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Pervasive non-random mitochondrial DNA heteroplasmy in the hybrid water frog *Pelophylax esculentus*

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Abstract

Background Heteroplasmy, the presence of more than one type of mitochondrial DNA (mtDNA) within an individual, is an exception to the maternal transmission of mtDNA and has been observed in several animal species. A central question is whether heteroplasmy among individuals and across generations is mainly influenced by genetic drift or by selection.

Results We quantified heteroplasmy in eight males, eight females and eight unfertilized eggs per female from a natural population of the hybrid frog species *Pelophylax esculentus* (between *P. ridibundus* and *P. lessonae*). After excluding sequencing error and potential sources of contamination, we found that all individuals and most of the eggs were heteroplasmic, containing 2–5 different haplotypes, from which one was very common and the rest appeared at very low frequencies (at maximum 2%). We observed a single *lessonae* haplotype, which was present in females and in their eggs but absent from all males. On the other hand, we observed four different *ridibundus* haplotypes that were present in males, females and eggs. Eggs had significantly lower heteroplasmy levels than their mothers.

Conclusions The distribution of haplotypes between males and females, the difference of heteroplasmy levels between mothers and their eggs, and results from simulations suggest that drift alone is not sufficient to explain the observed patterns of heteroplasmy.

Keywords Frogs, Pelophylax, Mitochondrial DNA, Heteroplasmy, Hybrids, Bottleneck, Selection

Introduction

Maternal transmission of mtDNA in animals implies that individuals contain a single type of mtDNA (homoplasmy). However, modern techniques, such as deep sequencing, have revealed that heteroplasmy - the presence of more than one type of mtDNA in an individual - is more common in populations than previously believed (only some reports for this evidence are: [1–8]. Alternative mtDNA haplotypes within a heteroplasmic individual can arise from *de novo* mutations, maternal inheritance of heteroplasmy and leakage of paternal mtDNA [9]. All three causes of heteroplasmy are considered neutral, that

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occur accidentally [10], either due to the mutational process or as mistakes of the mechanisms that guard maternal mtDNA transmission [11, 12].

The segregation of heteroplasmy across generations is determined by both drift and selection. Older experiments in cows, mice, and humans [13–15] have shown that heteroplasmy segregates stochastically across generations. More recent studies observed high variance of heteroplasmy levels among the progeny of heteroplasmic individuals in different animal species [16–23]. Based on these evidences it has been proposed that mtDNA copy number experiences a severe bottleneck during oogenesis. The few mitochondria that pass through the bottleneck propagate fast and populate the fully developed eggs [24–26]. Therefore, genetic drift plays a major role in the distribution of mtDNA haplotypes in the eggs.

Apart from drift, selection has also been observed in heteroplasmy levels. Heteroplasmy can be subject to purifying selection in order to remove disease-causing haplotypes in humans [27–29], mice [30], and *Drosophila* [31, 32]. Purifying selection also acts when heteroplasmy itself is harmful. As it has been shown in mice, the coexistence of two wild type haplotypes, without deleterious mutations, caused severe diseases [33, 34]. Finally, selection can act against heteroplasmy in order to prevent biparental mtDNA transmission.

The dynamics of heteroplasmy in natural populations remain largely unexplored, in part due to technical difficulties. Standard Sanger sequencing techniques may not detect low levels of heteroplasmy within individuals, and high-throughput sequencing may have difficulty distinguishing true heteroplasmy from sequencing errors [6, 25, 35]. However, increased sequence divergence between haplotypes can help to distinguish them from each other and from sequencing errors. This condition is often met in hybrids, where heteroplasmy typically arises from paternal leakage and the two mtDNA haplotypes originate from different species [36].

Water frogs of the genus *Pelophylax*, formerly known as *Rana*, present a valuable model for studying heteroplasmy due to their extensive interspecific hybridization [37]. The "*P. esculentus* complex" constitutes a hybrid system consisting of two parental species: *P. ridibundus* (genotype RR) and *P. lessonae* (genotype LL), as well as a hybrid species, *P. esculentus* (genotype RL) [38]. *P. esculentus* coexists with one of the parental species, typically *P. lessonae*, and is maintained through a genetic system called "hybridogenesis" [39, 40]. In this system, the hybrid's gametes contain only one species' nuclear genome and discard the other. *P. esculentus* displays various variants of the hybridogenic system, with the most common being the L-E system, where *P. esculentus* discards the *P. lessonae* chromosome set and retains only *the P. ridibundus* set. The hybrid *P. esculentus* is regenerated in each generation when its gametes with the *P. ridibundus* set of chromosomes are backcrossed with the gametes from the sympatric *P. le*ssonae [41]. Additionally, *P. esculentus* can also coexist with the *P. ridibundus* parental species (R-E system) [42]. The hybridogenic system in frogs is diverse, complex and not fully understood [43]. Several Southern European populations of the *Pelophylax* species exhibit extensive mtDNA heteroplasmy [44], indicating that the *Pelophylax* genus is prone not only to hybridization but also to mtDNA heteroplasmy. In this study, we focus on a population from Pancevo, Serbia, where all its individuals were heteroplasmic [44]. We investigated the qualitative and quantitative patterns of heteroplasmy among male and female individuals and the transmission of heteroplasmy in the eggs using amplicon-based next-generation sequencing. Our findings, based on sequencing data and computer simulations, suggest that the distribution and the transmission of heteroplasmy cannot be explained solely by drift.

Materials and methods

Samples used in this study

Eight females and eight males which were previously reported as heteroplasmic were randomly chosen from a diploid, hybrid population of the frogs *P. esculentus*, from Pancevo (Serbia) [44]. Also, two individuals, one *P. esculentus* from Orlovat (northern Serbia) and another *P. ridibundus* from Nis (southern Serbia), were used as controls. All tissue and DNA samples used for this study come from a previous study in which the samples originated from herpetological collections from Institute of Zoology, Faculty of Biology, University of Belgrade, Serbia [44]. DNA was extracted from somatic tissue (tongue) of each individual using the salt extraction protocol [45]. The same protocol was used to extract DNA from individual, unfertilized eggs. We used eight eggs from each female but we concluded with 61 unfertilized eggs, in total, because in one female we recovered DNA from 5 eggs only. To avoid contamination, the procedure of DNA extraction was performed separately for the adults and for the eggs.

