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# GENETIC DIFFERENTIATION AND RELATIONSHIP AMONG EGYPTIAN NILE DELTA LOCATED BUFFALO USING MICROSATELLITE MARKERS

**M. Attia, S. Abou-Bakr and A.A.Nigm**

***Animal Production Department, Faculty of Agriculture, Cairo University, Giza, Egypt***
***Corresponding author: mohamed.atya23@yahoo.com***

## SUMMARY

*The level of genetic differentiation, gene flow and relationship among six different populations of Egyptian buffalo located in the Nile Delta region were analyzed using nine microsatellite DNA markers. The nine microsatellites were BM1329; BMS483; BM143; AFR227; BMS2460; CSSM38; CSSM70; ETH02 and BM1706. The total number of animals sampled was 312, collected from six governorates; Behera, Menoufia, Kaliobia, Giza, Sharkia and Alexandria. The mean estimates of global F-statistics over all loci were 0.038±0.018 and 0.015±0.003 for $F_{IT}$ and $F_{ST}$, respectively, indicating a low level of inbreeding within and among populations. This also points towards low genetic differentiation between populations. All studied populations showed deviations from Hardy-Weinberg equilibrium in all studied loci (P < 0.01). Depending on the genetic distances and identify, there was a great genetic relationship among the different populations. Values of gene flow or migration between populations were high, the mean migration rate (Nm) found across all studied populations was 11.94, meaning that migration and admixture could have taken place between these populations.*

*Factorial Correspondence Analysis (FCA) revealed a low breed-specific clustering. Analysis of molecular variance (AMOVA) revealed that 1.48% of the total genetic variation is among populations, while the remaining 98.5% corresponded to differences within populations (P<0.001). It was concluded that the classification of the Nile Delta buffalo populations into different breeds does not have strong genetic support at the microsatellite polymorphism level due to the high gene flow and the low genetic differentiation assessed between populations. The results indicate that the Nile Delta located buffalo could be considered one breed.*

***Keywords: Genetic differentiation, Egyptian buffalo, microsatellite markers***

## INTRODUCTION

The Egyptian buffalo (*Bubalus bubalis*) contributes significantly to the agricultural economy and food security in Egypt. Also, buffalo is the main dairy animal in Egypt, in addition to being an important source of red meat. There are about four million buffalo heads raised in Egypt, providing about 2.6 million tons of milk and about 0.4 million tons of meat per year (FAOSTAT, 2011). Moreover, buffalo plays a vital role in socioeconomic life of small farmers in Egypt.

Buffaloes are spread all over Egypt, with the highest concentration in the peri-urban areas and the Nile delta (Moioli *et al.*, 2001), where feed is more abundant. Buffalo sub-populations (e.g. breeds) in Egypt were mainly based on geographical origin within the country with little or no documented phenotypic or genetic differences that may help in characterizing these sub-populations. Examples of the breed names mentioned in the Egyptian and international literature are the Baladi (Native), Beheri, Monoufi, Masri and Saiedi (Elbeltagy *et al.*, 2008).

Genetic characterization at the molecular level to assess existing biodiversity among different Egyptian buffalo populations is an essential prerequisite to any effective breeding programs. The investigation of the genetic relationships among buffalo populations will provide a useful tool in supporting conservation decisions and may contribute to the selection and preservation of genetic resources (El-Kholy *et al.*, 2007).

Microsatellite DNA markers, also known as Simple Sequence Repeats (SSRs) or short tandem (STRs), are regions of DNA that exhibit short repetitive sequence motifs. Because of their high degree of polymorphism, random distribution across the genotypes, microsatellite markers have been proved to be one of the most powerful tools for evaluating genetic diversity and estimating genetic distances among close populations of ruminant species (Ellegren *et al.*, 1997).

