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Identification of *SNPs* and copy number variations in mitochondrial genes related to the reproductive capacity of the cultured Asian yellow pond turtle (*Mauremys mutica*)

Yakun Wang[a,b], Jian Zhao[b], Xiaoli Liu[b], Wei Li[b], Shu Ouyang[b,c], Xinping Zhu[a,b,*]

[a] College of Life Science, Nanchang University, Jiangxi, Nanchang 330031, China
[b] Key Laboratory of Tropical & Subtropical Fishery Resource Application & Cultivation of Ministry of Agriculture and Rural Affairs, Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangdong, Guangzhou 510380, China
[c] College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, China

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ABSTRACT

Mitochondria function as an energy transfer organelle for cell metabolism with the energy being used for processes such as reproduction. To investigate whether mutations and copy number variations in mitochondrial genes are related to the reproductive capacity of the Asian yellow pond turtle, *Mauremys mutica*, there was exploration of the distribution frequency of 129 Single Nucleotide Polymorphism loci of six mitochondrial genes, *ND1*, *ND2*, *COX2*, *ND4*, *Cytb* andD-*loop*, of turtles from a relatively greater and lesser fecundity group by direct sequencing. The validation results for five candidate SNP loci in 83 female turtles indicated that only three SNP loci (C119T, A320G and A417C) in *ND1* were positively correlated with reproductive capacity in *M. mutica* ($P < 0.05$). In addition, by constructing linear regression equations of the copy numbers of *ND1*, *ND4*, *Cytb*, *D-loop*, *COX3*, and *ATP6* (log10 transformed) genes and the mean offspring number of different female turtles during a 4-year period, the copy numbers of *ND4* and *ATP6* (log10 transformed) genes were positively correlated ($P < 0.05$) with the fecundity of female turtles. Results from the present study may provide useful genetic markers for breeding *M. mutica* with greater reproductive capacity.

1. Introduction

An adequate amount of metaboloic energy is primarily maintained by oxidative phosphorylation in the mitochondria and is important for the reproductive success of an organism (Pfeiffer et al., 2001; Friedman and Nunnari, 2014). Mitochondrial genes have a greater mutation rate than nuclear genes, due to both direct exposure to free radicals produced by oxidative phosphorylation and a lack of protection as a result of the absence of histones (Richter et al., 1988; Wallace, 1992). The increased mutation frequency of mitochondrial genes and the variation caused by mutation effect reproductive function, including sperm motility (Patel et al., 2016), fertilization capacity (Handa and Nakajima, 1992; Torres et al., 2009; Clancy et al., 2011), and ovarian development (Ding et al., 2016). The effects of mitochondrial gene mutations on the reproductive capacity of animals have been reported in cattle, sheep, and pigs (Chen et al., 2017; Pradhan et al., 2018; Zhang et al., 2018); however, the effects of these mutations on reptile reproduction are less well studied than those in mammals.

In addition, copy number variation, a newly discovered type of genomic structural variation, is one of the factors affecting gene

* Corresponding author at: College of Life Science, Nanchang University, Jiangxi, Nanchang 330031, China.
E-mail address: zhuxinping_1964@163.com (X. Zhu).

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expression (Stranger et al., 2007), and these variations are important in determining the economic traits of a species (Xu et al., 2014; Goshu et al., 2018; Zhang et al., 2018). Mitochondrial genes are usually present in multiple copies (Tang et al., 2000; Barritt et al., 2002). The increase in mitochondrial DNA (mtDNA) copy numbers is associated with the transition of primordial to mature ovarian follicles and with normal fertilization rates and cleavage occurring (Murakoshi et al., 2013), while a lesser mtDNA copy number is associated with the function of normal sperm (Reynier et al., 2001; Wai et al., 2010). Furthermore, the appropriate threshold for energy (ATP contents ≥ 2 pmol/oocyte) is a prerequisite for maintaining oocyte division, embryonic implantation and development (Blerkom et al., 1995; Brenner et al., 2000). In plants, mitochondrial genes such as *Atp6* are associated with male fertilization capacity (Handa and Nakajima, 1992; Akagi et al., 1994; Howad and Kempken, 1997; Kim and Kim, 2006; Hu et al., 2013). Research on the effect of copy number variations of mitochondrial genes on fecundity has only been performed in mammals (Te-Sha et al., 2016). In turtles, mitochondrial studies have primarily focused on the stress response to hypoxia (Pamenter et al., 2008; Galli et al., 2013; Pamenter et al., 2016), but the effects of copy number variations of mitochondrial genes on reproductive functions have not been investigated

Mitochondria are primarily maternally inherited, and the metabolic energy needed for embryo development is derived from maternal mitochondria following fertilization. It is, therefore, important to analyze the relationship between mitochondrial gene mutations, copy number variations, and female reproductive capacity from the perspective of metabolic energy utilization, to elucidate the genetic mechanisms underlying differences among maternal reproductive capacities.