Artificial heteroplasmy experiment

In order to determine the accuracy of the dual indexing method for amplicon sequencing in quantifying a broad-scale amounts of mtDNA haplotypes we applied an artificial heteroplasmy dilution series. They were used as standard reference points for adjusting potential biases outlined by eventual PCR and sequencing errors. The initial step of PCR amplification could preferentially amplify the common haplotype and/or random substitutions in primers' region could reduce the affinity of the primers to one sequence relative to the other [46–48], which could both disregard the actual heteroplasmy

presence. Both the RL1 and RL2 loci respectively were amplified from two heteroplasmic female individuals and their PCR products were subsequently cloned into the pGEM vector using the pGEM®-T Easy Vector (Promega) kit according to the manufacturer's instructions. Clones were screened with restriction enzymes to identify the *lessonae* and *ridibundus* restriction fragments (see ref 44 for the experimental details). They were prepared for the extraction of the plasmid DNA using the NucleoSpin® Plasmid Kit. Plasmid DNA was quantified by Quant-iT™ PicoGreen™ dsDNA Assay Kit on the Qubit fluorometer (Invitrogen). Afterwards, we prepared serial dilutions with different concentrations of the plasmid DNA. Each set took a pure common haplotype of either the *ridibundus* or the *lessonae* and seven dilutions in a range from very low to high concentration of the other species rare (minor) haplotype starting from 0.1%, 0.2%, 0.5%, 1%, 5%, 10%, to 20%. We have also added samples with pure *ridibundus* and *lessonae* haplotypes as negative controls. The recovery of the method was tested with three technical replicates for each dilution.

Illumina dual indexing for amplicon sequencing at the MiSeq platform

We used the Illumina's two-step PCR protocol to measure heteroplasmy in somatic tissue and eggs. We designed primers for two loci, RL1 and RL2 that attach to both *ridibundus* and *lessonae* genomes (Table 1). For each sample we had two technical replicates. We added the tailed sequencing primers to the DNA in the first amplification step, while we added the dual indexing in the second round of amplification for easier demultiplexing. We used the KAPA HiFi HotStart PCR Kit (KapaBiosystems) for the first amplification step according to KAPA Biosystems protocol. Thermocycling conditions for the first PCR step included an initial denaturation at 95°C for 3 min, 27 cycles of denaturation at 98°C for 20 s, annealing at 55°C for 15 s and extension at 72°C for 30 s, following the final extension at 72°C for 5 min. The PCR products were cleaned with AMpure XP magnetic beads in a 1:1 bead-to-DNA ratio. The cleaned products were imaged on a 2% agarose gel. The software ImageJ was used to quantify the amount of DNA in each well. Approximately 15 ng of the DNA from the first PCR was used as a template for the second round of PCR, where primers containing indexes and adapters for Illumina flowcells were added. Thermocycling conditions for the second PCR step included an initial denaturation at 95°C for 3 min, 8 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, following the final extension at 72°C for 5 min. To minimize PCR bias and chimeras formation, the thermal cycler was programmed to use a fast ramping rate from denaturation to annealing [49], and a slow (1 degree/s) ramping rate from annealing to extension and extension to denaturation [50] in both PCR steps. Extraction and PCR blanks were included in the procedure for testing for potential contaminants. The products from the second PCR were imaged and quantified on a 2% agarose gel, then diluted to a common concentration and pooled in equimolar concentrations. This pool was cleaned with AMpure XP beads (0.9:1 bead-to-DNA ratio), then quantified by qPCR using a KAPA Illumina Universal qPCR kit.

Table 1 Mitochondrial DNA primers for the *ridibundus* and the *lessonae* genomes

Locus	Primer name	Primer	Sequenced fragment
RL1	RLFOR1	TCAGCACCCCAGTGAGAAC	205bp
RL1	RLREV1	CTTTAATCACGCTTAACGCC	203bp
RL2	RLFOR2	CTCCGCTTACACCGGAC	212bp
RL2	RLREV2	CATGATGCAAAAGGTACGAG	212bp

MiSeq platform read analysis and estimation of heteroplasmy levels

Paired-end sequencing was performed on the Illumina MiSeq using a v3-600 cycle sequencing kit. Since the insert size (242–249 bp) was shorter than the read length (300 bp), each fragment was read full-length in both directions. After sequencing, the sequencing primers and adapters were trimmed automatically, and the first level of demultiplexing was done automatically in the MiSeq apparatus based on the index reads. Quality filtering of the index reads was performed according to https://github.com/edm1/error-aware-demultiplexer and the discarded read IDs were also removed from the corresponding sequencing reads.

Processed reads were used to construct median-joining haplotype networks to assess whether natural or PCR recombination between the two main haplotypes has occurred, and to assess the degree of natural intraspecific sequence variation. For estimating the recovered levels of heteroplasmy, we divided the number of reads of the rare haplotype with the total number of reads of the two haplotypes (1 - (number of reads of the rare haplotype/total number of reads)) and presented this ratio as "heteroplasmy levels". All statistical analyses were performed in R [51].

Simulation for testing patterns of heteroplasmy transmission

Observed heteroplasmy levels found in eggs in relation to their mother were used to examine the possible patterns in the mtDNA heteroplasmy transmission. We implemented a simulation-based approach using the R programming language to test whether observed

heteroplasmy segregation patterns can be attributed solely to the bottleneck that happens during oogenesis [20, 52]. Bottleneck's effect has been confirmed in several species and its size seems to be conserved in distant phylogenetic taxa [21]. The mitochondrial content of each simulated egg was constructed by binomial sampling from the mitochondrial pool of its mother. The number of mtDNA that passed from the mother to the eggs varied between 20 and 10,000. This number represented the strength of the bottleneck. In each simulation we constructed a set of 61 virtual eggs: seven virtual females produced eight virtual eggs each, and one female produced five eggs. This is the number and the distribution of eggs per female in the actual experiment. The eight virtual females that we used as mothers of the simulated eggs contained levels of heteroplasmy equal to the real females. In each set of 61 simulated eggs, we counted the number of heteroplasmic eggs and the average levels of heteroplasmy accepting 5% error. For each bottleneck size we repeated the simulations 1000 times.

