3-16	LR	-	VIII	M.L.-3-60	LR	-
VI	M.L.-2-68	LR	-	VII	M.L.-3-17	LR	-	VIII	M.L.-3-61	LLR	-
VI	M.L.-2-69	LR	-	VII	M.L.-3-18	LR	-	VIII	M.L.-3-62	LLR	-
VI	M.L.-2-70	LR	-	VII	M.L.-3-19	LR	-	VIII	M.L.-3-63	LLR	-
VI	M.L.-2-71	LR	-	VII	M.L.-3-20	LR	-	VIII	M.L.-3-64	LLR	*bergeri*
VI	M.L.-2-72	LR	-	VII	M.L.-3-21	LR	-	VIII	M.L.-3-65	LLR	-

Pond	Sample	Genotype	mtDNA	Pond	Sample	Genotype	mtDNA	Pond	Sample	Genotype	mtDNA
VIII	M.L.-3-66	LR	-	X	M.L.-4-15	LL	-	XI	M.L.-4-59	RR	-
IX	M.L.-3-67	LR	-	X	M.L.-4-16	LL	-	XI	M.L.-4-60	RR	-
IX	M.L.-3-68	LL	-	X	M.L.-4-17	LL	-	XI	M.L.-4-61	LL	-
IX	M.L.-3-69	LL	-	X	M.L.-4-18	LL	-	XI	M.L.-4-62	RR	-
IX	M.L.-3-70	LR	-	X	M.L.-4-19	LL	-	XI	M.L.-4-63	LR	-
IX	M.L.-3-71	LR	-	X	M.L.-4-20	LL	-	XI	M.L.-4-64	RR	-
IX	M.L.-3-72	LR	-	X	M.L.-4-21	LL	-	XI	M.L.-4-65	LL	-
IX	M.L.-3-73	LL	-	X	M.L.-4-22	LL	-	XI	M.L.-4-66	LR	-
IX	M.L.-3-74	LL	-	X	M.L.-4-23	LL	-	XI	M.L.-4-67	LR	-
IX	M.L.-3-75	LR	-	X	M.L.-4-24	LL	-	XI	M.L.-4-68	RR	-
IX	M.L.-3-76	LR	-	X	M.L.-4-25	LL	-	XI	M.L.-4-69	RR	-
IX	M.L.-3-77	LR	-	X	M.L.-4-26	LL	-	XI	M.L.-4-70	RR	-
IX	M.L.-3-78		-	X	M.L.-4-27	LL	-	XI	M.L.-4-71	RR	-
IX	M.L.-3-79	LR	-	X	M.L.-4-28	LL	-	XI	M.L.-4-72	LR	-
IX	M.L.-3-80	LR	-	X	M.L.-4-29	LL	-	XI	M.L.-4-73	RR	-
IX	M.L.-3-81	LR	-	X	M.L.-4-30	LL	-	XII	M.L.-4-74	LR	-
IX	M.L.-3-82	LR	-	X	M.L.-4-31	LL	-	XII	M.L.-4-75	LR	-
IX	M.L.-3-83	LR	-	X	M.L.-4-32	LL	-	XII	M.L.-4-76	LR	-
IX	M.L.-3-84	LL	-	X	M.L.-4-33	LL	-	XII	M.L.-4-77	LR	-
IX	M.L.-3-85	LR	-	X	M.L.-4-34	LL	-	XII	M.L.-4-78	LR	-
IX	M.L.-3-86	LR	-	X	M.L.-4-35	LL	-	XII	M.L.-4-79	LR	-
IX	M.L.-3-87	LR	-	X	M.L.-4-36	LR	-	XII	M.L.-4-80	LR	-
IX	M.L.-3-88	LR	-	X	M.L.-4-37	LL	-	XII	M.L.-4-81	LR	-
IX	M.L.-3-89	LR	-	X	M.L.-4-38	LL	-	XII	M.L.-4-82	LR	-
IX	M.L.-3-90	LL	-	X	M.L.-4-39	LL	-	XII	M.L.-4-83	LR	-
IX	M.L.-3-91		-	X	M.L.-4-40	LL	-	XII	M.L.-4-84	LR	-