Elbeltagy *et al.* (2008) investigated genetic diversity of the Nile-Delta and Southern-Egypt buffalo populations in comparison with the Italian buffalo. The authors used two microsatellite multiplexes and found that a lower but significant level of genetic variation exists between Southern-Egypt and the Nile-Delta located buffalo. Therefore, Southern-Egypt buffalo could be considered as a distinct population from the Nile-Delta buffalo. The authors indicated that more studies need to be carried out to study probable variations among further putative sub-populations (breeds/types) of the Nile-Delta buffalo.

The objective of the present study was to investigate the genetic differentiation, gene flow and relationship among six different populations of Egyptian buffalo distributed in the Nile Delta region.

## MATERIALS AND METHODS

***Selection of animals and blood samples collection:***

Blood samples were collected from 312 buffaloes belonging to six herds. The herds were located in six different Nile Delta governments and recorded by Cattle Information Systems/Egypt (CISE). The six governments are: Behera, Menoufia, Kaliobia, Giza, Sharkia and Alexandria, with no recorded relationship between animals in the different studied governorates.

A volume of 10 ml peripheral blood was collected from the jugular vein in Falcon tubes supplied with EDTA.The samples were stored temporarily at -20°C before DNA extraction.

***DNA Extraction:***

The DNA was isolated from the peripheral leukocytes using Fermentas® kits, Cat. No. k0512, Fermentas Life Science, EU, according to Sambrook and Russel, 2000. The Yield, concentration and purity of DNA of the samples were quantified using ScanDrop® 200, Anyltikajena, UK. The quality of the isolated genomic DNA was also checked by running in 0.8% a garose gel through a horizontal gel electrophoresis system (mini gel, Biometra® EU). A 100 to 1000 bp ladder (Solis®) was used and all samples were brought at the same concentration (50 ng/ µl).

***Microsatellite DNA Markers Selection and PCR conditions***

Nine microsatellite DNA markers, namely: BM1329; BMS483; BM143; AFR227; BMS2460; CSSM38; CSSM70; ETH02 and BM1706 were utilized. The PCR was carried out on 100 ng of the genomic DNA in a 20 µl reaction volume of 50 mM KCL, 10 mM Tris-Hcl (pH 8.8), 200 µM dNTP, 1.5 mM $MgCl_2$, 5 pmol of each primer and 1.0 U Taq DNA polymerase. The amplification was realized using thermal cycler PCR machine (G-Storm®, Gene Technologies, UK). The standard PCR run cycle is usually as follows: primary denaturation: 95 °C for 3 min. then: 35 cycles as: 95 °C for 30 sec.; 58-58.7 °C for 60 sec. and 72°C for 60 sec. Final extension: 72 °C for 5 min., storage: 4 °C forever. The presence of PCR products was analyzed using horizontal gel electrophoresis system (mini gel, Biometra® EU) using agarose gel 2% and stained with ethidium bromide. The successful runs were subjected to the vertical electrophoresis run on 8-12% acrylamide depending on the fragment size. A 100 to 1000 bp ladder (Solis®) was loaded and ran at the same time. The polyacrylamide gels were stained with ethidium bromide and the images were captured using gel documentation system (Gel DocXR®, Bio-Rad®). The Quantity one® software is used to measure the unknown bands of buffalo samples with reference to the Ladder. The alignment matrix of the unknown size bands and the ladder resulted in the range for the base pair for each allele for each sample.

