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Female-dependent transmission of paternal mtDNA is a shared feature of bivalve species with doubly uniparental inheritance (DUI) of mitochondrial DNA

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Abstract

Several species from a number of bivalve molluscan families are known to have a paternally transmitted mitochondrial genome, along with the standard maternally transmitted one. The main characteristic of the phenomenon, known as doubly uniparental inheritance (DUI), is the coupling of sex and mtDNA inheritance: males receive both genomes but transmit only the paternal to their progeny; females do not have the paternal genome or they have it as a minority and do not transmit it to their progeny. In the families Mytilidae and Veneridae, both of which have DUI, a female individual is either female-biased (it produces only, or nearly so, female progeny), male-biased (it produces mainly male progeny) or non-biased (it produces both genders in intermediate frequencies). Here we present evidence for a same pattern in the fresh water mussel, *Unio delphinus* (Unionidae). These results suggest that the maternal control of whether a fertilized egg will develop into a male or a female individual (and the associated feature of whether it will inherited or not inherit the paternal mtDNA) is a general characteristic of species with DUI.

Introduction

Several species of molluscan bivalves have a peculiar mode of mitochondrial DNA transmission, known as doubly uniparental inheritance (DUI). In these species two mitochondrial genomes coexist in stable state. One of these genomes, the maternal or F (from female), is transmitted through the female lineage and the other, the paternal or M (from male), through the male lineage (Skibinski *et al.*, 1994a, b; Zouros *et al.*, 1994a, b). It is now known that DUI has a broad taxonomic distribution across bivalves (Theologidis *et al.*, 2008; Doucet-Beaupre *et al.*, 2010). In species with DUI, females are basically homoplasmic for the F genome and males are obligatory heteroplasmic, with their somatic tissues dominated by the F genome and their gonad dominated by the M genome. This tissue distribution of the two genomes is "leaky":small amounts of the M genome can be found in the somatic tissues of female and male individuals or even in eggs. Also, the F genome is usually found in DNA preparations from male gonads, most likely due to contamination from somatic cells (Garrido-Ramos *et al.*, 1998; Dalziel & Stewart, 2002; Obata *et al.*, 2006; Passamonti & Ghiselli, 2009). But no F genome has been found in pure extracts from sperm (Venetis *et al.*, 2006; Ghiselli *et al.*, 2011).

Within the same species, the divergence of the F and M genomes can be considerable. In Mytilidae, one of the most extensively studied families, this divergence varies from 23% for species of the *Mytilus edulis* complex (*M. edulis, M. galloprovincialis, M. trossulus*) to 40% for *Mytilus californianus*, but gene content and gene order are remarkably similar (Mizi *et al.*, 2005; Ort & Pogson, 2007). In the family Unionidae the divergence in primary sequence exceeds 50% (Doucet-Beaupre *et al.*, 2010) and there are also other notable differences, such as a 185-codon extension in the

cytochrome c oxydase II locus in the M genome (Curole & Kocher, 2005). The large sequence divergence between F and M genomes has, in fact, been used as a detection assay of DUI: when two highly divergent mtDNA sequences can be found in males of one species but not in females, it is taken as evidence that the species has the DUI mechanism of mtDNA transmission.

In pair-matings of species of the *Mytilus edulis* complex it has been observed that the sex ratio among progeny may deviate dramatically from the 1:1 ratio. More specifically, a female's brood can consist almost exclusively of females, be dominated by males or have the two sexes in intermediate ratios. This property depends entirely on the female parent, as evidenced from the fact that dams crossed to different sires produce broods with the same sex ratio (Saavedra *et al.*, 1997), and is apparently controlled by nuclear genes (Kenchington *et al.*, 2002). Direct evidence that the bias in sex ratio is independent from the male parent exists only for the mytilids. However, mother-dependent sex-bias has been also demonstrated in the venerid *Ruditapes philippinarum* (Ghiselli et al., 2012).