Results

Validating the heteroplasmy quantification

We conducted an artificial heteroplasmy experiment to test whether amplicon-based next-generation sequencing can detect very low levels of heteroplasmy (i.e. the ratio of the rare haplotype relative to the common one) and to assess how accurately it can quantify heteroplasmy levels. Initially, we applied the sequencing procedure in samples that included pure cloned haplotypes *lessonae* and *ridibundus* for both loci RL1 and RL2 in order to test for contamination. In none of these samples we retrieved haplotypes other than those expected. Next, we mixed two cloned haplotypes, *lessonae* and *ridibundus*, in a wide range of heteroplasmy levels (ranging from 0.01% to 20% of the rare haplotype relative to the common one) for each of the two mitochondrial loci (RL1 and RL2). We designed two series of dilutions, one where the rare haplotype was the *ridibundus* and one where it was the *lessonae*, and we created four standard curves by plotting the expected heteroplasmy levels against the recovered ones (Fig. 1). Despite the broad range of the initial heteroplasmy levels (0.1–20%), the recovery curves matched

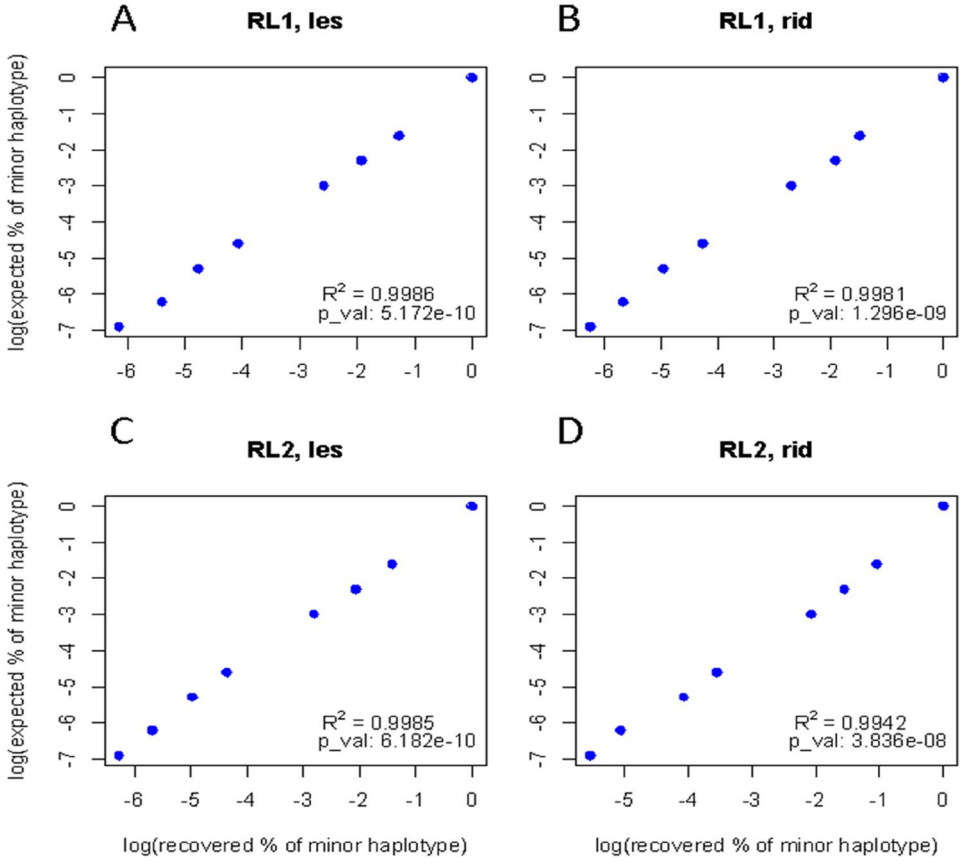

Fig. 1 The recovery curves from the artificial heteroplasmy experiment. The plots show the initial proportion of the rare haplotype (y-axis) versus the recovered proportion of the rare relative to the common haplotype (x-axis) in logarithmic scale. In the first column (**A**, **C**) the rare haplotype is *lessonae* (les) and in the second column the rare haplotype is *ridibundus* (rid) (**B**, **D**). The plots of the first row show the results for the first locus (RL1) (**A**, **B**) and the plots of the second row show the results for the second locus (RL2) (**C**, **D**)

closely the expected data ($R^2 > 0.994$, p-value $< < 0$, Fig. 1) supporting the reliability of our method.

Using the artificial heteroplasmy experiment, we were also able to quantify the error rate resulting from the PCR and sequencing procedures, as the number of reads that differed in sequence from the initial, cloned sequences. We estimated the error rate to be 9.17×10^{-5}, which is lower than the reported error rate for the Illumina MiSeq platform for genomic data (median 0.00473, standard deviation 0.938 [53]). Based on this rate, we developed a likelihood function to calculate the probability that the number of reads of a specific haplotype recovered in each sample were genuine and not technical artifacts. In each sample we only retained haplotypes for which this probability was equal to or less than 0.001. For instance, in individual F120, at locus RL2, we identified 14,957 reads of haplotype R2b (common haplotype), 52 reads of haplotype L2, 22 reads of haplotype R2d, and 4 reads of haplotype R2a. The error estimation excluded R2a from further analysis as the number of reads for this haplotype was lower than the error rate, and thus, these reads could be PCR artifacts or sequencing errors.

Heteroplasmy in males and females

For each of the two mitochondrial loci (RL1 and RL2) and for each of the eight males and eight females, we performed two technical replicates. The number of reads per replicate per locus ranged from 2297 to 16,446 (mean: 6286.1, median: 5835.5). These numbers correspond to the sequencing depth. Heteroplasmy levels between the RL1 and RL2 loci among the 16 individuals were not significantly different (Mann-Whitney U test, p-value = 0.067). This observation indicates that our experimental approach was not biased towards one of the two loci. Therefore, the data for the two loci were combined in the subsequent analyses.