***Statistical analysis:***

Microsatellite toolkit (Park, 2001) and Convert software version 1.3.1 (Glaubitz, 2004) were used to prepare input files for all other genetic software that were used. POPGENE 3.2 software package (Yeh *et al.*, 1999) was used to estimate the gene flow across all loci. To assess the population genetic structure of the six populations under study, Wright's F-statistic was estimated. The fixation indices per locus ($F_{IT}$ and $F_{ST}$) were calculated according to Weir and Cockerham (1984) using the FSTAT 2.9.3.2 software package (Goudet, 2002). The pairwise $F_{ST}$ estimates among the analysed populations were obtained with the ARLEQUIN 3.11(Excoffier *et al.*, 2005) computer program. GENETIX 4.05 software (Belkhir *et al.*, 1996-2004) was used to obtain the estimated gene flow beween pairs of the populations. GENEPOP 4.0 software (Raymond and Rousset, 1995) was used to carry out a test for Hardy-Weinberg equilibrium at each locus over all populations. To quantify the population structure within and between the analyzed genetic populations of buffalo, the Analysis of Molecular Variance (AMOVA) was obtained using ARLEQUIN 3.11 software. Factorial correspondence analysis (FCA) (Benzécri, 1982), carried out with GENETIX 4.05, was used to further investigate the differentiation of the populations. To detect the genetic relationship among the six buffalo populations, two methods were used. First, unbiased genetic identify and Nei's unbiased genetic distance DA (Nei, 1978). Secondly, genetic identity and Nei's standard genetic distance (Nei, 1972) were estimated using the POPGENE 3.2 software. Phylogenetic trees were constructed using Neighbor Joining (Saitou and Nei, 1987) based on Nei (1972) genetic distance using POPULATIONS1.2.28 software (Langella, 2002). TREEVIEW software (Page, 1996) was used to draw the dendrogram presentations. Bootstrap analyses with 1000 replicates were used to evaluate the internal consistency of the suggested groupings, as well as the magnitude of the sampling errors.

## RESULTS AND DISCUSSION

***Genetic differentiation among populations:***

Population differentiation was examined by fixation indices $F_{IT}$ and $F_{ST}$ for each locus and across all loci. The two fixation indexes ($F_{IT}$ and $F_{ST}$) could reflect the extent of inbreeding in populations. Table (1) shows F- statistic values of the whole populations at each locus and migration rate (Nm). The mean estimates of global F-statistics over all loci were 0.038±0.018 and 0.015±0.003 for $F_{IT}$ and $F_{ST}$, respectively. For the total inbreeding coefficient of individual related to whole population ($F_{IT}$), values ranged from -0.023 (BM143) to 0.145 (CSSM38). The $F_{ST}$ estimated indicate that the most informative marker as far as genetic differentiation is concerned was BMS2460 (0.031) and the least was BMS483 (0.003) for the studied populations. The low $F_{IT}$

values which are close to zero indicated a low level of inbreeding within and among populations and also pointed towards low genetic differentiation between populations. The $F_{ST}$ is the average inbreeding of the breed related to the whole population and the measure differentiation among populations (Falconer and Machay, 1996). $F_{ST}$ is very commonly used in diversity studies to detect selection through differences between subpopulations, and is often reported as the amount of genetic variation that can be explained by the difference between populations.

The average genetic differentiation between populations ($F_{ST}$) was 0.015 indicating that 1.5% of the genetic diversity can be explained by the genetic differentiation between populations whereas 98.5% can be explained by differences among individuals within the population. According to Wright (1978) $F_{ST}$ value below 0.05 is generally considered as a very low level of genetic differentiation, thus the populations under the present study showed a very low level of genetic differentiation.

A high level of inter population gene flow due to the migration and the admixture taking place between these populations might have played the major role for the low level of genetic differentiation observed in the studied populations. In addition, the lack of breed improvement and the uncontrolled mating practices could result in gene flow among populations. These results are in agreement with the average value of the migration rate (Nm) found across all the studied populations (11.94) presented in Table (1). The values of migration rate were positive and ranged from 7.2378 (BMS2460) to 23.0024 (BMS483). When Nm value was over 1, it shows no significant genetic differentiation and less than 1 shows genetic differentiation, while those over 4 show a great deal of gene exchange (Zhao *et al*., 2010).