The Asian yellow pond turtle *Mauremys mutica* is an important freshwater turtle species in China and is an emerging aquaculture species. With continuous expansion of the scale of artificial breeding, the small yield of eggs due to *M. mutica* reaching sexual maturity at about 5 years of age, with only four to five hatchlings resulting from each female turtle every year, has become a problem hindering development of this industry (Zhu et al., 2001b). In actual breeding production, more eggs can be obtained by increasing the quantity and quality of feed (Zhu et al., 2001a). Considering the differences in reproductive capacity among individuals, however, there may be opportunities to screen individuals to identify those with greater reproductive capacity in a breeding population to promote sustainable development of the pond turtle industry.

The goal with the present study was to explore the relationship between mutations (single nucleotide polymorphisms, SNPs) or copy numbers of mitochondrial genes and the reproductive capacity of female turtles from the perspective of metabolic energy utilization. Molecular markers related to reproductive capacity were developed, which can be applied to promote the development of lineages of *M. mutica* with greater reproductive capacity.

2. Materials and methods

2.1. Samples and DNA extraction

In 2005, hatchlings were maintained in the Pearl River Fisheries Research Institute of the Chinese Academy of Fishery Sciences, Guangzhou (23.0350°N, 113.1311°E). A total of 126 turtles (43 males and 83 females) were randomly selected for breeding in the same outdoor concrete pond (26.3 m^2 total area containing 16.5 m^2 of water area and 9.8 m^2 of sandy area for nesting). All the females reached sexual maturity and ovipositing of eggs began in late April 2010. During the experimental period (2013–2016), the number of eggs for which there was an oviposition every year was counted, and fertilized eggs were artificially hatched using a previously reported method (Zhu et al., 2006). The physical movements of all the female turtles and the hatchlings that had been cultured for at least 1 month after incubation were tracked using passive integrated transponders (PIT) for 4 years (Fontaine et al., 1987; Parmenter, 1993). Nail samples were collected from all the female turtles and the offspring. The DNA samples were extracted using the microtissue kit (OMEGA) and the quality of samples was assesed using electrophoresis.

The number of offspring from each female turtle based was determined based on paternity tests for females and their offspring for 4 years using the method of Zhang et al. (2017). All the female turtles were arranged in descending order based on the total number of hatchlings produced during the 4-year period. The 21 female turtles with the largest number of offspring were selected to represent the group with relatively greater fecundity, and the 21 female turtles with the fewest progeny were selected to represent the group with the relatively lesser fecundity. Thus, candidate SNP loci related to reproductive capacity were screened and subsequently validated in all the female turtles.

2.2. Primer design, amplification and mutation site detection

Primers were designed for amplification and mutation site detection for *ND1*, *ND2, COX2*, *ND4*, *Cytb*, and *D-loop* genes using the previously published procedures (Zhao et al., 2015). Accession numbers and primer sequences are shown in Table 1. After custom synthesis by TsingKe (Guangzhou, China), the primers were diluted to 10 pmol/μL using deionized water before PCR amplification. Samples for each PCR assay contained 1 μL of genomic DNA (˜0.1 μg), 25 μL of 2 × T5 Super PCR Mix, 2 μL of each primer (10 pmol/μL), and double-distilled water to a final volume of 50 μL. Amplification reactions were performed with initial denaturation at 95 °C for 2 min; followed by 35 cycles of denaturation at 98 °C for 40 s, annealing for 40 s at the temperature defined for couples of primers used (Table 1), and extension at 72 °C for 50 s; with a final extension at 72 °C for 10 min. The PCR products were electrophoresed in 1% agarose gels, and purified using agarose gel DNA fragment recovery kit protocols (Takara), and subcloned into the pMD19T-Simple vector, followed by transformation into *Escherichia coli* for bidirectional sequencing by TsingKe (Guangzhou, China).'