Heteroplasmy levels for each replicate were not correlated with the number of reads (Spearman correlation coefficient 0.06, p-value = 0.5832), meaning that replicates with a higher number of reads were not associated with higher levels of heteroplasmy. This observation suggests that the sequencing depth of our samples was enough to accurately estimate the observed levels of heteroplasmy.

All females and three males were heteroplasmic at both mitochondrial loci (Table 2). Five males were homoplasmic for the RL1 locus and heteroplasmic for the RL2 locus. Each heteroplasmic individual contained two to four different haplotypes, of which one had a frequency greater than 98% and was the common haplotype (Table 2). The recovered haplotypes were compared to the published mtDNAs of *P. ridibundus* and *P. lessonae* (Accession numbers JN627422 and JN627426, respectively). All recovered haplotypes (four for RL1 and five for RL2, Fig. 1S) were grouped either with *lessonae* or with *ridibundus* haplotypes. The *lessonae* group had a single haplotype for each locus (L1 and L2). The *ridibundus*

Table 2 Heteroplasmy in males, females and their eggs. Levels of heteroplasmy per individual (and the standard errors) are presented. Also, the average levels of heteroplasmy among eight eggs per female are shown. The data for the mother and her eggs appear in the same row, which also shows the haplotypes for each mitochondrial locus that were found in each individual or the eggs of each female. The common haplotype is indicated with an asterisk (*)

Adults					Eggs			
individual	Heteroplasmy levels	SE	RL1 locus	RL2 locus	average heteroplasmy	SE	RL1 locus	RL2 locus
F103	0.01207	0.00116	L1*/R1a/R1b	L2*/R2b/R2c	0.00195	0.00067	L1*/R1a/R1b/R1c	L2*/R2a
F104	0.01086	0.00038	L1/R1a*/R1b/R1c	L2/R2a*/R2b	0.00145	0.00013	L1/R1a*/R1b	L2/R2a*/R2b
F118	0.00367	0.00063	L1*/R1a/R1b	L2*/R2a	0.00053	0.00028	L1*/R1a/R1b	L2*/R2a
F119	0.00038	0.00010	L1*/R1a	L2*/R2a	0.00031	0.00009	L1*/R1a/R1b	L2*/R2b
F120	0.00510	0.00010	L1/R1a/R1b*	L2/R2b*/R2d	0.00148	0.00005	R1a/R1b*	L2/R2a/R2b*/R2d
F122	0.00071	0.00029	L1*/R1b	L2*/R2b	0.00013	0.00008	L1*/R1b	L2*/R2b
F124	0.00229	0.00030	L1*/R1b	L2*/R2b	0.00196	0.00025	L1*/R1b	L2*/R2a/R2b
F125	0.00077	0.00011	L1*/R1b	L2*/R2b	0.00056	0.00034	L1*/R1a/R1b	L2*/R2a/R2b
average	0.00448	0.00073			0.00105	0.00012		
M101	0.00106	0.00060	R1a*	R2b/R2c*				
M102	0.00320	0.00072	R1a/R1b*	R2a/R2b*/R2c/R2d				
M109	0.00095	0.00054	R1b*	R2b*/R2d				
M111	0.00148	0.00075	R1b*	R2a/R2b*/R2d				
M112	0.00363	0.00040	R1a*/R1b	R2a*/R2b				
M113	0.00102	0.00059	R1b*	R2b*/R2d				
M114	0.00199	0.00047	R1a/R1b*	R2a/R2b*/R2d				
M115	0.00163	0.00070	R1b*	R2a/R2b*/R2d				
average	0.00187	0.00001						

group was more diverse, containing three haplotypes for RL1 and four for RL2, which differed by one to six nucleotides (Fig. 2SA-B). Each of the nine rare haplotypes was the common haplotype in at least one individual from nature, either in the individuals included in this study (haplotypes L1, R1a, R1b, L2, R2a, R2b, R2c) (Table 2) or in two individuals from other populations that were not included in this study. Specifically, haplotype R1c was found as a common haplotype in a male from Orlovat and R2d was found as a common haplotype in a female from Nis, Serbia. This suggests that the rare haplotypes are not sequencing errors.

All males shared the R1a and R1b *ridibundus* haplotypes and lacked the *lessonae* L1 haplotype, which was present in all females (Table 2). Seven females also contained the R1a and R1b haplotypes. The haplotype R1c was observed only in one female individual (F104) at low frequency (9 reads out of 4735), yet it exceeded the threshold for sequencing error.

The second mitochondrial locus (RL2) contained five haplotypes: one *lessonae* (L2) and four *ridibundus* (R2a, R2b, R2c, R2d). The R2a haplotype appeared as the common haplotype in individuals F104 and M112 (Table 2). Given that these individuals had the R1a haplotype as their common haplotype in the first locus, we can assume that the R1a and R2a haplotypes from both loci represent the same mtDNA haplotype. The R2b haplotype was the common haplotype in individuals F120, M102, M109, M111, M113, M114, and M115 (Table 2). These individuals had the R1b haplotype as their common haplotype in the first locus. Therefore, R1b and R2b represent the same haplotype. Haplotype R2d is a variant of R2b, differing in a single indel (Fig. 2S). It was found at a low frequency in individuals F120, M102, M109, M111, M113, M114, and M115 (Table 2), but it was the common haplotype in a male individual *P. esculentus* from Nis (Serbia). Therefore, we can link R2d with R1b haplotype. Finally, haplotype R2c was the common haplotype in M101 and appeared in other individuals in low frequency (Table 2). It differed from R2b in one nucleotide and from R2a in two nucleotides (Fig. 2S). In M101, the common haplotype for the first locus was R1a, which is linked with R2a in other individuals. Therefore, R2c can be considered as a variant of R2a. The L2 *lessonae* haplotype was present in all females and was the common haplotype in six of them, but was absent from males (Fig. 2S-C). On average, the levels of heteroplasmy in the 16 individuals were 0.0031 (± 0.00054), ranging between 0.0003 (± 0.00009) in F119 to 0.012 (± 0.00116) in F103 (Fig. 2).