**Table 1. F- Statistics ($F_{ST}$ and $F_{IT}$) for each locus across the whole populations and migration rate (Nm)**

| Locus | $F_{IT}$ | $F_{ST}$ | Nm |
|---|---|---|---|
| **BM1329** | 0.021 | 0.004 | 20.8912 |
| **BMS483** | 0.005 | 0.003 | 23.0024 |
| **BM143** | -0.023 | 0.006 | 18.8719 |
| **AFR227** | -0.004 | 0.005 | 20.2745 |
| **BMS2460** | 0.008 | 0.031 | 7.2378 |
| **CSSM38** | 0.145 | 0.016 | 10.7634 |
| **CSSM70** | 0.080 | 0.022 | 9.1747 |
| **ETH02** | 0.076 | 0.019 | 10.1415 |
| **BM1706** | 0.036 | 0.027 | 7.9662 |
| **Mean** | 0.038 | 0.015 | 11.9415 |
| **SE** | 0.018 | 0.003 | |

**Table 2. Estimated pair-wise $F_{ST}$ as a measure of the between populations differentiation (below diagonal) and the gene flow (above diagonal) between each pairs of the populations**

| | Behera | Menoufia | Kaliobia | Giza | Sharkia | Alexandria |
|---|---|---|---|---|---|---|
| **Behera** | **** | 15.89 | 18 | 17.16 | 13.56 | 17.52 |
| **Menoufia** | 0.016 | **** | 15.04 | 19.60 | 13.19 | 19.93 |
| **Kaliobia** | 0.014 | 0.017 | **** | 27.34 | 13.88 | 24.32 |
| **Giza** | 0.015 | 0.013 | 0.009 | **** | 14.47 | 14.80 |
| **Sharkia** | 0.018 | 0.019 | 0.018 | 0.017 | **** | 15.62 |
| **Alexandria** | 0.014 | 0.013 | 0.010 | 0.017 | 0.016 | **** |

Moioli *et al*. (2001) in their study for evaluating the genetic diversity between Italian, Greek and Egyptian buffalo populations using 13 microsatellites, found that the mean estimate for $F_{ST}$ was 0.057±0.015, the total inbreeding estimate was 0.240±0.042 and the mean estimate for $F_{ST}$ between the Italian and Greek populations was 0.031±0.015, while it was 0.070±0.020 between the Egyptian and both the Greek and Italian groups. Also, Elbeltagy *et al*. (2008) in their study to investigate the genetic diversity in the Nile-Delta and Southern-Egypt buffalo populations in comparison with the Italian buffalo using two microsatellite multiplex, found that a high level of genetic differentiation ($F_{ST}$ estimate) between the Italian group and each of the Delta and Southern Egypt group (0.083 and 0.076, respectively) was observed while the Southern Egypt group showed a lower level of genetic differentiation with the Delta group (0.014).

The pair-wise comparisons of population differentiation shown in Table (2) (below diagonal), indicate low genetic differentiation between the investigated populations. The $F_{ST}$ ranged from (0.009) between Kaliobia and Giza populations to the

highest genetically different populations with value of (0.019) between Menoufia and Sharkia populations.

***Deviations from Hardy- Weinberg equilibrium (HWE):***

All the studied populations showed deviations from HWE, based on genotypic frequencies for all combinations among loci and genetic populations with highly significant level in all the studied loci ($P<0.01$). These results are expected due to the selection applied for genetic improvement of economic traits, mainly milk production, in addition to the transfer of the animals from one place or market to another. These results are in agreement with the results obtained by Elbeltagy *et al.* (2008) in their study to investigate biodiversity in Mediterranean buffalo using microsatellite markers, who found that both the Italian and the Delta populations deviated significantly from HW equilibrium.

***Genetic distance and identity:***

Unbiased genetic identity and Nei's unbiased genetic distance DA (Nei, 1978) between the different populations under study are presented in Table (3). The lowest genetic distance value was (0.154) between Giza and Kaliobia populations. This is because the two regions are close to each other. In contrast, the highest genetic distance value was (0.266) between Menoufia and Sharkia populations. These results are in agreement with the highest genetic identify (0.857) between Giza and Kaliobia populations and the lowest genetic identify (0.766) between Menoufia and Sharkia populations.