IX	M.L.-3-92	LR	-	X	M.L.-4-41	LL	-	XII	M.L.-4-85	LR	-
IX	M.L.-3-93	LR	-	X	M.L.-4-42	LL	-	XII	M.L.-4-86	LR	-
IX	M.L.-3-94	LR	-	X	M.L.-4-43	LL	-	XII	M.L.-4-87	LR	-
IX	M.L.-3-95	LR	-	XI	M.L.-4-44	RR	-	XII	M.L.-4-88	LR	-
IX	M.L.-4-01	LR	-	XI	M.L.-4-45	LL	-	XII	M.L.-4-89	LR	-
IX	M.L.-4-02	LR	-	XI	M.L.-4-46	RR	-	XII	M.L.-4-90	LR	-
IX	M.L.-4-03	LR	-	XI	M.L.-4-47	LR	-	XII	M.L.-4-91	LR	-
IX	M.L.-4-04	LR	-	XI	M.L.-4-48	RR	-	XII	M.L.-4-92	LR	-
IX	M.L.-4-05	LL	-	XI	M.L.-4-49	RR	-	XII	M.L.-4-93	LR	-
IX	M.L.-4-06	LR	-	XI	M.L.-4-50	RR	-	XII	M.L.-4-94	LR	-
IX	M.L.-4-07	LL	-	XI	M.L.-4-51	LR	-	XII	M.L.-4-95	LR	-
X	M.L.-4-08	LL	-	XI	M.L.-4-52	LR	-	XII	M.L.-5-01	LR	-
X	M.L.-4-09	LL	-	XI	M.L.-4-53	LR	-	XII	M.L.-5-02	LR	-
X	M.L.-4-10	LL	-	XI	M.L.-4-54	RR	-	XII	M.L.-5-03	LR	-
X	M.L.-4-11	LL	-	XI	M.L.-4-55	RR	-	XII	M.L.-5-04	LR	-
X	M.L.-4-12	LL	-	XI	M.L.-4-56	LL	-	XII	M.L.-5-05	LR	-
X	M.L.-4-13	LL	-	XI	M.L.-4-57		-	XII	M.L.-5-06	LR	-
X	M.L.-4-14	LL	-	XI	M.L.-4-58	RR	-	XII	M.L.-5-07	LR	-

Pond	Sample	Genotype	mtDNA	Pond	Sample	Genotype	mtDNA	Pond	Sample	Genotype	mtDNA
XII	M.L.-5-08	LR	-	XIV	M.L.-5-52	RR	-	XV	M.L.-6-01	LR	-
XII	M.L.-5-09	LR	-	XIV	M.L.-5-53	RR	-	XV	M.L.-6-02	LR	-
XII	M.L.-5-10	LR	-	XIV	M.L.-5-54	RR	-	XV	M.L.-6-03	LR	-
XII	M.L.-5-11	LR	-	XIV	M.L.-5-55	RR	*ridibu.*	XV	M.L.-6-04	LR	-
XII	M.L.-5-12	LR	-	XIV	M.L.-5-56	LLR	*bergeri*	XV	M.L.-6-05	LR	-
XII	M.L.-5-13	LR	-	XIV	M.L.-5-57	RR	-	XV	M.L.-6-06	LR	-
XIII	M.L.-5-14	RR	-	XIV	M.L.-5-58	RR	-	XV	M.L.-6-07	LL	-
XIII	M.L.-5-15	RR	-	XIV	M.L.-5-59	RR	-	XV	M.L.-6-08	LR	-
XIII	M.L.-5-16	LRR	*bergeri*	XIV	M.L.-5-60	RR	-	XV	M.L.-6-09	LR	-
XIII	M.L.-5-17	RR	*bergeri*	XIV	M.L.-5-61	RR	-	XV	M.L.-6-10	LR	-
XIII	M.L.-5-18	RR	-	XIV	M.L.-5-62	RR	-	XV	M.L.-6-11	LR	-
XIII	M.L.-5-19	RR	-	XIV	M.L.-5-63	RR	-	XV	M.L.-6-12	LL	-
XIII	M.L.-5-20	RR	-	XIV	M.L.-5-64	RR	-	XV	M.L.-6-13	LR	-
