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Differentiation between four Bovidae species using two transfer RNAs

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ABSTRACT

Objectives: Advances in molecular techniques and the great information in the Genbank database offer new approaches to study evolution and genetic relationships in different populations. In the present work a small fragment of mtDNA was used for the differentiation between six animal groups representing four species from family Bovidae.

Methodology and Results: Small fragment of mitochondrial DNA (12bp of CYTB + MT-TT + MT-TP) was amplified and sequenced in 48 Egyptian river buffalo samples. Multiple sequence alignment with the corresponding fragment sequences in other river buffalo counterparts, swamp buffalo, domestic cattle, zebu cattle, sheep and goat in Genbank database indicated that the investigated fragment had a unique sequence for each of the investigated species or subspecies. Two phylogenetic trees were constructed to compare the performance of the investigated fragment in delineation of the six investigated groups with the performance of the CO1 DNA barcode in delineation of the same groups.

Conclusions and application of findings: This study revealed that the investigated fragment (12bp of CYTB + MT-TT + MT-TP) has a unique sequence for each of the studied species or subspecies and could be used for the identification of the investigated groups. The results of the two constructed phylogenetic trees also indicated that CO1 barcode-based tree was not able to discriminate between river buffalo and swamp buffalo subspecies, whereas, the phylogenetic tree constructed using the150 bp mitochondrial DNA fragment showed 100% success in delineation of the investigated groups. These results suggest that the investigated segment has a barcode character and could be used as a complementary locus to CO1 barcode in identifying and discriminating between the investigated groups and especially between the two subspecies of water buffalo species.

Key words: Bovidae, Egyptian river buffalo, Mitochondrial DNA, CYTB gene, MT-TT gene, MT-TP gene, CO1, Species identification, DNA bar-coding

INTRODUCTION

Advances in molecular techniques offer new approaches to study evolution and genetic relationships in different populations. DNA bar-coding as a genetic tool focuses on delineation of species rather than their relationships (Hajibabaei et al., 2007). It provides efficient method for species level identification through the use of a short genetic marker in an organism's DNA to identify it as belonging to a particular species. It differs from molecular phylogeny in that the main goal is not to determine classification but to identify an unknown sample in terms of a known classification (Kress et al., 2005). DNA bar-coding seeks to assemble a standardized reference library

for DNA-based identification of eukaryotic species (Kerr et al., 2007). The utility and limitations of this approach need to be tested on well-characterized taxonomic assemblages. This approach will contribute powerfully to taxonomic and biodiversity research (Hajibabaei et al., 2007), life history, ecological studies (Baker, 2008) and forensic analysis (Kress et al., 2005).

DNA bar-coding is based on the premise that a short standardized sequence can distinguish individuals of a species because genetic variation between species exceeds that within species (Hebert et al., 2003a; 2004a). The 5' half (648-bp region) of the mitochondrial cytochrome C oxidase subunit 1 (CO1) gene was proposed as the standard marker for DNA bar-coding in animal species (BOLD, 2003). The use of CO1 bar-coding in animals has proven to discriminate among species in most groups tested. It has proven to discriminate species of birds (Hebert et al., 2004a), fish (Ward et al., 2005; Hübert et al., 2008) lepidoptera (Hajibabaei et al., 2006) skipper butterfly (Hebert et al., 2004b) and freshwater Stingray (Toffoli et al., 2008).

Although CO1 has been accepted as the barcode region in animal species, researchers have suggested that other loci might also serve as a basis for species identification. Mitochondrial markers other than CO1 have been employed to identify samples to the species level. Small sequence (150 bp) of mtDNA cytochrome b gene (CYTB) was used for species identification of canned sardine (Jerome et al., 2003). Yan et al. (2005) also used the cytochrome b gene (CYTB) to identify Chinese alligators from fresh and partially cooked meat found in Chinese markets. Also, a nuclear gene 18S rDNA has been used for the identification of soil nematodes and other small organisms (Blaxter, 2004).