According to the genetic identity and Nei's standard genetic distance (Nei, 1972) method, Table (4), the same results were obtained. The lowest genetic distance value was (0.201) between Giza and Kaliobia populations. The highest genetic distance value was (0.313) between Menoufia and Sharkia populations and the genetic identies were (0.818) and (0.732), respectively.

Genetic distance and genetic identity showed small differences and high similarity between each two populations. Genetic distance shows compatible results with the genetic differentiation measure $F_{ST}$.

***Gene flow***

The lowest value of genetic differentiation between populations is supported by high level of gene flow between each two population, Table (2). The gene flow between different pairs of buffalo populations varied between 13.19 (Sharkia - Menoufia) and 27.34 (Giza - Kaliobia), while the global gene flow across different populations overall loci was found to be 11.94. Laval *et al.*, (2000) reported that migration has a great effect on the reduction of genetic differentiation. The highest value of gene flow was observed between Giza and Kaliobia populations, which also have a lowest value of $F_{ST}$, indicating very high rate of transferring animals between these governorates. This could be explained by the geographical proximity between Kaliobia and Giza. The lowest value of gene flow was observed between Menoufia and Sharkia populations, which also have the highest value of $F_{ST}$ as a genetic differentiation.

**Table 3. Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal)**

| | Behera | Menoufia | Kaliobia | Giza | Sharkia | Alexandria |
|---|---|---|---|---|---|---|
| **Behera** | **** | 0.793 | 0.808 | 0.799 | 0.769 | 0.812 |
| **Menoufia** | 0.232 | **** | 0.788 | 0.825 | 0.766 | 0.834 |
| **Kaliobia** | 0.213 | 0.238 | **** | 0.857 | 0.774 | 0.853 |
| **Giza** | 0.224 | 0.193 | 0.154 | **** | 0.779 | 0.790 |
| **Sharkia** | 0.263 | 0.266 | 0.256 | 0.249 | **** | 0.801 |
| **Alexandria** | 0.209 | 0.181 | 0.159 | 0.238 | 0.222 | **** |

**Table 4. Nei's standard measures of genetic identity (above diagonal) and genetic distance (below diagonal)**

| | Behera | Menoufia | Kaliobia | Giza | Sharkia | Alexandria |
|---|---|---|---|---|---|---|
| **Behera** | **** | 0.757 | 0.771 | 0.763 | 0.735 | 0.776 |
| **Menoufia** | 0.278 | **** | 0.753 | 0.787 | 0.732 | 0.798 |
| **Kaliobia** | 0.259 | 0.284 | **** | 0.818 | 0.739 | 0.816 |
| **Giza** | 0.271 | 0.239 | 0.201 | **** | 0.744 | 0.753 |
| **Sharkia** | 0.308 | 0.313 | 0.301 | 0.295 | **** | 0.766 |
| **Alexandria** | 0.254 | 0.226 | 0.204 | 0.284 | 0.266 | **** |

***Factorial Correspondence Analysis (FCA).***

Factorial Correspondence Analysis (FCA) was used to detect admixtures between the six Egyptian buffalo populations and to understand the genetic structure of the studied populations. Graphical presentation of FCA plotted the individuals of the six populations of Egyptian buffalo in an overlapped state and exhibited low breed-specific clustering (Figure 1). These results support the fact that the studied populations showed a very low level of genetic differentiation.

