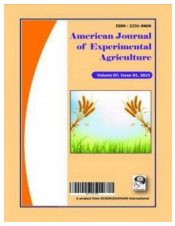

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# Genetic Diversity of Prolactin Gene in Japanese Quail (*Coturnix coturnix japonica*) as Affected by Location in Nigeria

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author FOE designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors AES and SEA reviewed the experimental design and all drafts of the manuscript. Author FOE managed the analyses of the study. Authors FOE and OHO performed the statistical analysis. All authors read and approved the final manuscript.*

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## ABSTRACT

Restriction fragment length Polymorphism (RFLP) marker was used to investigate the effect of location on polymorphism, relationship and population structure with respect to prolactin gene (PRL gene) in Japanese quails in Nigeria. Blood samples were collected from thirty quails each from 5 different regions (North, South, West, East and North Central), for PRL loci analysis. DNA was extracted from the samples. Polymerase chain reaction (PCR) and electrophoresis was used to characterize a 24 base pair (bp) insertion/deletion (Indel) in a 358 bp PCR product. The populations were characterized for their genetic variability using allele frequencies, polymorphic information content, observed heterozygosity (*Ho*), genetic distance (D), F-statistics ($F_{IT}$, $F_{IS}$, $F_{ST}$), analysis of molecular variance, test for Hardy-Weinberg Equilibrium (H-WE) and cluster analysis. Two alleles A (0.35 to 0.63) and B (0.37 to 0.65) were observed at the PRL gene loci. The highest $F_{IT}$ was recorded between 0.10 (East) and 0.19 (North) indicating inbreeding within the population. The $F_{IS}$

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among populations were between 0.09 (North central) and 0.14 (North) while $F_{ST}$ ranged from 0.001 (North central) to 0.06 (North), indicating moderate genetic differentiation among populations. Chi Square result indicated that the population were not in H-WE. The phylogenetic relationships showed that the population from the 5 regions had common descent. Clusters from the combined population showed that PRL gene is based on individual genotype and not location. AMOVA analysis showed that 3% of the total genetic variation was explained by population difference, 19% by variation among individuals and 77% within individuals. The results showed that study of prolactin gene diversity is useful for decision making for selective breeding and conservation strategies for Japanese quails irrespective of the location.

*Keywords: Prolactin; polymorphism; conservation; inbreeding; Japanese quail.*

## 1. INTRODUCTION

Prolactin (luteotropic hormone or luteotropin) is a protein, which plays an essential role in the induction and maintenance of brooding behaviour in poultry [1,2,3], crop-sac development in columbiforms, regulation of the gonad and immune responses in many species [4]. High circulating prolactin may interfere with the functions of the ovaries and decrease sperm production in the testis [5].

Japanese quail (*Coturnix coturnix Japonica*) have been used by man for meat and egg since 1910 [6]. Studies have shown that Japanese quail is closely related to chicken, about 96.5% [7]. It consists of micro and macro chromosomes that cannot be morphologically and cytologically differentiated [8].

Molecular markers are very useful in the study of population structure and gene flow on traits of economic importance, especially traits that are difficult to select for on the field. In Nigeria there has been the move towards encouraging the production of micro-livestock as likely source of animal protein with the hope that the utilization of micro-livestock will provide an alternative to conventional chicken. Studies have shown that the 24-bp insert-deletion is significantly associated with broody behaviour and egg production [3,9], indicating its usefulness as a molecular marker for egg production. Various studies on characterization of prolactin gene have been reported in commercial chickens [9,10,11,12,13,14,15]. But the effect of environment on the variability of prolactin gene especially in Nigeria has not been reported. This is important because the phenotypic variability differs under different environmental conditions and genetic characterization may be more applicable for studying long-term environmentally induced variation. The genetic diversity of Japanese quail in Nigeria has not been investigated and the pressure for alternative protein source to chicken has increased the need for genetic improvement and understanding of the genetic variation of traits of economic importance in Japanese quail in Nigeria. The genetic diversity studies of PRL gene that have been carried out covered only a limited geographical area. Therefore a more detailed study that cuts across the entire country is still require to better understand the genetic structure of Japanese quail in Nigeria. In this study, the molecular data from quails sampled across the entire country were used to evaluate genetic diversity of PRL gene in Nigeria.