Other markers complementary to CO1 barcode have been used where CO1 sequences are not produced robustly or are found to be divergent within species as was shown in amphibians (Vences et al., 2005). They have also been used as further molecular evidence in the discovery of cryptic species as in case of some of parasitoid flies (Smith et al., 2006).

In a study conducted to evaluate the effectiveness of CO1 bar-coding in 18 species of family bovidae, Cai et al. (2010) showed that the only exception was in the case of water buffalo (*bulalus bubalis)* where intra-specific divergences were observed within the species using CO1 sequence as DNA bar-coding. These findings suggest the use of other loci complementary to the CO1 barcode in identifying the water buffalo and other members of family bovidae.

The domestic water buffalo is a species of a great economic potential, especially in the developing countries. It is a major source of milk and meat. Domestic water buffalo includes both river buffalo subspecies (*Bubalus bubalis bubalis)* and swamp buffalo subspecies (*Bubalus bubalis carabanesis) (*Roth, and Myers. 2004). Buffalo in Egypt are of the river type. According to Elbeltagy et al. (2008) Egyptian river buffalo are divided into two subpopulations the northern and the southern. They have been in Egypt for hundreds of years and have since become the most important livestock (Galal and Elbeltagy, 2006).

In the present work, the study explored the use of a fragment of mitochondrial DNA (150 bp), consisting of a partial sequence of CYTB gene (1-12 bp) and two successive transfer RNA genes; MT-TT (17-85 bp) and MT-TP (85-150 bp) coding for tRNA-Thr and tRNA-Pro respectively (12bp of CYTB + MT-TT + MT-TP), for the identification of four species from family Bovidae. Two of these species, water buffalo {*Bubalus bubalis*} and cattle {*Bos primigenius*} belong to subfamily Bovinae and the other two species sheep {*Ovis aries*} and Goat {*Capra aegagrus*} belong to subfamily Caprinae.

Water buffalo (*Bubalus bubalis*) in one hand includes two subspecies; the river buffalo (*Bubalus bubalis bubalis*) and swamp buffalo (*Bubalus bubalis carabenesis*). Cattle (*Bos primigenius*) on the other hand, has two subspecies which are the domestic cattle (*Bos primigenius taurus*) and zebu cattle (*Bos primigenius indicus*).

The performance of the investigated segment (12bp of CYTB + MT-TT + MT-TP) in delineation of the four bovidae species was compared with the performance of CO1 barcode in delineation of the same investigated species, since CO1 has been

accepted as the DNA barcode in animals. Therefore, two phylogenetic trees have been constructed. One of these constructed trees based on the sequence of Egyptian river buffalo fragment (12bp of CYTB + MT-TT + MT-TP) which has been determined in the present study and the corresponding sequences from GenBank database in each of the investigated species or subspecies. The other tree based on the published sequences in Genbank database for the 5' half of CO1 (650 bp) in the same investigated species or subspecies.

MATERIALS AND METHODS

Samples collection: A total of 48 samples were collected from the two main subpopulations of Egyptian river buffalo. Twenty-seven of these samples from unrelated buffaloes represent the Northern subpopulation and the other 21 unrelated samples represent the southern subpopulation. Blood samples were collected from the jugular vein into vacuutainer tubes containing ethylenediamine tetra-acetic acid (EDTA). DNA was isolated from blood samples using the extraction method of Sambrook and Russell (2001).

Sequencing of mitochondrial DNA: A mitochondrial DNA segment consisting of: a partial sequence of CYTB gene, two successive transfer RNA genes; MT-TT and MT-TP, D-loop and partial sequence of transfer RNA gene; MT-TF respectively was amplified in each buffalo DNA sample. Nucleotide sequences of the forward (5'TAGTGCTAATACCAACGGCC-3') and the reverse (5'AGGCATTTTCAGTGCCTTGC-3') primers were designed based on a published water buffalo mtDNA sequence (Parma et al., 2004).