Table 1
Primer sequence, product size, annealing temperature and Genbank ID used for sequencing, mutation site detection and copy number variations analysis of mitochondrial genes in *Mauremys mutica*.

Gene	Primer sequence (5' → 3')	Annealing temperature (°C)	Product size (bp)	Genbank ID
Sequencing and mutation site detection				
ND1	F1: ATAATCCCAATCCTCATCGC	54	925	4962493
	R1: GTATTGGCGGGAGTCCTGAT			
ND2	F: ATTTCCAGCGACCATTGAGT	54	783	4962492
	R: TGTAGTGGCGGTGAAGCATA			
COX2	F: TTAGGATTTCAAGACGCAA	58	566	4962490
	R: TGTCCGTAAAATACCCCTG			
ND4	F1: TTTTCTCCAACTCCTACCTT	58	802	KP938958
	R1: ATAAGTGTGATAGCCCCAGT			
Cytb	F1: AGCATTCTCATCAGTAGCCC	54	953	4962481
	R1: CGATTAGTCCTATGAGGGGTA			
D-loop	F1: ACTATTTTACTCTCCCGTGC	54	794	NC_009330
	R1: TGTGTCAGTTTAGTTGCTCTC			
Variation of the copy number				
ND1	F2: TCTAATCGCCATCTCCAGT	60	286	4962493
	R2: ATCAAAAGGTGCTCGGTTA			
ND4	F2: ACCCACCTCACGAAAACG	60	217	KP938958
	R2: TGGCAGAGACCCGATAAG			
Cytb	F2: GGGTTCTCAGTAGACAATGC	60	295	4962481
	R2: GAGTGGATAGGGGGTTAGC			
D-loop	F2: ACGAGAGATAAGCAACCCT	60	298	NC_009330
	R2: CTTGAACGAAAGTCCAGTCT			
COX3	F: ACAAAAAGGACTACGATACGG	60	198	4962487
	R: CCTGAGGCTAATAAGACTGCT			
ATP6	F: CCCAACTCTCAATAAACATAGG	60	171	4962488
	R: GTCGGATAAAAAGGCTGATT			

2.3. Copy number detection

Primers were designed for the detection of copy numbers of *ND, ND4*, *Cytb*, *D-loop*, *COX3*, and *ATP6* genes (Table 1). A standard curve was constructed by serially diluting plasmids containing the target genes using previously described protocols (Mukherjee et al., 2013). The copy numbers of the six genes in all the female turtles were calculated using absolute quantitative real-time RT-PCR. These experiments were performed using the StepOnePlus real-time PCR system (Applied Biosystems) in a final volume of 20 μL, containing 1 μL of 100 ng/μL genomic DNA, 10 μL of 2 × SYBR® Green Realtime PCR Master Mix (Toyobo), 0.5 μL of each primer (10 pmol/μL), and 8 μL of double-distilled water. The amplification conditions were as follows: initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 40 s, annealing at 60 °C for 45 s, and extension at 72 °C for 30 s; with a final cycle of 95 °C for 5 s, 60 °C for 30 s, and 95 °C for 15 s to obtain the dissolution curve.

2.4. Statistical analysis

Bioedit v7.0.9 software (Hall, 1999) was used for sequence alignment, and redundant bases on both sides of the sequence were deleted to ensure alignment between the two ends of the sequence. Nucleotide mutation site analysis was performed using MEGA5.0 software (Tamura et al., 2011).

The T-tests were performed to compare differences in maternal size, offspring mass, and offspring number between the relatively greater and lesser fecundity groups. Before analysis, the Kolmogorov-Smirnov test method was used to determine whether the values of the traits were normally distributed. The Fisher precise probability method (Fisher's exact probability test) was used to evaluate the distribution of candidate SNP loci with a minimum theoretical frequency of less than five according to chi-square tests between the two groups.

Association analysis between candidate polymorphisms and the offspring numbers of all the female turtles using a general linear model (GLM) program of SPSS 19.0 for Windows (SPSS, Chicago, IL, USA) was performed using the following equation:

$$Y_{ij} = \mu + B_i + e_j,$$

where $Y_{ij}$is the offspring number; μ is the overall mean; B_i is the effect of genotype; and e_j is the residual error.