Unionids represent the third group of species in which DUI has been extensively studied (Hoeh *et al.*, 2002; Curole, 2004; Soroka, 2008). Here we present evidence that the female-dependent sex-bias occurs in the unionid *Unio delphinus*. We have taken advantage of the fact that in fresh water mussels of the family Unionidae males release sperm in the water, which then is captured by downstream gravid females. Fertilization occurs in the interlamellar spaces of the female's external gills, where the resulting embryos develop into "glochidia". Glochidia are later released into the water and attach to the gills or fins of the appropriate host fish before metamorphosing into

juveniles. This system of reproduction assures that all glochidia carried by a female are her own progeny, even though they might have been fertilized by sperm from more than one male. If we could score the mtDNA content of a number of glochidia from single females, we would expect that glochidia from some females will be in their vast majority homoplasmic (they will contain only the F mtDNA type), glochidia from some other females will be in their majority heteroplasmic (they will contain the F and the M mtDNA types), and glochidia from still other females will be homoplasmic and heteroplasmic in intermediate numbers. The first type of mothers will correspond to the female-biased type, the second to the male-biased and the third to the intermediate sex-ratio mothers that are well defined in mytilids and venerids.

Materials and Methods

Species used and DNA extraction

Fifteen specimens belonging to *Unio delphinus*, a species that occurs in the Atlantic rivers of the Iberian Peninsula and of North Africa, were collected from different rivers of Morocco. This species is gonochoristic. Females are distinguished macroscopically by the presence of eggs in the two external demibranchs, unlike males which have a voluminous whitish gonad. Sex of adults was determined by eye examination and only individuals with mature gonads were included in the study. This restricted the number of animals used to seven females and two males. A small piece of foot from adult males and females, and of gonad from adult males, was excised and preserved in absolute ethanol. Glochidia were obtained from excised marsupial gills of gravid females, stored initially at -80ºC and, then, transferred and preserved in absolute ethanol. Single glochidia were isolated with the help of a Pasteur pipette under the stereoscope and placed in a 1.5 ml tube with lysis buffer and proteinase K for DNA extraction. DNA from adult tissues, from batches of glochidia and from single glochidia was extracted following the protocol recommended by ChargeSwitch gDNA Tissue Kit (Invitrogen).

PCR amplifications

Amplification and detection of the female mitotype (the F genome), which is expected to be present in all preparations, was performed in all the samples as a control of DNA quality and PCR amplification. We first used the primers COI-H (Machordom *et al.*, 2003) and COI-L (Folmer *et al.*, 1994). This pair of primers amplified a 658-bp fragment of the *cox1* gene from all adult tissues of both sexes. The PCR product from

foot and gonads was purified and sequenced. The Sequencer 4. 6. software (Gene Codes Corp.) was used for the alignment and comparison of *cox1* sequences. Sequences that were different in the F and M genomes, yet conserved within each genome, were used to design F or M specific primers. As F-specific primers we used the primer pair COIF_Unio 5′-GTTTTGGTTGCTTGTGCCAG-3′ and COIR_Unio 5′- AAGATTTCGATCCGTAAGTAG-3′, which gives a 322-bp product. The following primers were used for the detection of the M genome: FW1 5′-GTCTTTAAGGGTTATTATTCGG-3′, RV1 5′- RAATCTCACATTATTYAA-3′ and COIM-F 5′- CAATGAGCAGCTTTACTATTC-3′. The pair FW1/RV1 amplifies a fragment of 194 bp and the pair COIM-F/RV1 amplifies a 142 bp fragment from the *cox1* gene. As the M genome dominates the male gonad but is rarely found in other tissues, we used total DNA from male gonads as a positive control for the M specific primers. The M genome, on the other hand, is effectively absent from female and male somatic tissues, thus we used total DNA from the foot of females as a negative control of M specific primers.

PCR reactions were carried out in a total volume of 12.5 µl, each containing 2 to 6 µl of genomic DNA, 600 nM of each primer, 0.16 mM of dNTP mix, 2.5 mM of $MgCl_2$, 2.5 µl PCR 5x buffer and 1 U of GoTaq® Polymerase (Promega). The concentration of DNA was optimized to obtain the best results. The reactions were performed under the following conditions: 94ºC for 5 min, 35 cycles of 94ºC for 45 sec, 45ºC for 1 min, 72ºC for 1 min and 30 sec and a final extension for 10 min at 72ºC. PCR products were detected after electrophoresis in 1.5% agarose gels after staining with SBYR Safe (Invitrogen).