## 2. MATERIALS AND METHODS

### 2.1 Sample Size and Blood Collection

Blood samples were randomly collected from 30 quails each from 5 different regions in Nigeria viz; North, South, West, East and North Central (Fig. 1) for PRL loci analysis. The samples were collected through jugular venepuncture into 2 ml vacutainers treated with K3-Ethylenediaminetetra acetic acid (EDTA) and inverted several times to ensure proper mixing in order to prevent coagulation. Ethical permission was obtained prior to the sampling (see ethics statement below).

### 2.2 DNA Isolation

Genomic DNA was extracted manually from 200 µl of individual blood samples using a commercial kit (GeneJET Whole Blood Genomic DNA Purification Mini kit). The DNA yield was assessed and quantified using Nanodrop ND-1000 UV/Vis Spectrophotometer (Nanodrop Technologies, Inc., DE) and and gel electrophoresis on 1% agarose. DNA concentration was adjusted to 50 ng/µl.

### 2.3 PCR and Electrophoresis Procedure

Polymerase Chain Reaction (PCR) was carried out, using the Applied Biosystems Veriti™ GeneAmp® thermocycler and the PCR Master Kit (Thermo Scientific). The kit of master mix consisted of 2.5 ul of 10X PCR buffer, 2 mM of $MgCl_2$ and 2 mM dNTPs (each). Each reaction mixture consisted of 12.5 µl of the master mix, 0.5 U/µl of *Taq* DNA polymerase, 1 µl of the DNA solution (50 ng/µl), 1 µl of each primer (5 pmol/µl) and deionized water to make up to 25 µl in PCR tubes. The tubes were positioned in the interchangeable blocks of 96 wells of 0.2ml. Amplification of PRL gene fragment (130 or 154 bp, containing the 24 bp indel at np 358) using the thermo-cycler ABI9700 was carried out using primers described by Cui et al. [9], 2006, PRL-F (5´-TTT AAT ATT GGT GGG TGA AGA GAC A-3´) and PRL-R (5´-ATG CCA CTG ATC CTC GAA AAC TC-3´), with an initial incubation and enzyme activation of 94℃ for 5 min; followed by 35 cycles of 30 sec at 94℃, 30 sec at 61℃, and 45 sec at 72℃; and a final extension of 5 min at 72℃. The PCR-products of the 24 bp were run on 6% polyacrylamide gel, staining was done using Silver nitrate.

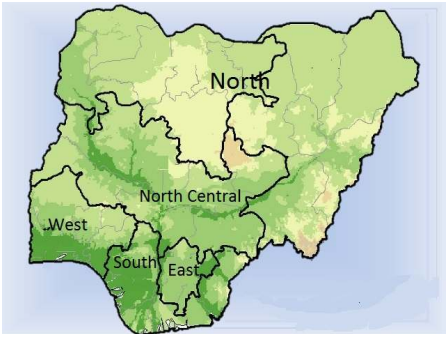


**Fig. 1. Map of Nigeria showing the five regions of Nigeria from which Japanese Quails were sampled for use in this study**

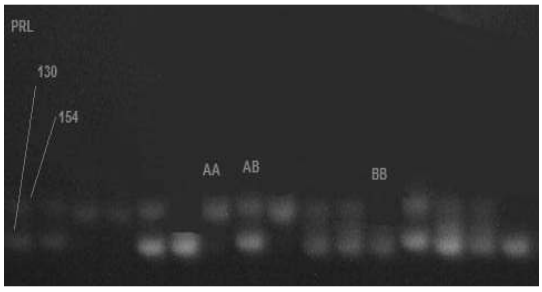


**Plate 1. Gel pictures of primers on 6% polyacrylamide gel electrophoresis (PAGE)**

### 2.4 Statistical Analysis

Measures of genetic diversity, such as total number of alleles, allele frequencies, mean number of alleles and Polymorphism information content (PIC), a measure of how PRL loci are informative in relation to expected heterozygosity were computed using Powermaker version 3.25 software [16]. POPGENE version 1.31 software [17] was used to determine Hardy-Weinberg Equilibrium and Heterozygosity. Genetic distance with calculated pairwise $F_{ST}$ and gene flow values were calculated using GENPOP software version 4.13 [18]. Genetic distance and identity was obtained according to the method Nei [19].