Linear regression analysis was conducted to evaluate the trends of variation in the cycle threshold (CT) values of the genes (Table 1) with the 4-year numbers of progeny (mean ± SE). The copy number (log10 transformed) of genes with CT values significantly related to the 4-year numbers of progeny was determined using the standard curve described in 2.3. Ultimately, there was construction of a linear regression equation of gene copy number (log10 transformed) and the average 4-year mean offspring number in GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

Table 2
Comparative analysis in phenotype characteristics for the female turtles and their offspring between the relatively greater and lesser fecundity groups during the 4 years of the study.

Traits	Greater fecundity	Lesser fecundity	t	df	*P*
Average turtle body mass (g)	827 ± 122	750 ± 132	1.973	40	0.055
Average clutch size	1.96 ± 0.22	0.77 ± 0.51	9.72	40	0.0002**
Average clutch mass (g)	59.67 ± 11.33	8.62 ± 6.56	17.89	40	0.0001**
Average offspring number	28.14 ± 4.78	3.86 ± 2.67	20.33	40	0.0003**
Average offspring body mass (g)	8.47 ± 0.58	8.04 ± 2.22	0.859	40	0.396

Number of female turtles were both 21 in the relatively greater and lesser fecundity groups with a total 591 and 81 offspring in 4 years for the two groups, respectively.
** Indicates highly significant differences between groups.

3. Results

3.1. Comparison of reproductive characteristics between the relatively greater and lesser-fecundity groups

The body mass of the 21 female turtles in the relatively greater fecundity group ranged from 686 to 1102 g and the number of progeny produced ranged from 18 to 42 during the 4 years; the body mass of these offspring ranged from 7.17 to 9.73 g. The body mass of the 21 female turtles in the relatively lesser fecundity group ranged from 611 to 1146 g; the 4-year progeny numbers were 0–8; and the offspring body mass ranged from 5.96 to 10.74 g. The T-test results indicated that there was no significant difference between the relatively greater and lesser fecundity groups with respect to the average maternal body mass or average offspring weight. There were, however, significant differences in average clutch size (the average number of offspring in a nest), average clutch mass (Ratio of the total body mass of all the offspring in all nests to the number of nest), and the average number of progeny per turtle during the 4 year period (Table 2).

3.2. Correlation analysis and verification of SNP loci and reproduction capacity

A total of 129 SNP loci were screened for the *ND1*, ND2, *COX2*, *ND4*, *Cytb* and *D-loop* genes (Table 3), but only five loci (C119T, A320G, G371A, and A417C in *ND1* and G792A in *Cytb*) had a different distribution between the relatively greater and lesser fecundity groups. Verification results for the five candidate SNP loci screened in all the females indicated that only three SNP loci (C119T, A320G, and A417C in *ND1*) were significantly correlated with reproductive capacity (Table 4, Fig. 1). At the C119T locus, female turtles with a T had significantly more progeny than those with a C. At the A320G locus, females with an A were significantly inferior to females with a G with respect to offspring number. At the A417C locus, females with a C had a greater number of offspring than females with an A (Table 4).

3.3. Regression analysis of associations of copy number with average offspring number

The linear regression model between the CT values of *ND1*, *ND4*, *Cytb*, *D-loop*, *COX3*, and *ATP6* and the mean offspring number of female turtles during the 4 years data were collected indicated that only the CT values of *ND4* and *ATP6* were associated negatively with the average offspring number during the 4 years data were collected (Fig. 2). Together with the standard curve for the copy numbers (log10 transformed) and CT values of *ND4* and *ATP6*, linear regression equations were developed for the relationship between copy number (log10 transformed) and the 4-year number of offspring of female turtles numbers as follows:

$$ND4\text{: } Y = 3.99 * X - 24.40$$

$$ATP6\text{: } Y = 3.12 * X - 17.86$$

where Y is the mean annual offspring number and X is the copy number (log10 transformed).

Results indicate the 4-year mean offspring number of females was positively correlated with the copy numbers of both *ND4* and *ATP6* (log10 transformed; Table 5; Fig. 3).