XIII	M.L.-5-21	RR	-	XIV	M.L.-5-65	RR	*ridibu.*	XV	M.L.-6-14	LR	-
XIII	M.L.-5-22	LR	*bergeri*	XIV	M.L.-5-66	RR	-	XV	M.L.-6-15	LR	-
XIII	M.L.-5-23	RR	-	XIV	M.L.-5-67	RR	-	XV	M.L.-6-16	LR	-
XIII	M.L.-5-24	RR	*bergeri*	XIV	M.L.-5-68	RR	-	XV	M.L.-6-17	LR	-
XIII	M.L.-5-25	LRR	*bergeri*	XIV	M.L.-5-69	LLR	*bergeri*	XV	M.L.-6-18	LR	-
XIII	M.L.-5-26	LR	*bergeri*	XIV	M.L.-5-70	LLR	*bergeri*	XV	M.L.-6-19	LR	-
XIII	M.L.-5-27	RR	-	XIV	M.L.-5-71	RR	-	XVI	M.L.-6-20	LR	-
XIII	M.L.-5-28	RR	-	XIV	M.L.-5-72	LR	*ridibu.*	XVI	M.L.-6-21	LR	-
XIII	M.L.-5-29	LR	*bergeri*	XIV	M.L.-5-73	LLR	*bergeri*	XVI	M.L.-6-22	RR	-
XIII	M.L.-5-30	LRR	*bergeri*	XIV	M.L.-5-74	LLR	*ridibu.*	XVI	M.L.-6-23	RR	-
XIII	M.L.-5-31	LR	*bergeri*	XIV	M.L.-5-75	RR	-	XVI	M.L.-6-24	LR	-
XIII	M.L.-5-32	RR	*bergeri*	XIV	M.L.-5-76	RR	*ridibu.*	XVI	M.L.-6-25	LR	-
XIII	M.L.-5-33	RR	*bergeri*	XIV	M.L.-5-77	RR	-	XVI	M.L.-6-26	LR	-
XIII	M.L.-5-34	RR	-	XIV	M.L.-5-78	LLR	*bergeri*	XVI	M.L.-6-27	LR	-
XIII	M.L.-5-35	RR	-	XV	M.L.-5-79	LR	-	XVI	M.L.-6-28	RR	-
XIII	M.L.-5-36	RR	-	XV	M.L.-5-80	LR	-	XVI	M.L.-6-29	RR	-
XIII	M.L.-5-37	LRR	*bergeri*	XV	M.L.-5-81	LR	-	XVI	M.L.-6-30	LR	-
XIII	M.L.-5-38	RR	-	XV	M.L.-5-82	LR	-	XVI	M.L.-6-31	RR	-
XIII	M.L.-5-39	RR	-	XV	M.L.-5-83	LR	-	XVI	M.L.-6-32	LR	-
XIII	M.L.-5-40	LR	*bergeri*	XV	M.L.-5-84	LR	-	XVI	M.L.-6-33	RR	-
XIII	M.L.-5-41	RR	-	XV	M.L.-5-85	LR	-	XVI	M.L.-6-34	LR	-
XIII	M.L.-5-42	RR	-	XV	M.L.-5-86	LL	-	XVI	M.L.-6-35	LR	-
XIV	M.L.-5-43	LLR	*bergeri*	XV	M.L.-5-87	LR	-	XVI	M.L.-6-36	RR	-
XIV	M.L.-5-44	RR	-	XV	M.L.-5-88	LR	-	XVI	M.L.-6-37	LR	-
XIV	M.L.-5-45	LLR	*bergeri*	XV	M.L.-5-89	LR	-	XVI	M.L.-6-38	LR	-
XIV	M.L.-5-46	RR	*ridibu.*	XV	M.L.-5-90	LR	-	XVI	M.L.-6-39	RR	-
XIV	M.L.-5-47	RR	*ridibu.*	XV	M.L.-5-91	LR	-	XVI	M.L.-6-40	LR	-
XIV	M.L.-5-48	RR	-	XV	M.L.-5-92	LR	-	XVI	M.L.-6-41	RR	-
XIV	M.L.-5-49	RR	-	XV	M.L.-5-93	LR	-	XVI	M.L.-6-42	LR	-