$$Ds= (1-J_{xy})-1/2[(1-J_x) + (1-J_y)]$$
$$Ds=In\ (J_{xy}/\sqrt{J_xJ_y})$$

Where; $J_x=(2nx\sum x_i^2-1)/(2n_x-1)$
$J_y=(2n_y\sum y_i^2-1)/(2n_y-1)$
$J_{xy}=\sum_{xy}$
n =population size

Analysis of Molecular Variance (AMOVA) was done using GenAlEX 6.5 [20]. Phylogenetic tree based on Nei genetic distance, using the neighbour joining tree method [21] was constructed using the PHYLIP version 3.69 software program and then bootstrapped 1000 times across all loci [22].

## 3. RESULTS

A total of 278 alleles were observed at the PRL gene loci. The PIC observed among the population ranged from 0.35 (East) to 0.37 (North) as shown in Table 1. The *Ho* among populations ranged from 0.37 (North) to 0.59 (West). Gene diversity ranged from 0.46 (South and East) to 0.50 (North Central) Table 2. The farthest genetic distance was recorded between 0.0025 (North Central) to 0.10 (North) while the closest was between 0.009 (West) and 0.003 (North). The highest $F_{IT}$ was recorded between 0.10 (East) and 0.19 (North) as shown in Table 3, indicating inbreeding within the population ($F_{IS}$=0.01) and resulted in heterozygote deficient and low genetic differentiation while among populations $F_{IS}$ were between 0.09 (North central) to 0.14 (North), and $F_{ST}$ ranged from 0.001 (North central) to 0.06 (North), indicating moderate genetic differentiation among populations. Chi Square result indicated that the populations were not in Hardy–Weinberg equilibrium. Phylogenetic relationships showed

two major clusters with North central branching off, South and North clustering at point one while East and West clustered at point two with all two clusters originating from a source as shown in Fig. 2. Results of Analysis of Molecular Variance (Table 4) revealed that 3% of the total genetic variation was explained by population difference 19% by variation among individuals and 77% within individuals. Based on present results two alleles, [insertion (A) or deletion (B)] and three genotypes, AA, AB, and BB were observed in the population. The observed frequencies of alleles and genotypes for the PRL gene are shown in Table 2. The allele frequencies and genotypes of Japanese quail in Nigeria as well as gene diversity are as shown in Table 2, respectively. Heterozygosity values were calculated to determine the level of genetic variation within the populations. Results is as shown in Table 3.

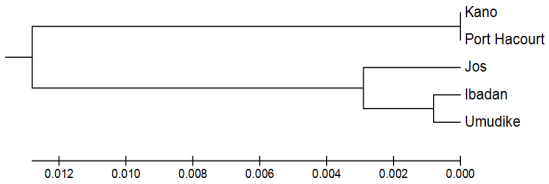


**Fig. 2. Neighbour-joining tree of different populations constructed using Nei's genetic distance (1978)**

## 4. DISCUSSION

### 4.1 Polymorphism and Polymorphic Information Content

The efficiency of PRL was determined both as the amount of polymorphism and PIC (polymorphic information content) coefficient. The average level of polymorphism across the populations ranged from 0.35 to 0.38 (Table 1). In the majority of the studied populations PRL marker was reasonably informative according to classification by [23] highly informative markers have PIC values >0.50, the reasonably informative markers have PIC value between 0.25-0.50 and the slightly informative markers have PIC value <0.25. However [6] recorded lower PIC values than those obtained in this study while [24] reported higher PIC values between 0.427 and 0.815 in a study using four strains of Japanese quail. [25] recorded PIC value between 0.523-0.702 in a study of 19 Chinese native chicken breeds. [17] reported values between 0.560-0.641 in a study of 12 indigenous chicken populations in Southern China, [26] reported 0.426-0.599 in a study with Turkish native chicken breeds and [27] reported values ranging from 0.172-0.847 in a study with 13 Spanish chicken breeds population.