4. Discussion

Animal reproduction (spawning) is a process regulated by many factors, including metabolic energy availability for physiological processes. A lack of energy impairs the reproductive functions, thus reducing the number of offspring per female. The number of offspring resulting from hatching of eggs, rather than the total number of eggs, reflects the actual reproductive capacity of spawning animals (e.g., turtles). As a site for cellular oxidative phosphorylation, mitochondria have important functions in maintaining sperm motility, ensuring ovulation and embryonic development (Brenner et al., 2000; Patel et al., 2016; Li et al., 2018a). In the present study, the SNP loci and copy number variations of mitochondrial genes related to the reproductive capacity of *M. mutica* were screened and detected from the perspective of energy utilization, these results will provide more effective markers for screening

Table 3
Fisher exact test for mitochondrial genes between the relative greater and lesser fecundity groups.

Gene	Locus	*P*(Fisher)	Gene	Locus	*P*(Fisher)	Gene	Locus	*P*(Fisher)
ND1	G65A	0.107	*ND2*	T52C	0.505	*D-loop*	T55C	0.606
	C119T	0.048*		T67C	0.197		T67C	1.000
	C317T	0.488		T79C	0.197		T77C	0.697
	A320G	0.048*		C94T	1.000		G80A	0.697
	G371A	0.030*		C111T	0.197		C82T	0.215
	T394C	0.184		C112T	1.000		T85C	0.697
	C410A	0.232		G128A	0.197		T101C	0.756
	A417C	0.048*		T145C	0.215		A103G	1.000
	A514G	1.000		C155A	0.197		G105A	0.663
	T594C	1.000		T157C	0.197		A106G	1.000
	C661A	0.108		A158G	1.000		A123G	0.744
	C673T	0.107		C161T	0.488		C126T	1.000
	C686T	0.663		G170A	0.197		G128A	0.697
	G717A	0.232		A172C	0.159		T155C	1.000
	A726G	0.606		C193T	0.197		C159T	0.751
	C774T	0.107		G208A	0.197		C161T	1.000
Cytb	A96G	1.000		A229G	0.350		A162G	1.000
	C126A	0.410		C250T	0.326		C169T	0.663
	G157A	0.410		G251A	0.197		T178C	0.410
	C210T	0.410		A292G	1.000		G191A	0.130
	C321T	0.697		T305C	0.197		A210G	0.232
	G334A	0.697		A313G	1.000		A228G	1.000
	T432C	1.000		C328T	0.197		A271G	0.538
	G459A	1.000		T367C	1.000		280insT	0.697
	T465C	0.697		T388C	0.197		A344G	1.000
	C549T	0.697		A394G	1.000		A399G	0.326
	A609C	1.000		A406G	0.197		C419T	1.000
	G732A	0.410		C427T	0.215		C423T	0.734
	T762C	0.751		C442T	0.118		C425T	0.751
	C786T	0.410		A448G	0.484		C474T	1.000
	G792A	0.004**		T457C	0.197		A549G	0.606
COX2	A112G	0.663		A460T	0.197		G567A	0.238
	C184T	0.663		T475C	0.197		T600C	0.410
	T187C	0.663		T484C	0.111		C609T	0.410
	G214A	0.277		G490A	0.197		G627A	0.238
	T370C	0.488		T508C	0.326			
	G382A	0.130		G515A	0.215			
	C403T	0.663		T523C	0.197			
	A445G	0.663		A526G	0.181			
ND4	T58C	0.067		G542A	0.197			
	A66G	0.663		A544G	0.326			
	T149C	0.697		564insG	0.488			
	C378T	1.000		A566G	0.197			
	G446A	0.184		A590G	0.197			
	C550T	1.000		C671T	0.197			
	C560A	0.697		A677G	0.306			
	G609A	1.000						
	T623C	0.697						

* Indicates significant differences in the distribution of SNP loci.
** Indicates highly significant differences of the distribution of SNP loci.

individuals with relatively greater reproductive capacity for breeding populations.

Results of previous studies indicate that mutations in *ND2, ND4*, *Cytb*, and *D-loop* genes are associated with changes in reproductive function, primarily as a result of affects on oocyte development, oogenesis, and reproductive capacity (Shamsi et al., 2013; Te-sha et al., 2016; Chen et al., 2017; Pradhan et al., 2018). In the present study, however, there were not any SNP loci that were significantly associated with reproductive capacity in these four genes as well as in the *COX2* gene. Furthermore, mutations occurring in introns do not result in changes in the amino acid sequence and, thus, do not affect the function of proteins. The number of samples examined in the present study may not have been adequate to ascertain the potential SNP sites that were missed, because some mutations may occur in only a few individuals and these may not have been detected as a result of the small sample size in the present study. To improve the efficiency and accuracy of screening, therefore, a larger number of female turtles for which offspring numbers are known, as compared to the numbers of animals assessed in the present study, are needed to more precisely screen SNP loci that are associated with reproductive capacity.