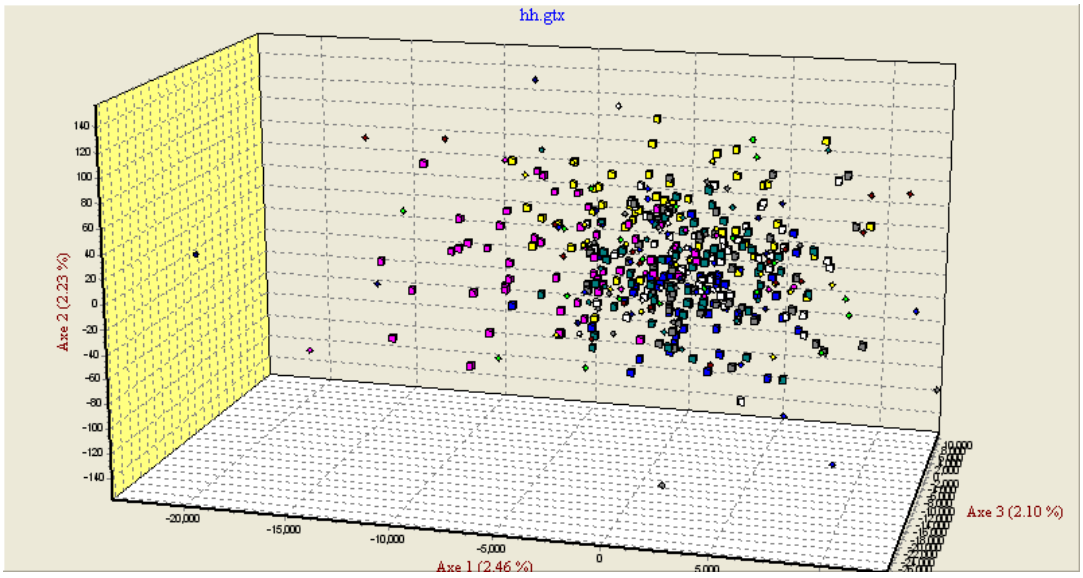


**Fig. 1. Graphical presentation of factorial correspondence analysis (FCA) showing distribution of individual animals of six Egyptian buffalo populations (blue: Giza; yellow: Behera; grey: Menoufia; white: Kaliobia; green: Alexandria; pink: Sharkia). Percentage value in each axe indicates contribution to the total genetic variation.**

**Table 5. Analysis of Molecular Variance (AMOVA)**

| Source of variation | d.f. | Sum of squares | Variance components | Percentage of variation |
|---|---|---|---|---|
| **Among populations** | 5 | 53.003 | 0.06163 | 1.48 |
| **Among individuals within populations** | 306 | 1282.356 | 0.09455 | 2.27 |
| **Within individuals** | 312 | 1248.5 | 4.0016 | 96.24 |
| **Total** | 623 | 2583.859 | 4.15779 | |

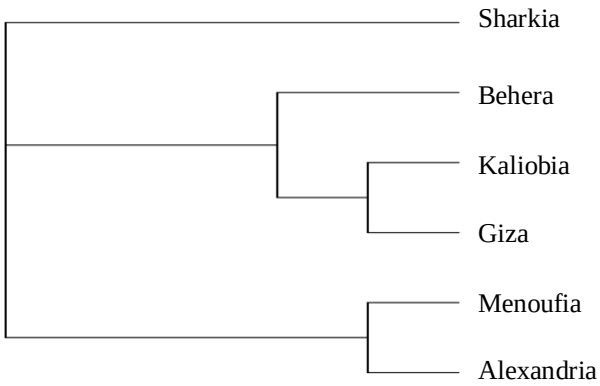


**Fig. 2. Neighbor joining of the six buffalo populations according to Nei's (1972) genetic distance**

***Analysis of molecular variance (AMOVA)***

In order to understand the partitioning of the level of genetic diversity of six Egyptian buffalo populations, an AMOVA analysis was conducted, Table (5). Percentages of variation among populations, among individuals within populations and within individuals were estimated ($p < 0.001$). The highest percentage of variation (96.17%) corresponded to within individual s component. Components among populations and among individuals within populations showed low magnitudes (1.48% and 2.34%, respectively). AMOVA indicated that only 1.48% of the total genetic variation is among populations, while the remaining 98.5% corresponded to differences within populations. AMOVA results agree with $F_{ST}$ results. Most of the genetic diversity in buffalo lies within breeds, and estimates of the percentage of diversity between populations vary between 2.8% in Chinese swamp populations (Zhang *et al*., 2007), 3.4–9.69% in Indian river breeds and local populations (Kumar *et al*., 2006 and Vijh *et al*., 2008), and 5.7% in Italian, Greek and Egyptian river breeds (Moioli *et al*., 2001).