Males and females had similar levels of heteroplasmy (Mann-Whitney U test, $p = 0.573$), but they differed in their common haplotype (Mann-Whitney U test, $p = 0.003$). The *lessonae* haplotype was the common haplotype in six females (Table 2), while in males, the *lessonae* haplotype was absent. All females were heteroplasmic for two to four haplotypes, whereas five males appeared homoplasmic for RL1, and three contained two *ridibundus* haplotypes. For RL2, all males were heteroplasmic for two to four *ridibundus* haplotypes. This qualitative difference (presence/absence of heteroplasmy) between the two mtDNA loci suggests that when heteroplasmy is at such low levels, the rare allele(s) might not be revealed due to the stochasticity of the amplification process and sequencing methods.

Heteroplasmy in eggs

Forty-six out of 61 eggs were found to be heteroplasmic in at least one locus, with an average overall level of heteroplasmy of 0.00105 ± 0.00012 (Table 2). The average heteroplasmy levels for each mother's eight eggs ranged from 0.00013 ± 0.00008 for female F122 to 0.00196 ± 0.00025 for female F124. The levels of heteroplasmy in the eggs were significantly lower than those in the mothers (Wilcoxon paired test, $p = 0.007$), indicating the presence of purifying selection. There was no significant correlation between the levels of heteroplasmy of the mothers and their eggs ($p = 0.163$), meaning that higher levels of heteroplasmy in a mother did not result in higher heteroplasmy levels in her eggs. All heteroplasmic eggs shared the same common haplotype with their mother. The number of different haplotypes observed in eggs did not always match those observed in their mothers.

For instance, at locus RL1, female F104 had the R1c haplotype and female F120 possessed the haplotype L1, which were not present in their eggs. At locus RL2, female F103 possessed the haplotypes R2b and R2c that were not present in her eggs. Similarly, female F119 had the haplotype R2a, which was not present in her eggs. On the other hand, some haplotypes appeared in the eggs but not in their mothers. For example, at locus RL1, the eggs from females F103, F119, and F125 contained the haplotypes R1c, R1b, and R1a, respectively, which were not detected in their mothers.

The observed heteroplasmy is not a technical error

The low levels of heteroplasmy observed in both adults and eggs (below 2%, Table 2) make it essential to exclude the possibility that our findings were due to contamination or sequencing errors. We can rule out the possibility of contamination because the experiments involving tissues and eggs were conducted at distinct locations and times, yet yielded similar levels of heteroplasmy. Also, we took all the necessary precautions to avoid contamination. Additionally, if contamination were the cause, one would expect a random pattern of heteroplasmy rather than a clear distinction between sexes and between mothers and eggs (for heteroplasmy levels). Most importantly,

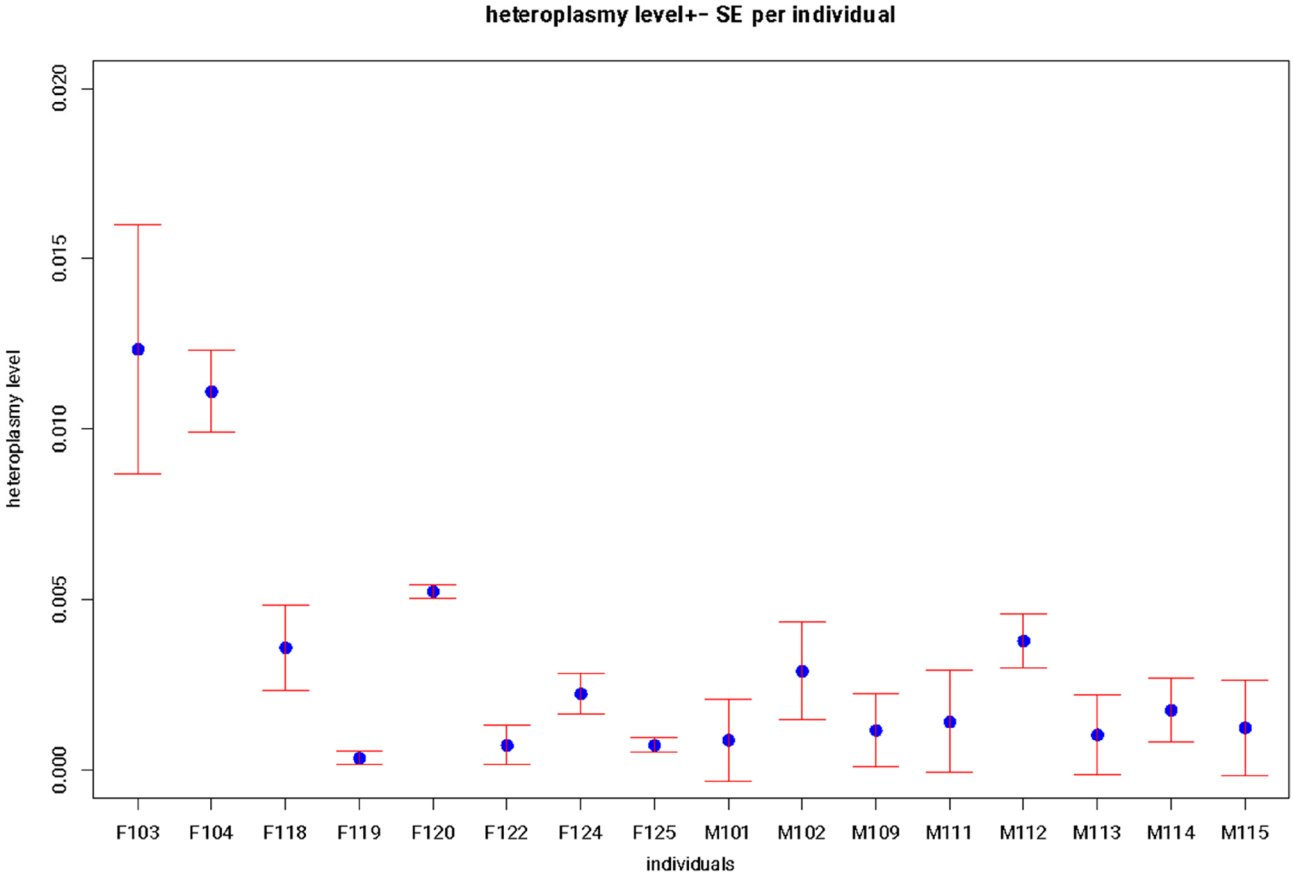

Fig. 2 Histogram of heteroplasmy levels (proportion of the rare haplotype relative to the common one) in eight females (the first eight points) and eight males (the following points) from the Pancevo natural population of water frogs. The vertical bar shows the standard error, calculated from the technical replicates. On the x-axis are shown the names of the individuals