Sex assignment to glochidia

There is, at present, no direct way for detecting the sex of a glochidium. Thus we used an indirect way for sex determination, based on the observation that presence of the M genome is tightly associated with maleness in all bivalve families in which DUI has been detected, including Unionidae. For each glochidium we used three different primer pairs: the F-specific pair COIF-Unio/COIR-Unio, the M-specific pair FW1/RV1 and the M-specific pair COIM/RV1. The F-specific primers were used as a control for DNA quality. For each pair of primers we performed three PCR amplifications. A glochidium that did not produce a PCR product with the F-specific primers was excluded from further analysis. A glochidium was classified as "male" if it was positive in all three reactions with one or the other M-specific primer pair (or both), or if it was positive in two reactions with one primer pair and in at least one reaction with the other primer pair. A glochidium was classified as "female" if it was negative for all three reactions for both primer pairs or positive only in one reaction for one or the other primer pair. The remaining three cases, when the reaction was positive for two of the three reactions with one M-specific primer pair but negative in all three for the other M-specific primer pair or was positive in only one reaction for both primer pairs, were declared as doubtful. Doubtful cases were subjected to further examination by performing three more PCR reactions for each pair of M-specific primers. This gave us 3+3 tests for each male-specific pair and 12 tests for both male-specific pairs. If more than six of the twelve reactions were positive, the glochidium was classified as a "male". Otherwise it was classified as a "female".

Chi-square tests for deviation from the 1:1 sex ratio among glochidia from a female parent and for homogeneity of sex ratio among glochidia from different female

parents were implemented with the EXCEL software program or by Fisher's exact test. The binomial distribution was used to place standard errors for the frequency of females or males among glochidia from the same female.

Results and Discussion

We examined the mtDNA content of the foot of one male and of two females and of the gonad of two males of *Unio delphinus*. We recovered only the F mtDNA type in the foot of females and both the F and the M types in the foot and the gonad of males (Figure 1). These results are in agreement with what we expect from a species with the DUI mode of mtDNA transmission. As stated in the Introduction, the landmark of DUI is that females are virtually homoplasmic for the maternal mtDNA type (the F type), however the M genome can be occasionally found in their somatic tissues (Kyriakou *et al.*, 2010; Batista *et al.*, 2011; Ghiselli *et al.*, 2011; Obata *et al.*, 2011). The somatic tissues of males are also dominated by the F mtDNA type, but presence of the M type in somatic tissues is more common than in females (Kyriakou *et al.*, 2010; Batista *et al.*, 2011; Ghiselli *et al.*, 2011; Obata *et al.*, 2011). In the male gonad, in contrast, the F genome occurs only in cells of somatic origin and the M is the dominant mtDNA in the germ cell line - and the exclusive one in the mature sperm (Venetis *et al.*, 2006). We may confidently conclude that *Unio delphinus* has the DUI system, a conclusion consistent with the fact that DUI was observed in all Unionidae species that were examined for this purpose (Walker *et al.*, 2006; Doucet-Beaupre *et al.*, 2010) and could, therefore, be used to examine whether among mothers there is a strong difference in the proportion of progeny with the paternal mtDNA genome, and by extension, in sex-ratio.

We aimed at characterizing the mtDNA content of 25 glochidia from each of seven gravid *Unio delphinus* females that could be examined for this purpose. An example of scoring PCR products is shown in Figure 2. Our scheme of characterizing the sex of a glochidium on the presence or absence of the M genome (see Materials and

Methods) produced the following results (Table 1). In five mothers there was an excess of glochidia exhibiting the mtDNA pattern that corresponds to the female gender. In one of these mothers no progeny could be characterized as a male. In the other four, the frequency of glochidia that could be characterized as males ranged from 12% to 32%, and the null hypothesis of equal ratio between female to male glochidia could be rejected in all of them, except in one in which the probability for the null hypothesis is between 5% and 10%. The sex ratio bias among these five female-biased mothers varies significantly (the chi-square value from the test of homogeneity is 11.364 on four degrees of freedom, P = 0.023).

In two mothers the sex ratio was in favour of males. One of these mothers was daughterless and in the other the observed frequency of female glochidia was 32% (Table 1). Again, the bias is different between the two male-biased mothers (P = 0.002 from Fisher's exact test for homogeneity) and the upper 95% confidence limit for the frequency of daughters between the two mothers is 14% in the first and 50% in the other.