Both synonymous and nonsynonymous mutations in genes lead to functional changes (Hasegawa et al., 1998; Gao et al., 2004; Reamonbuettner et al., 2013). In the present study, three SNP loci, C119T, A320G, and A417C, located within the coding region of the *ND1* gene, were associated with reproductive capacity. For both the C119T and A320G loci, synonymous mutations occurred. The

Table 4
Validation of candidate SNP loci in *ND1* and *Cytb* related to fecundity in 83 females.

SNP ID	Loci	Genotype	No. female	No. offspring[a]	*F*	*P*
ND1	C119T	*C*	77	14.22 ± 1.06	$F_{1, 81} = 7.084$	0.007**
		T	6	25.00 ± 2.35		
	A320G	*A*	77	14.22 ± 1.06	$F_{1, 81} = 7.804$	0.007**
		G	6	25.00 ± 2.35		
	G371A	*A*	20	12.05 ± 1.87	$F_{1, 81} = 2.61$	0.110
		G	63	15.94 ± 1.22		
	A417C	*A*	77	14.22 ± 1.06	$F_{1, 81} = 7.084$	0.007**
		C	6	25.00 ± 2.35		
Cytb	G792A	*A*	21	16.67 ± 1.90	$F_{1, 81} = 0.869$	0.354
		G	62	14.44 ± 1.24		

[a] Total number of offspring produced by females during 4 years is presented as mean ± standard error (SE) of phenotypic values.
** Indicates highly significant differences.

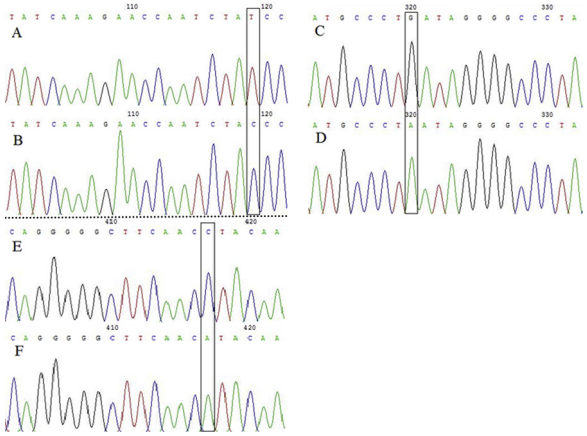

Fig. 1. Chromatograms depicting peak values at the three loci for the *ND1* gene that were associated with reproductive capacities of *M. mutica*; At locus C119T (top left), the T and C mutations are associated with the relatively greater and lesser fecundity groups, respectively; At locus A320G (top right), the G and A mutations are associated with the relatively greater and lesser fecundity groups, respectively; At locus A417C (bottom left), the C and A mutations are associated with the relatively greater and lesser fecundity groups, respectively.

amino acids encoded before and after the mutation are, therefore, invariant (these two sites encode tyrosine and leucine, respectively). The average offspring numbers of female turtles with a T at locus C119T and a G at locus A320G were approximately two times greater than those of female turtles with a C at locus C119T and an A at locus A320G, respectively. It is assumed that the mutations may enhance the translation and editing of *ND1* mRNA and increase protein abundances, facilitating the electron transmission through electron chains and generating more energy (Kimchisarfaty et al., 2007). This hypothesis is not consistent with the findings of Pratley (2008), where synonymous mutations in *ND1* were not associated with metabolic efficiency of mitochondria in muscle at rest. In addition, in some animals in the present study there was a nonsynonymous mutation at the A417C locus in the *ND1* gene, where the amino acid was an isoleucine before mutation and a leucine after mutation. In the females with this mutation, reproductive capacity was greater implying that leucine insertion in the protein leads to an enhanced reproductive capacity of female pond turtles. This improvement may have occurred because the change to leucine further promotes energy uptake and utilization in the mitochondria. The results from the present study differ from those of a study with small tail Han sheep where there were nonsynonymous mutations detected in the *ND1* gene that had no significant effect on litter size (Chen et al., 2017).