### 4.2 Allele Frequencies

A total of 278 alleles were identified in the populations of Japanese quail assessed at one Polymerase Chain reaction, Restriction fragment length Polymorphism loci. In this study, two alleles and three genotypes were detected for this locus respectively. The allele frequencies among populations ranged from A 0.35 (East) to 0.63 (North and South) and B 0.37 (North and South) to 0.65 (East) respectively (Tables 2). Present results are in agreement with previous report by [14] and [13], that reported two alleles (I = 0.52 D = 0.48) in a study with Japanese quail. In a study with native hens, [10] reported two alleles (I=0.76 and D=0.24), [11] also observed two alleles T and C with frequencies 0.67 and 0.33 in chicken. In addition, [28] reported two alleles (I and D) and three genotypes (II, ID and DD) in a study with chicken. [9] reported the frequencies of alleles I and D to be 1 and 0 in White Leghorn, 0.20 and 0.80 in Taihe Silkies, 0.05 and 0.95 in Yangshan, 0.17 and 0.83 in Nongdahe and 0.22 and 0.78 in White Rock, in a study with native and commercial chickens, [29] in a study of polymorphism of PRL gene in duck as well as [3] in a study of PRL gene also observed two alleles A and B with frequencies 0.59 and 0.41, in Rhine geese while Wan-xi White recorded 0.73 and 0.27 frequencies. Based on individual populations from five regions in Nigeria as shown in Table 2, the allele frequency for A; North and South had the highest frequencies of (0.63) while East had the least frequency of (0.35) respectively for allele B; East (0.65) had the highest while North and South (0.37) had the least frequencies respectively.

The genotype frequencies obtained at the 24-bp indel site of prolactin gene were (insertion insertion) AA 0.11 (West) to 0.44 (North), (insertion deletion) AB 0.39 (North) to 0.59 (West) and (deletion deletion) BB 0.13 (South) to 0.44 (East) respectively. The frequency of heterozygous genotype (AB) was higher compared to homozygous (AA) and (BB) genotypes among populations as shown in Table 2. This result agrees with that reported by [30] and [14] in Japanese quail with heterozygote genotypes frequencies of (ID 0.85). [12] also reported heterozygote frequency of (ID 0.40) in Iranian indigenous breeder hens.

### 4.3 Hardy-Weinberg Equilibrium (HWE)

Hardy-Weinberg equilibrium test for this region of PRL gene was conducted for the populations. The result indicates that all the populations were not in HWE ($P<0.05$) for this region of the PRL gene Table 3. This result is in line with that reported by [31] on microsatellite study in 64 chicken populations from different continents. Among populations, West, North central and south showed significant deviations under the heterozygote excess assumption while North and East showed significant deviations under heterozygote deficit assumption. On the average there were significant deviations from Hardy-Weinberg equilibrium, which agrees well with the results on observed heterozygosity.

The observed heterozygote deficit assumption, the deviations from HWE ($P<0.05$) from the overall population may be indicative of excess of individual migration, less mutation rate in PRL loci and less artificial selection and random breeding of birds in the population based on the study PRL gene. The heterozygote excess may be indicative of excess heterozygote than homozygote individual migration, high mutation rate in PRL loci and reduced artificial selection and non-random breeding in the population [32]. According to the history of the studied lines, they were under selection for different economic traits and thus researchers concluded that the marker was informative to determine the genetic variation in different populations of Japanese quail effectively with respect to PRL gene.

### 4.4 Gene Diversity and Heterozygosity Estimate

According to [33], marker should be in the range of 0.3 to 0.8 in a population in order for marker to be useful in measuring genetic variation. Gene diversity among populations ranged from 0.46 in South and East populations respectively to 0.50 in North central population while North population recorded gene diversity of (0.47). This confirms the usefulness of this marker in measuring genetic variation of PRL gene in Japanese quail. These values were higher compared to those reported by [34].