If we ignored the heterogeneity among mothers, we may calculate an average frequency of 0.176 (SE = 0.034) of sons among the five female-biased mothers and 0.16 (SE = 0.052) of daughters between the two male-biased mothers. If the seven mothers were assumed to represent a random sample of females from the population from which they were derived, the overall sex ratio among glochidia will be significantly in favour of females (frequency of female glochidia 0.63, chi-square test for the null hypothesis of equal sex ratio 12.623 on one degree of freedom, P = 0.0004). This would not be a justified conclusion given the small number of mothers

we examined and the large variation in sex ratio among mothers. In addition, the number of female-biased versus male-biased mothers is statistically not different from 1:1 (the probability that the 5:2 split is different from 1:1 is 0.226).

It is worth comparing these results with the results that Kenchington *et al*. (2002) obtained for females from a natural population of *Mytilus edulis*, the only comparable study available in the literature. Of the 31 *M. edulis* mothers listed (Kenchington *et al.*, 2002), 13 were female-biased, 15 were male-biased and in 3 the sex-ratio was not different from 1:1. This distribution is not different from the one we report here for *Unio delphinus* (5 female-biased, 2 male-biased, 0 non-biased; chi-square 2.227, d.f.= 2, P= 0.328). The mean sex-ratio among female-biased mothers of *M. edulis* was 97% in favour of daughters, (compared to 82% in *Unio delphinus*) and the among-mothers heterogeneity was significant, with three mothers producing 88% daughters on average and the remaining being totally sonless. The mean sex-ratio among male-biased mothers was 82% in favour of sons (compared to 84% in *Unio delphinus*). The heterogeneity of sex ratio among these mothers varied from 66% to 100% in favour of sons. These figures are broadly comparable with the results from *Unio delphinus* reported here for the presence or absence of the M genome in glochidia. Given that the almost perfect association between M genome inheritance and maleness that occurs in Mytilidae is also known to occur in many unionid species (Hoeh *et al.*, 2002; Curole, 2004; Breton *et al.*, 2011) we may conclude that the unionid females may also be of three types, female-biased, male-biased and unbiased.

It must be noted that in the *M. edulis* study, progeny were scored directly for sex by observing the presence of sperm or eggs and for presence or absence of the M genome

by PCR amplification. Because glochidia cannot be sexed, the gender of a glochidium was in this study inferred from the presence of the M mitochondrial genome. This appears to be in fact the only way that our question – whether there is a sex-bias in cohorts from single mothers – can be answered in a unionid species, where it is practically impossible to raise single-mother families. The inference of maleness from the presence/absence of the M genome has, however, several difficulties. One of these relates to the high mutation rate of the M genome (see, for example, Stewart *et al.*, 1996; Ort & Pogson, 2007). If one of the unknown male parents of our single-mother cohorts of glochidia carried a mutation that obliterated the recognition site of one M-specific primer, male glochidia fathered by this male will be scored as lacking the M genome and will be miss-classified as females. This was in fact the case in a study of pair-matings of *Mytilus galloprovincialis* in which all male progeny of a specific male were characterized as lacking the M genome on the basis of one specific primer-pair (Saavedra *et al.*, 1997). The same progeny were found positive for the M genome when a different set of primers was used (Theologidis *et al.*, 2007). To minimize this possibility of false negatives we have employed two M-specific primer pairs with a different forward primer. This eliminates this likelihood for the forward binding site, but leaves a small probability that it may happen with the reverse site.

Another reason is that the glochidium is an immature stage in which the gonad represents a small part of the animal's total cell population. Consequently, the amount of the M genome in the mtDNA pool of a male glochidium is small compared to the amount of the F genome. As a result, failure to detect the M genome in a glochidium may not necessarily imply the glochidium is a female (another cause of false negatives). This problem can be serious when the same pair of primers is being used

for amplification of both the F and the M genomes and the products are recognized by their different restriction fragment length profiles (the RFLP assay) (Saavedra *et al.*, 1997). In such cases the more abundant F genome may out-compete the less frequent M genome, with the result that the M product may not be detectable with the RFLP assay. In our PCR reactions we used separate primers for amplifying the F and the M genomes. In addition, we did multiple PCR reactions for the same glochidium and doubled the number of these reactions in cases the first set of reactions did not produce a clear result.

Another possibility for a false negative scoring is masculinization, the reversal of transmission route an F genome from maternal to paternal (for a review a see Zouros, 2013). A male glochidium with a masculinized paternal genome (i.e. a genome whose coding sequence is similar to the F genome) could be erroneously scored as homoplasmic for the maternal genome and, thus, as a female. There is good evidence that masculinization does not occur, or is extremely rare, in unionids (Hoeh *et al.*, 2002; Theologidis *et al.*, 2008), therefore the possibility that masculinization may affect our results is negligible.