Results of the most recent research indicate that *ND4* gene expression is associated with meiosis and that expression of this gene is less in triploid male-sterile fish (Li et al., 2018b). In addition to being a site of energy storage for maintaining the integrity of spermatozoa and sperm motility, the capacity to balance the energy supply and demand from ATP is considered to be the most important factor affecting oocyte fertilization and embryonic development (Blerkom et al., 1995). The expression of the *ATP6* gene depends on copy number (Tan et al., 2017), and an increased copy number of the *ATP6* gene is associated with induction of fat metabolism when the cellular energy available for physiological functions is less than optimal in vivo (Kai et al., 2016). In the present study, there was a positive correlation between the mean offspring number and the copy number of the *ND4* and *ATP6* genes. Because females with larger copy numbers for these two genes produced more offspring, it is suggested that they have a greater reproductive capacity and, therefore, were able to more effectively adapt to the negative effects of lesser than optimal dietary energy consumption during the period of the year when reproduction is occurring. This adaptation capacity may be due to the increase in copy numbers and thus greater expression of the *ND4* and *ATP6* genes, which is important for electron transport (Gu et al., 2013) and energy utilization (Lu and Hanson, 1994; Narita et al., 2002) to meet the energy requirements for reproduction. Findings in the present study

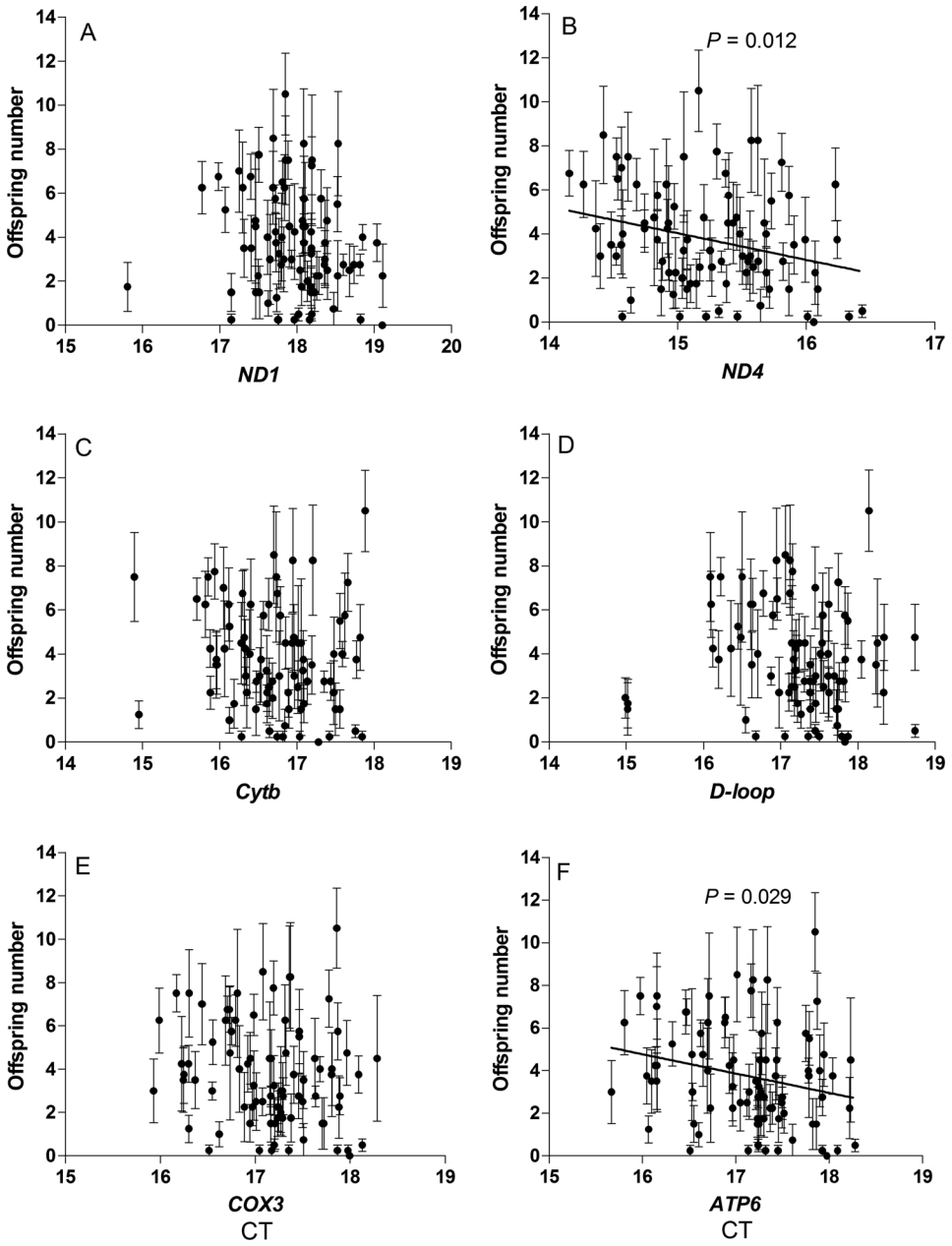

Fig. 2. Correlation analysis between CT values obtained conducting absolute quantitative PCR of mitochondrial genes and offspring numbers (mean ± SEM) during the 4-year period of the study; $P < 0.05$ indicates a significant association.

Table 5