we used the male from Orlovat as a negative control as it appeared homoplasmic in the PCR. After sequencing, this individual remained homoplasmic for the haplotype R1c for RL1 and R2e for RL2.

The observed heteroplasmy cannot be the result of sequencing error because each of the rare haplotypes that we observed was a common haplotype in another individual and therefore it cannot have been created by error. The sequencing process produced erroneous haplotypes, which formed a star phylogeny around the real haplotypes (rare and common ones). Using the artificial heteroplasmy experiment, we quantified sequencing error and we excluded from further analyses the haplotypes below the error threshold.

When studying heteroplasmy, one has to exclude the possibility that the observed haplotypes are mtDNA parts that have been translocated to the nucleus (NUMTs) [54]. Several observations indicate that the heteroplasmy we have observed is not due to NUMTs. First, different rare haplotypes appear in different individuals (Table 2). This would happen only in the case that all rare haplotypes were different NUMTs that have been transferred to the nucleus recently (because none of them is fixed), as a wave (all together) and only once (all rare haplotypes are identical to the common ones). This sounds very unlike.

Second, while the main haplotype of the mother is the main haplotype of the eggs, this does not always happen for the rare haplotypes (see the last two paragraphs in "heteroplasmy in eggs" above, and Table 2). Given that the eggs share the nuclear DNA with their mother, at least the haplotypes of the eggs (which have fewer copies of nDNA in the extracted DNA pool than their mother) should appear in the mother's nDNA. Instead, the pattern we observed is expected if the rare haplotypes are true mtDNAs with low but variable frequencies, which sometimes can escape the random amplification of the PCR.

Third, the presence of the *lessonae* haplotype in females but not in males cannot be attributed to NUMT located in sex chromosomes basically because it is the common haplotype in most females and therefore it must be real mtDNA. Also, the sex determination system in *Pelophylax esculentus* (previously known as *Rana esculenta*) is XX/XY [55]. In case that the *lessonae* haplotype was an autosome or an X-linked NUMT, it should appear in both males and females. In case that it was a Y chromosome

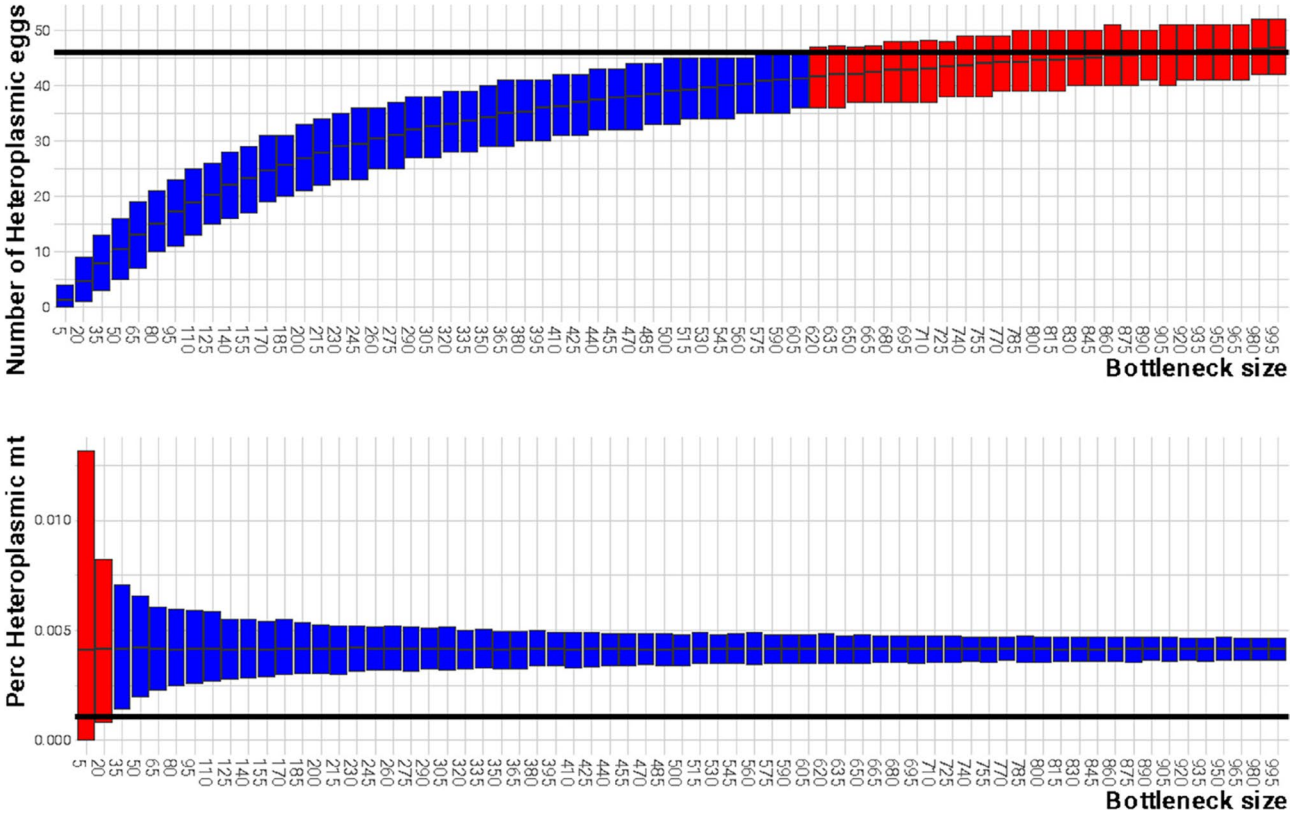

Fig. 3 Simulations of the transmission of heteroplasmy from mothers to eggs assuming a bottleneck during oogenesis. The colored boxes contain the 95th quantile of heteroplasmic eggs. Upper part: the number of heteroplasmic eggs in the set of 61 eggs, per bottleneck size. The black line indicates the actual number of heteroplasmic eggs [46]. The color of the boxes changes to red in the bottleneck sizes that include the 46 eggs. Lower part: Levels of heteroplasmy in each bottleneck size in the simulated eggs. The black line represents the actual average level of heteroplasmy. The red boxes indicate the bottleneck sizes, which include the actual heteroplasmy level

linked NUMT, it should appear only in males. Yet, it only appears in females.