***Phylogenetic relationship***

The results of the Neighbor Joining method are presented in Figure (2). The results of Neighbor Joining are consistent with the geographical location of these populations and support the genetic distance estimates. The dendrogram showed that the Nile Delta located populations resulted from mixing three main clusters. The tree indicated the close relationship between Giza and Kaliobia populations, and Behera population was clustered with the Giza / Kaliobia cluster. The close genetic relationship between Giza and Kaliobia and their geographic proximity suggest the possibility of admixture between the two populations. Likewise, Menoufia and Alexandria populations form a cluster. While Sharkia population is the most genetically and geographically distant and formed a separate cluster. The results revealed that geographically adjacent populations were genetically related, perhaps due to founder effects and mixing of the populations near bordering areas.

## CONCLUSION

The classification of the Egyptian Nile delta buffalo populations into different breeds does not have strong genetic support at the microsatellite polymorphism level. This was due to the high between-populations gene flow and consequently low genetic differentiation. Results indicated that the Nile Delta buffalo could be considered one type. The results obtained will further help for making effective breeding policies for genetic improvement and conservational activities considering microsatellite data.

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## الإختلافات والعلاقات الوراثيه بين جاموس دلتا النيل بإستخدام واسمات التوابع الوراثيه الدقيقه

**محمد عطية ، سامى أبو بكر ، علي عطيه نجم**

***قسم الإنتاج الحيوانى، كلية الزراعة، جامعة القاهرة،الجيزة، جمهورية مصر العربية***

أجريت هذه الدراسه بهدف دراسة الإختلافات والعلاقات الوراثية والهجرة بين ست من عشائر الجاموس في دلتا النيل وذلك بإستخدام تسعه من واسمات التوابع الوراثيه الدقيقه وهي: ETH02، CSSM70، CSSM38، BMS2460، AFR227، BM143، BMS483، BM1329 ، BM1706. تم جمع عينات الدم من 312 حيوان من ست محافظات مختلفه وهي : البحيره والمنوفيه والقليوبيه والجيزه والشرقيه والأسكندريه. كانت متوسطات معامل التربيه الداخليه داخل وبين العشائر($F_{IT}$ ، $F_{ST}$) 0.038±0.018 و0.015±0.003 بالترتيب مما يدل علي إنخفاض التربيه الداخليه داخل وبين العشائر وعدم وجود فروق وراثيه بينها حيث كان تقدير التفريق الوراثي ($F_{ST}$) ألاكثرمعلوماتيه (0.019) بين الشرقيه والمنوفيه بينما كان الأقل (0.009) بين الجيزه والقليوبيه. كما أظهرت جميع العشائر إنحراف عن اتزان هاردي فاينبرج ($p < 0.01$). أوضحت المسافات الوراثيه قيماً منخفضه بين كل العشائر تحت الدراسه والتي تعكس تماثلاً وراثياً بين العشائر. كانت قيم التدفق الجيني والهجره عاليه حيث كان معدل الهجره بين العشائر 11.94 مما يعني حدوث الهجره أو الخلط بين العشائر تحت الدراسه. أوضح تحليل عامل التماثل بين العشائر تحت الدراسه (FCA) تشابهاً وراثياً عالياً بين العشائر وعدم وجود سلاله مميزه. أظهر تحليل التباين الجزيئي أن 1.48% من مجموع الإختلافات الوراثيه يقع بين المجاميع وأن 98.5% تقع داخل المجموعات بين الأفراد ($p < 0.001$). تبين النتائج عدم وجود فروق وراثيه بين العشائر تحت الدراسه علي المستوي الجزيئي مما يؤكد أن عشائر الجاموس المصري في دلتا النيل تنتمي إلي عشيره واحده.