Output of single linear regression analysis identifying predictors of the mean offspring number during the 4 years of the study.

Factors	Coefficent[a]	*R*	95% confidence interval	*t*	*P*
ND4	3.99	0.273	0.887–7.099	2.558	0.012*
ATP6	3.12	0.240	0.327–5.922	2.222	0.029*

[a] Coefficent of linear regression equation between copy numbers (log10 transformed) for the *ND4* and *ATP6* genes and mean offspring number, respectively.

* Indicates differences at $P \leq 0.05$.

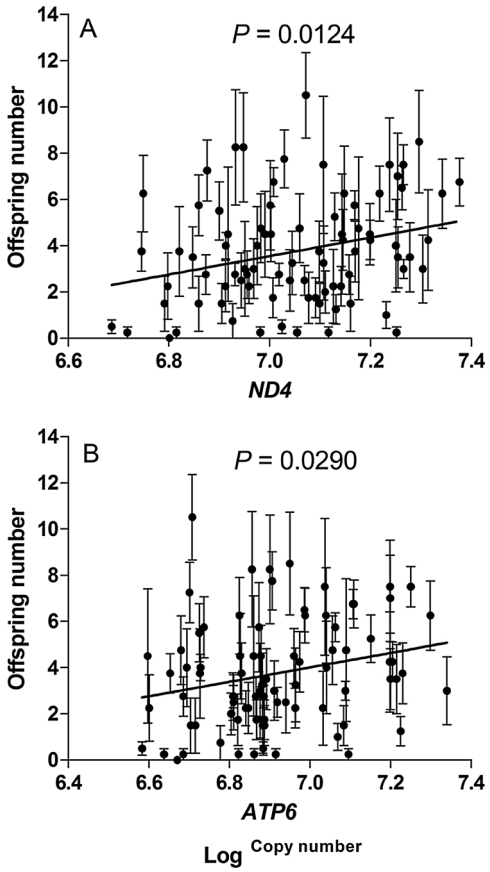

Fig. 3. Linear regression analysis of the relationship between the copy numbers (log10 transformed) for the *ND4* and *ATP6* gene and offspring numbers (mean ± SEM) during the 4-years period of the study; $P < 0.05$ indicates a significant association.

are consistent with those of Feng et al. (2015) where there was an attempt to ascertain the genetic basis of litter size using suppression subtractive hybridization techniques and it was found that *ATP6* was the most abundant gene expressed in the hypothalamuic-pituitary-gonadal axis. This gene was considered, therefore, as a candidate gene related to litter size in small tail Han sheep.

5. Conclusions

In the present study, there was identification of three SNP loci (C119T, A320G and A417C) for the *ND1* gene for the first time. The female turtles with a T, G and C had more progenythan those with a C, A and A at locus C119T, A320G and A417C, respectively. Furthermore, there was an association of a greater reproductive capacity with increasing copy numbers of the *ND4* and *ATP6* genes in *M. mutica*. Results of the present study provide some new and effective genetic resources for the breeding of animals from strains with relatively greater fecundity that may contribute to sustaining development of the *M. mutica* industry.

Conflicts of interest

The authors have no conflicts of interest to declare.

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