Finally, the observation that 15 eggs were homoplasmic suggests that the rare haplotypes are real mtDNA because otherwise all eggs should have the rare haplotypes as they all have nuclear DNA. It also agrees with the existence of a bottleneck in the mtDNA during oogenesis, where drift dictates the distribution of the haplotypes in the eggs.

Together these observations strongly suggest that the observed haplotypes are not a technical artifact or NUMTs but are real mtDNA haplotypes.

The effect of genetic bottleneck size in heteroplasmy inheritance

We conducted simulations to examine the role of drift on the transmission of heteroplasmy from mothers to eggs, assuming a genetic bottleneck during oogenesis, which results in a reduction in the number of randomly distributed mtDNA variants. In our dataset, 46 out of 61 eggs were heteroplasmic, for which the average level of heteroplasmy was 0.0010453. To test whether these numbers can be explained by the genetic bottleneck alone, we simulated 1000 sets of 61 eggs that originated from eight mothers, which had the same level of heteroplasmy as the mothers in our data. For example, let us suppose that each of the 8 females had x1, x2,…x8 levels of heteroplasmy. We assumed a bottleneck with size y (y was the number of mtDNAs that passed from the mother to each of her eggs). We took a sample of 8 eggs per mother (apart from one from which we took 5 eggs), reconstructing a simulated set of 61 eggs (which was the number of our actual set). In this set we counted the number of heteroplasmic eggs and the average levels of heteroplasmy. For each bottleneck size we identified the 5% upper and lower limit for the number and the average heteroplasmy of the eggs, which represents the effect of the bottleneck alone in the distribution of heteroplasmy. We found that we needed a bottleneck size of at least 600 mtDNA molecules in order to obtain 46 heteroplasmic eggs out of 61 (Fig. 3, upper part). On the other hand, we needed a much stricter bottleneck (size of 20) in order to achieve the actual levels of heteroplasmy (i.e., 0.0010453) in the simulated sets (Fig. 3, lower part). In fact, we could achieve the actual heteroplasmy levels only because genetic drift increases the variance without altering the

mean, and in stricter bottlenecks the variance is higher and thus includes the actual levels of heteroplasmy.

Discussion

The current understanding of heteroplasmy in natural populations is that it occurs sporadically across individuals and that there is high variance in its levels within individuals (ranging from less than 1% to more than 20% - see, for example, [7, 17]). The heteroplasmy we observed in frogs has three major characteristics. First, it is widespread in the population. All the individuals studied in the Pancevo population from which we took our sample, were heteroplasmic [44]. Indeed, many hybridogenic populations within the genus *Pelophylax* appear heteroplasmic [44, 56]. This phenomenon could be an unstudied side-characteristic of the hybridogenic system or because *P. esculentus* is a hybrid species and heteroplasmy is common in hybrids. There is ample evidence in natural and laboratory populations that heteroplasmy is more common in hybrids [36, 44, 57–61]. Second, most individuals have more than two haplotypes, which is uncommon but not absent from natural populations [62]. Third, there is a single very common haplotype (with a frequency of more than 98%) within each individual, and the rest are very rare, as has been observed in other organisms [7, 17, 63]. Each of these characteristics has been previously observed elsewhere, but it is the first time that all three appear in a single species.

Hybridization and the hybridogenic system of reproduction in frogs could explain the observed pattern of heteroplasmy, assuming recurrent paternal leakage and maternal transmission. According to the hybridogenic system, individuals of the hybrid *P. esculentus* (RL) coexist with those of the paternal *P. ridibundus* species (RR). During gametogenesis, the *esculentus* individuals discard the R genome and maintain the L genome in their gametes, which is clonally transmitted [39]. A plausible scenario would have as a first step the initial cross between a pure *lessonae* female (LL) and a *ridibundus* male (RR). All hybrids (RL) would contain the maternal mtDNA (L). Second, the *esculentus* hybrid (RL) would produce eggs that would carry the *lessonae* nuclear genome (L) and the mtDNA content of their mother, which would be the predominant L mtDNA of their grandmother (Lm1), and perhaps the leaked R mtDNA from their grandfather (Rm1). Third, in the next generation, the females would produce eggs with an L nuclear genome but would be heteroplasmic for Lm1/Rm1 mitochondria. These eggs would be fertilized by an R sperm from the pure RR population. If the RR population was mitochondrially variable, the sperm could contain a second haplotype, Rm2. If there was paternal leakage, then the mtDNA pool of the offspring would be Lm1/Rm1/Rm2. Repeating cycles of paternal leakage and heteroplasmy transmission through females could supply individuals with all the different *ridibundus* haplotypes that exist in the pure RR population.

This scenario has two weaknesses. Due to the size and behavior of the two species, it has been suggested in the literature that the primary cross of the first step should have occurred between a female *ridibundus* and a male *lessonae* [41], rather than the opposite as we suggest here. Also, our model cannot easily explain the absence of the *lessonae* haplotype from males and its persistence as the common haplotype in most females. For both of these reasons, it would not be surprising if our population is a peculiarity of the hybridogenic system, which appears to be extremely variable [43] and not fully understood. However, the R-E system that we propose in this model (i.e., a *ridibundus* individual mates with an *esculentus* one) has been observed in nature [64] and has several interesting exceptions, such as a few XY females and a sex ratio bias towards females [41].