The average direct count among the population was less than the expected heterozygosity. The observed heterozygosity (Ho) values ranged from 0.27 to 0.62 while the expected (He) value ranged from 0.42 to 0.52 respectively. When compared to other studies, the observed heterozygosity of Japanese quail in Nigeria was 0.47, which higher than that reported by [3]. The estimate observed heterozygosity suggests the presence of variation in Japanese quail population. High values of observed heterozygosity (0.62) within the populations could be attributed to the number of allele detected in the test loci [35]. The less observed heterozygosity in the population may be attributed to the level of inbreeding in the case of inbreeding the deficit affects all or most of the loci in a similar way. Wahlund effects may contribute to less heterozygosity, due to the presence of substructure within the populations, genotyping errors can be a contributing factor and the presence of null allele can also contribute to deficit heterozygote in the population, genetic drift, less mutation in PRL gene, selective mating within the population, small population size (population bottleneck or population dynamics that severely reduces the level of genetic variation related to that expected. High heterozygosity values in this study may be an indicator of higher reproductive characteristic, may also implies the presence of Isolate-break effect (mixing of two previously isolated populations) and the presence of store of genetic diversity irrespective of the low level of differentiation within close relatives. This agrees with the prediction of [36].

### 4.5 Genetic Identity and Genetic Distance

The genetic distance estimate [19] was calculated using allele frequencies. The distance between populations ranged from 0.0025 to 0.10. Genetic identity indicates that West (0.99) and North central (1.00) populations were more genetically alike while the North central (1.00) and North (0.91) population were the least genetically identical (Table 4). Phylogenetic Analysis was done from the genetic distance estimate and was used to construct the phylogenetic trees (Fig. 2) which corresponds with the genetic distance estimates.

### 4.6 Genetic Distance Estimate and Phylogenetic Relationship

Genetic distance was calculated using [19] genetic distance method to evaluate inbreeding relationship between populations of Japanese quail in Nigeria. The bias due to unequal sample size was corrected using the bootstrap procedure by [37]. The results from Nei genetic distance revealed that there were considerable distances among the populations. The West 0.01 and North central 0.00 had similar distance value indicating that they are equally close.

The neighbour-joining tree (Fig. 2) among populations shows two main branching from the ancestral root, Phylogenetic relationships revealed two major clusters, however some sub-clusters were also observed, with North and South branching off to cluster at point one while North made up the second cluster with West and East as sub clusters. The degree of closeness shown in clusters among populations may be explained considering that they are characterized by a common breeding system. Populations did not follow uniform clustering as shown in Fig. 2. Based on neighbour-joining tree clustering assessment, high level of similarities between populations may be indicative that the variability of PRL gene in Japanese quail in Nigeria is not dependent on geographical location rather on individual genotypes.

## 4.7 Genetic Differentiation

Population differentiation was examined by fixation indices $F_{IT}$, $F_{IS}$ and $F_{ST}$ across Japanese quail population in Nigeria Table 3. Wright's F-statistics and other similar indices that describe the partitioning of genetic variations at different levels can be estimated for natural populations using molecular marker data [19]. F statistics value $F_{IT}$, and $F_{ST}$ are measure of deviation from HWE proportion and total population respectively. Positive values indicate a deficiency in heterozygotes while negative value indicates an excess of heterozygotes. $F_{IS}$ can be interpreted as a measure of inbreeding. Positive $F_{IS}$ shows the deficiency of heterozygote in the population and the level of relationship between mates compared to the average relationship of the population. The highest $F_{IT}$ was recorded between 0.10 (East) and 0.19 (North) indicating inbreeding within the population. Among populations $F_{IS}$ were between 0.09 (North central) to 0.14 (North) and pair wise $F_{ST}$ value across populations ranged from 0.001 (North central) to 0.06 (North), indicating moderate genetic differentiation among populations. [38] reported that a pair wise $F_{ST}$ value up to 0.05 indicates moderate differentiation between populations. Gene flow from one population to another ranged from 4.09 (North central) to 195.5 (North), this indicates an important genetic isolation, which may be due to the distance created by geography.

The estimated $F_{ST}$ value, correspond with the amount of genetic variability accounted for by the difference in breeding practise, the similarity in environment and to a large extent due to past gene flow among them. The genetic differentiation may also be due to mutation, genetic drift, selection, sampling error, genetic bottleneck of severe reduction in population size, small re-occurring population size, inbreeding, non-random breeding, selective mating among individuals within the population, freely interbreeding, migration pattern and wahlund effect in the PRL gene.