Each PCR reaction was carried out in a total volume of 10 µl using 50 ng of isolated DNA, 5 pmol of each primer and 5 µl of 1× Ampli Taq Gold PCR Master Mix (Applied Biosystems). The reaction mixture was cycled in a thermocycler using the following reaction conditions: 95 °C (5 min), 35 cycles of 95 °C (1 min), 59 °C (45 s), 72 °C (2 min), followed by a final step at 72 °C (10 min).

PCR products were purified using Exo SAP-IT PCR Purification Kit (Applied Biosystems) following the manufacturer's recommended protocol. Sequencing reactions were performed using Big Dye TM terminator Cycle Sequencing Kit (Applied Biosystems). Sequences were determined using ABI3700 and 3730 automated DNA sequencers (Applied Biosystems).

Analysis of sequence data: The DNA sequences were edited manually using an AUTOASSEMBLER (Perkin Elmer). A fragment of mitochondrial DNA (150 bp), consisting of a partial sequence of CYTB gene (1-12 bp) and two successive transfer RNA genes; MT-TT (from 17-85 bp) and MT-TP (from 85-150 bp), was selected from all of the tested Egyptian river buffalo sequences and was investigated in the present study. The D-loop region has been studied elsewhere (Hassan et al., 2009). The nucleotide sequences of the investigated segment (12bp of CYTB + MT-TT + MT-TP) in all samples were analyzed. They were then compared with the corresponding sequences in the whole mitochondrial genome, published in Genbank of other river buffalo (accession numbers AY488491.1, AF547270.1), swamp buffalo (accession numbers NC_006295.1, AY702618.1), zebu cattle (accession numbers AY126697.1, AF492350.1, NC_005971.1), domestic cattle (accession numbers NC_006853.1, HM045018.1),sheep (accession numbers NC_001941.1, EF490456.1, EF490454.1, EF490452.1, EF490451.1), and goat (accession numbers AF533441.1, NC_005044.1, GU229280.1, GU229279.1, GU229278.1). The CLUSTAL-X program (Thompson et al., 1997) was used for multiple alignments.

A Bayesian phylogenetic tree was constructed using the 48 Egyptian buffalo sequences of the selected segment (12bp of CYTB + MT-TT + MT-TP) and the corresponding segment sequences in other river buffalo, swamp buffalo, zebu cattle, domestic cattle, sheep, and goat taken from the above accession numbers in GenBank. The corresponding segment sequence was taken from the whole mitochondrial genome (accession number: NC_001788.1) of Equus asinus (donkey) and was used as the out group. The tree was constructed by the Markov chain Monte Carlo (MCMC) method as implemented in the MRBAYES 3.1 package (Ronquist and Huelsenbeck, 2003) using the general time reversible substitution model with the invariant site plus eight gamma categories. Initial runs were performed to find out the fluctuating value of the likelihoods of Bayesian trees and stationarity was reached at 450,000 generations. The trees were sampled every 100 generations. The first 1150 sampled trees were discarded (burnin=1150) and the remaining tree samples (whose log likelihoods converged to stable values) were used to generate a 50% majority rule consensus tree. Bayesian analyses were repeated

four times. A second Bayesian barcode based-phylogenetic tree was constructed using the 5' half of CO1 gene sequence (650 bp) from the available Genbank database for the same investigated species or subspecies. The corresponding segment sequence (5' half of CO1 gene sequence) in Equus asinus (donkey) was used as the out group. Accession Numbers of river buffalo, swamp buffalo, domestic cattle, zebu cattle, sheep, goat and donkey whole mitochondrial genome sequences were the same as those used for the construction of the first Bayesian phylogenetic tree. Stationary was reached at 140,000 generations and the first 375 sampled trees were discarded (burnin=375).