Our scheme of sex assignment to a glochidium on the presence or absence of the M genome was designed to reduce the possibility of error, but obviously it is not error-proof. The probability of miss-assignment should, however, be random and would not be expected to cause a systematic bias in favour of one or the other gender among different mothers. The very pronounced differences that we have seen among mothers cannot, therefore, be attributed to difficulties with scoring.

A model for the coupling of maleness with the presence of the paternally transmitted mitochondrial genome was proposed by (Zouros, 2000), Kenchington et al. (2009) and Zouros (2013). The results we report here do not imply that the molecular machinery that controls transmission of the paternal mtDNA and sex determination is identical in sea mussels, clams and fresh water mussels in all its details. They do, however, reinforce the hypothesis of a single and very old origin of DUI that has conserved its basic characteristics for at least 400 million years.

Titles and legends to figures

Figure 1. PCR products from total DNA extracted from female and male foot (F) and male gonad (M). A) Amplification by the F-specific primers COIF_Unio/COIR_Unio, B) Amplification by the first pair of M-specific primers COIMF/RV1, C) Amplification by the second pair of M-specific primers FW1/RV1. L: 100 bp ladder. The lower band (size between 100-200bp) appearing in the foot (panel C) is probably a product from the F genome.

Figure 2. PCR products from nine glochidia from female N1318. A) Amplification with F-specific primers COIF_Unio/COIR_Unio, B) Amplification with the first pair of M-specific primers COIMF/ RV1, C) Amplification with the second pair of M-specific primers FW1/RV1. L: 100 bp ladder. The second band in all panels corresponds to the primer dimmers. An F-specific product was obtained from all glochidia. An M-specific product was obtained from glochidia # 1, 2, 3 and 5 with either M-specific primer pair. Glochidia # 6 and 8 also produced a weak reaction with the first pair of M-specific primers. The protocol for sex assignment (see Material and Methods) classified glochidia # 1, 2, 3 and 5 as males and # 4, 6, 7, 8 and 9 as females (see Table 1).

Table 1. Inferred sex distribution among the glochidia of seven females of *U. delphinus*. See Results and Discussion for the characterization of a glochidium as female or male and for and statistical tests. CL: 95% confidence limits.

Mother	No of female glochidia	No of male glochidia	Frequency of males (CL)	Probability of no sex bias
N1315	18	7	0.28 (0.16-0.40)	0.0280
N1316	17	8	0.32 (0.13-0.51)	0.0720
N1317	22	3	0.12 (0.00-0.25)	0.0001
N1318	21	4	0.16 (0.01-0.31)	0.0007
N1320	25	0	0.00 (0.00-0.14)	0.0000
N1321	8	17	0.68 (0.49-0.87)	0.0720
N1322	0	25	1.00 (0.86-1.00)	0.0000
Total	111	64	0.37 (0.30-0.44)	0.0040