XIV	M.L.-5-50	RR	-	XV	M.L.-5-94	LR	-	XVI	M.L.-6-43	LR	-
XIV	M.L.-5-51	RR	-	XV	M.L.-5-95	LR	-	XVI	M.L.-6-44	LR	-

Pond	Sample	Genotype	mtDNA	Pond	Sample	Genotype	mtDNA	Pond	Sample	Genotype	mtDNA
XVI	M.L.-6-45	RR	-	VIII	M.L.-7-12*	LLR	*bergeri*	XIV	M.L.-7-33*	LR	-
XVI	M.L.-6-46	LR	-	VIII	M.L.-7-13*	LLR	*bergeri*	XIV	M.L.-7-34*	RR	*ridibu.*
XVI	M.L.-6-47	RR	-	VIII	M.L.-7-14*	LLR	*bergeri*	XIV	M.L.-7-35*	RR	-
XVI	M.L.-6-48	LR	-	VIII	M.L.-7-15*	RR	*ridibu.*	XIV	M.L.-7-36*	LLR	*bergeri*
XVI	M.L.-6-49	RR	-	VIII	M.L.-7-16*	LLR	-	XIV	M.L.-7-37*	LR	-
XVI	M.L.-6-50	RR	-	VIII	M.L.-7-17*	LLR	-	XIV	M.L.-7-38*	LR	-
XVI	M.L.-6-51	LR	-	VIII	M.L.-7-18*	LLR	*bergeri*	XIV	M.L.-7-39*	LLR	*bergeri*
XVI	M.L.-6-52	LR	-	VIII	M.L.-7-19*	LLR	*bergeri*	XIV	M.L.-7-40*	LLR	*bergeri*
XVI	M.L.-6-53	LR	-	VIII	M.L.-7-20*	LLR	*bergeri*	XIV	M.L.-7-41*	RR	*ridibu.*
			-	VIII	M.L.-7-21"	LLR	*bergeri*	XIV	M.L.-7-42*	RR	-
VIII	M.L.-7-01*	LR	-	VIII	M.L.-7-22"	LLR	*bergeri*	XIV	M.L.-7-43*	LLR	*bergeri*
VIII	M.L.-7-02*	LR	*bergeri*	VIII	M.L.-7-23"	LLR	*bergeri*	XIV	M.L.-7-44*	LLR	*bergeri*
VIII	M.L.-7-03*	LR	*bergeri*	VIII	M.L.-7-24"	RR	*ridibu.*	XIV	M.L.-7-45*	LLR	*bergeri*
VIII	M.L.-7-04*	LR	*bergeri*	VIII	M.L.-7-25"	LR	*bergeri*	XIV	M.L.-7-46*	LLR	*bergeri*
VIII	M.L.-7-05*	LR	*bergeri*	VIII	M.L.-7-26"	LLR	*bergeri*	XIV	M.L.-7-47*	LLR	*bergeri*
VIII	M.L.-7-06*	LR	-	VIII	M.L.-7-27"	LR	-	XIV	M.L.-7-48*	RR	-
VIII	M.L.-7-07*	LR	-	XIV	M.L.-7-28*	LR	*bergeri*	XIV	M.L.-7-49"	LLR	*bergeri*
VIII	M.L.-7-08*	LR	*bergeri*	XIV	M.L.-7-29*	LR	*bergeri*	XIV	M.L.-7-50"	LLR	*bergeri*
VIII	M.L.-7-09*	LR	*bergeri*	XIV	M.L.-7-30*	LR	*bergeri*	XIV	M.L.-7-51"	LLR	*bergeri*
VIII	M.L.-7-10*	LLR	*bergeri*	XIV	M.L.-7-31*	LR	*bergeri*	XIV	M.L.-7-52"	RR	*bergeri*
VIII	M.L.-7-11*	RR	*ridibu.*	XIV	M.L.-7-32*	RR	*bergeri*	XIV	M.L.-7-53"	LLR	*bergeri*