The levels of heteroplasmy observed in all adult frogs are comparable to those observed in natural populations of Drosophila, which were estimated to be 0.8% [65], but only 6% of individuals were heteroplasmic, and in humans [66]. In another study in *Drosophila simulans,* this proportion was 0.66% [67], while in the Pancevo frogs' population, all individuals were heteroplasmic [44]. Higher variation in heteroplasmy levels has been observed in humans and mice, ranging from less than 1% to more than 20% [17]. Recently, stable heteroplasmy was observed in the ambrosia beetle, with levels ranging between 0.25 - 0.75 [68].

In some cases, we observed that a haplotype was present in the eggs but absent in the mother or vice versa. For example, eggs from F103, F120, F124, and F125 contained the haplotype R2a at locus RL2, which was not present in their mothers. There are two, not mutually exclusive, possible explanations for this observation. The first is that different haplotypes are not equally distributed across the tissues of females. This has been observed in other species [69–71]. Therefore, the tongue, which was used to detect heteroplasmy in mothers, might sometimes contain different haplotypes than the gonads, particularly those haplotypes that are in lower frequency. The second explanation is that during oogenesis, there is random sampling of the mothers' mitochondria, and haplotypes that exist in low frequency -lower than the detection limit of our method- in the mother, appear in detectable frequencies in the eggs. This is consistent with the hypothesis of a genetic bottleneck in mitochondria during oogenesis, which has been observed in several organisms [18, 19, 21].

The heteroplasmy levels that we observed in the eggs were significantly lower compared to their mothers, indicating the presence of purifying selection during

oogenesis. Selective suppression of heteroplasmy levels has been previously observed in mammals [27, 30, 32, 72] and in *Drosophila* [26, 31], where selection acted in embryos or in adults rather than in unfertilized eggs. Our simulations showed that the combination of the number of heteroplasmic eggs with the observed levels of heteroplasmy cannot be explained by bottleneck alone, but selection should also be involved. Two scenarios could explain the non-random transmission of heteroplasmy in the eggs.

The first scenario proposes a severe bottleneck during oogenesis, similar to what has been observed in other animals. This bottleneck would result in the observed levels of heteroplasmy in the eggs, but positive selection would be needed to increase the number of heteroplasmic eggs. This idea is supported by several studies that demonstrate a severe bottleneck during oogenesis [16–19] and by the observation of adaptive heteroplasmy in *Drosophila* [31]. The second scenario proposes a relaxed bottleneck, allowing the observed number of heteroplasmic eggs, but purifying selection would act to reduce the average levels of heteroplasmy in the eggs.

Selection on heteroplasmy has been reported so far to act against haplotypes carrying deleterious mutations or favoring beneficial mutations of mtDNA [24]. There is mounting evidence that mitochondria carrying defective mtDNA mutations are removed during the mtDNA bottleneck phenomenon in oogenesis and that process takes place in the Balbiani body [73, 74]. However, since each of the haplotypes observed in this study is common in some individuals, there is no evidence that they contain very harmful mutations. Thus, one available explanation is that selection acts on heteroplasmy levels themselves rather than against deleterious haplotypes. This type of selection may be associated with mito-nuclear interactions. It has been shown that mtDNAs interact differently with different nuclear genomes [75]. But also specific combinations of distinct mtDNA haplotypes (i.e., heteroplasmy), rather than single haplotypes, could be more beneficial in a specific nuclear background than others; then this is expected to affect the levels of heteroplasmy. Another possible explanation could be that low levels of heteroplasmy might be beneficial since it would enable interlineage recombination without jeopardizing maternal transmission of mtDNA [76]. While a theoretical study has suggested that heteroplasmy could be an evolvable trait [77] and an experimental study has shown stable heteroplasmy in *C. elegans* [78], two empirical studies have reported severe fitness reduction resulting from the coexistence of two non-detrimental haplotypes in mice [33, 34].

Although selection appears to be involved in the transmission of heteroplasmy levels from mothers to their eggs, we cannot rule out a non-selective explanation for the non-random distribution of haplotypes between males and females. Such an explanation could be a peculiar feature of the hybridogenic system in frogs or an unknown process that occurs in hybrids.

Conclusions

We have used a quantitative approach to characterize the extensive heteroplasmy that was previously qualitatively observed in the hybrid frog *P. esculentus*. We report that each adult individual has a single, very common haplotype and a few very rare haplotypes and that there is unequal distribution of the haplotypes between sexes. The unfertilized eggs have, on average, lower levels of heteroplasmy than their mother. We know that a bottleneck takes place in the mtDNA during the egg formation in many organisms. This bottleneck increases the effect of genetic drift by reducing the number of mtDNA that passes from the primordial germ cells to the eggs. Under the bottleneck, a rare haplotype has a lower probability of passing into eggs compared to a common one. Assuming a bottleneck, the heteroplasmy levels and the number of heteroplasmic eggs are expected to decrease with the increase of the strength of the bottleneck. With simulations we could quantify the combination of the number of heteroplasmic eggs and the level of heteroplasmy in those eggs that we would expect under specific sizes of bottleneck. These two numbers (i.e., the levels of heteroplasmy and the number of heteroplasmic eggs) do not agree in our data, suggesting that the bottleneck alone (i.e., the genetic drift that follows) cannot explain the observed patterns of heteroplasmy, implying the involvement of selection.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

E.D.L. and J.R. designed the experiments. J.B.K. performed the next generation sequencing experiment and the bioinformatic processing of the sequences. J.R. and E.-S.P. performed the experiments. P.P. performed the simulations experiments. E.D.L., J.R. and P.P. wrote the paper.

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Data availability

All primary amplicon sequences for this study have been deposited in the SRA database (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1223815).

Declarations

Ethics approval and consent to participate

The tissues and the DNA used for this study come from the previous study by Radojicic et al. 2015 (ref. [44]) in which samples come from herpetological collections from the Institute of Zoology, Faculty of Biology, University of Belgrade, Serbia. There was no primary collection of animals for this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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