**Table 1. Major allele frequencies, gene diversity, observed heterozygosity among populations and polymorphic information content (PIC) of Japanese quail PRL in Nigeria**

| Gene | Regions | Major allele frequency | Gene diversity | Heterozygosity (Ho) | PIC |
|---|---|---|---|---|---|
| PRL | Ibadan | 0.59 | 0.48 | 0.59 | 0.36 |
| | Jos | 0.52 | 0.50 | 0.54 | 0.37 |
| | Kano | 0.63 | 0.47 | 0.37 | 0.37 |
| | Port Harcourt | 0.63 | 0.46 | 0.47 | 0.36 |
| | Umudike | 0.65 | 0.46 | 0.41 | 0.35 |

**Table 2. Summary of alleles and genotypes frequency for Hardy-Weinberg equilibrium among populations for PRL gene in Japanese quail in Nigeria**

| Region | Allele frequency (%) | | Genotype frequency (%) | | |
|---|---|---|---|---|---|
| | A | B | AA | AB | BB |
| Ibadan | 0.41 | 0.59 | 0.11 | 0.59 | 0.30 |
| Jos | 0.48 | 0.52 | 0.21 | 0.54 | 0.25 |
| Kano | 0.63 | 0.37 | 0.44 | 0.37 | 0.19 |
| Port Harcourt | 0.63 | 0.37 | 0.40 | 0.47 | 0.13 |
| Umudike | 0.35 | 0.65 | 0.15 | 0.41 | 0.44 |

*A = Insertion allele; B = Deletion allele; AA = Insertion-insertion; AB = Insertion-deletion; BB = Deletion- deletion*

**Table 3. Summary of fixation indices ($F_{IT}$, $F_{ST}$ and $F_{IS}$) genetic distance, genetic identity and gene flow (Nm) among populations**

| Locus | Region | $F_{IS}$ | $F_{IT}$ | $F_{ST}$ | Genetic distance (Ds) | Genetic identity | Nm* |
|---|---|---|---|---|---|---|---|
| PRL | Ibadan | -0.15 | -0.14 | 0.005 | 0.01 | 0.99 | 51.62 |
| | Jos | -0.07 | -0.07 | 0.001 | 0.0025 | 1.00 | 195.5 |
| | Kano | 0.14 | 0.19 | 0.06 | 0.10 | 0.91 | 4.09 |
| | Port Harcourt | -0.02 | -0.005 | 0.02 | 0.03 | 0.97 | 12.81 |
| | Umudike | 0.09 | 0.10 | 0.01 | 0.02 | 0.98 | 17.03 |

**Table 4. Summary of analysis of molecular variance among populations of Japanese quail from 5 regions in Nigeria**

| Source | df | S | MS | Est. Var | % |
|---|---|---|---|---|---|
| Among populations | 9 | 5.453 | 0.606 | 0.009 | 3% |
| Among individuals | 140 | 46.333 | 0.331 | 0.055 | 19% |
| Within individuals | 150 | 33.000 | 0.220 | 0.220 | 77% |
| Total | 299 | 84.787 | | 0.285 | 100% |

### 4.8 Analysis of Molecular Variance (AMOVA)

The results obtained from AMOVA provided an estimate of the measure of population genetic differentiation within and between populations as shown in Table 4 above. The AMOVA carried out for the PRL data showed that only 3% of the total genetic variation was explained by population difference, 19% by variation among individuals and 77% within individuals. The hierarchical analysis of variance revealed an $F_{ST}$ value of significant ($P<0.001$) Indicating the presence of genetic differentiation in the population. Variance components within individuals were highly significant ($P<0.001$). The result from AMOVA and pairwise computation indicates differentiation relative to a random collection of genotypes and reflects differences in the spatial distribution in genetic variation. [28] reported similar results of higher variations within populations. This implies that PRL gene is based on individual genotype and not determined by location.

## 5. CONCLUSION

The goal of this study was to investigate the effect of location on polymorphism, relationship and population structure with respect to prolactin gene (PRL gene) in Japanese quails in Nigeria. The results suggest that moderate genetic differentiation exists in these populations, indicating little or no effects of location on the population with respect to PRL gene in Japanese quails in Nigeria. The result indicated the presence of two alleles for this locus. This study also suggests that there were no isolate rather all the populations were from the same decent. The result from this study opens interesting prospects for future selective breeding and conservation strategies. Since Prolactin is an important gene for reproduction, segregation of the RFLP marker could be assessed for reproductive capacity.

## ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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