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Fig. 1

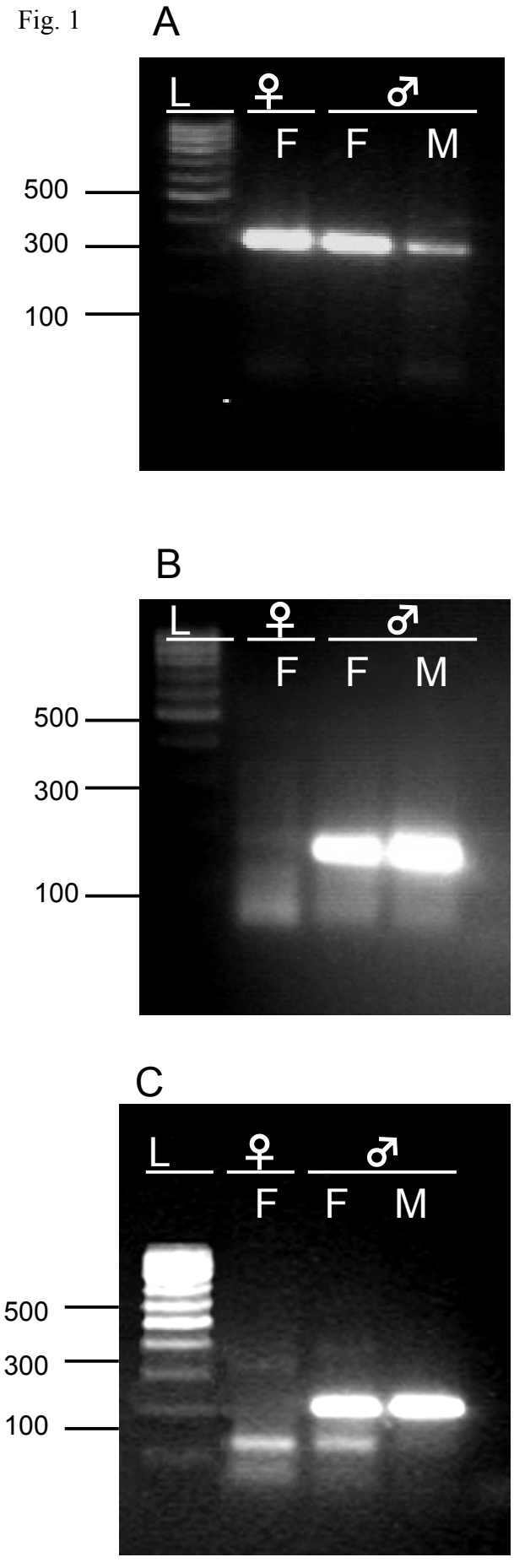
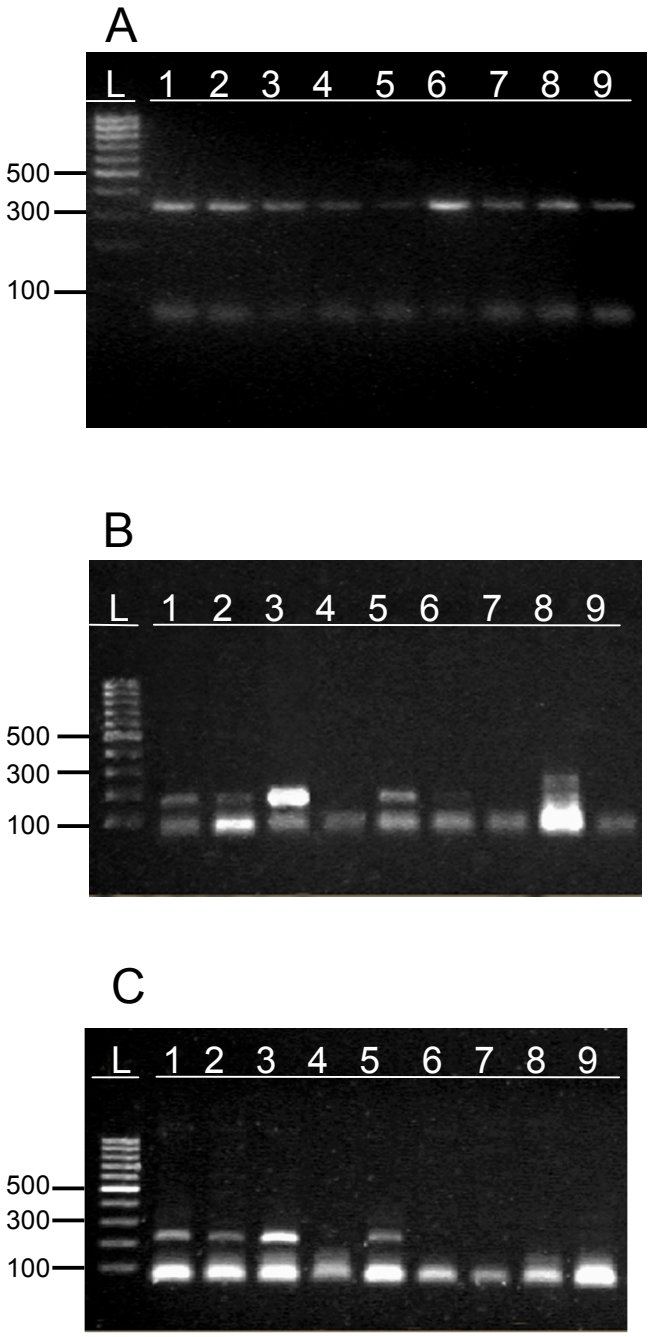


Fig 2.


A
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500
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B
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