RESULTS

The DNA sequences of 150 bp segment of the mitochondrial DNA (12bp of CYTB + MT-TT + MT-TP), which represents partial sequence of CYTB gene (1-12 bp) and two successive transfer RNA genes; MT-TT (17-85 bp) and MT-TP (85-150 bp), were determined in 48 Egyptian river buffalo samples and were analyzed. Multiple sequence alignment indicated that this segment (12bp of CYTB + MT-TT + MT-TP) is 100% conserved in all river buffalo samples from northern and southern Egypt and other counterparts, from Italy (accession number: AY488491.1) and India (accession number: AF547270.1). Sequences of the 48 Egyptian river buffalo DNA segments were submitted to Genbank with accession numbers from HM461918-HM461965. Alignment of Egyptian river buffalo sequence of the investigated segment (12bp of CYTB + MT-TT + MT-TP) and the corresponding sequences in the Genbank database for other river buffalo, swamp buffalo, domestic cattle, zebu cattle, sheep and goat is presented in table 1.

Table1: Alignment of Egyptian river buffalo sequence {150 bp: partial sequence of CYTB gene (from 1-12 pb), MT-TT gene (from 17-85 pb) and MT-TP (from 85 to 150 pb)} and the corresponding sequences in the data base for river buffalo, swamp buffalo, domestic cattle, zebu cattle, sheep and goat. The identical nucleotides between Egyptian river buffalo sequence and other investigated sequences are presented by dots.

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egy.riverbuffalo              1  TTAAAATGAAGACAAGTCTTTGTAGTATATTAAATACACTGGTCTTGTAAACCAGAAAAGGAGAACAACCAACCTCCCCA 80
riverbuffalo_AY488491.1          ................................................................................
riverbuffalo_ AF547270.1         ................................................................................
swampbuffalo_NC_006295.1         ..........................C.....................................................
swampbuffalo_ AY702618.1         ..........................C.....................................................
goat_AF533441.1                  C.......................C.A.C.................................T.G....T.....T.
goat_ NC_005044.1                C.......................C.A.C.................................T.G....T.....T.
goat_GU229280.1                  C.......................C.A.C.................................T.G....T.....T.
goat_GU229279.1                  C.......................C.A.C.................................T.G....T.....T.
goat_GU229278.1                  C.......................C.A.C.................................T.G....T.....T.
sheep_ NC_001941.1               C.......................C.A.C.....................G...................T.
sheep_ EF490456.1                C.......................C.A.C.....................G...................T.
sheep_ EF490454.1                C.......................C.A.C.....................G...................T.
sheep_ EF490452.1                C.......................C.A.C.....................G...................T.
sheep_ EF490451.1                C.......................C.A.C.....................G...................T.
domestic cattle_NC_006853.1      C............G..........C..CT.....................G...........T........T.
domestic cattle_ HM045018.1      C............G..........C..CT.....................G...........T........T.
zebu cattle_ AY126697.1          C............G..........C..CT.....................G...........T........T.
zebu cattle_ AF492350.1          C............G..........C..CT.....................G...........T........T.
zebu cattle_ NC_005971.1         C............G..........C..CT.....................G...........T........T.

egy.riverbuffalo             81  AGACTCAGGGAAGAGGCTATAGCCCCACTACCAACACCCAAAGCTGAAGTTCTATTTAAACTACTCCCTG  150
riverbuffalo_AY488491.1          ..........................................................................
riverbuffalo_ AF547270.1         ..........................................................................
swampbuffalo_NC_006295.1         ..........................................................................
swampbuffalo_ AY702618.1         ..........................................................................
goat_AF533441.1                  ......A.........C......T.....T..G.............A.............T......
goat_ NC_005044.1                ......A.........C......T.....T..G.............A.............T......
goat_GU229280.1                  ......A.........C......T.....T..G.............A.............T......
goat_GU229279.1                  ......A.........C......T.....T..G.............A.............T......
goat_GU229278.1                  ......A.........C......T.....T..G.............A.............T......
sheep_NC_001941.1                ......A......A..............T.....................C........T......
sheep_ EF490456.1                ......A......A..............T.....................C........T......
sheep_ EF490454.1                ......A......A..............T.....................C........T......
sheep_ EF490452.1                ......A......A..............T.....................C........T......
sheep_ EF490451.1                ......A......A..............T.....................C........T......
domestic cattle _NC_006853.1     ......A......AA..GC..T.T...C.T....C.....................T......
domestic cattle _ HM045018.1     ......A......AA..GC..T.T...C.T....C.....................T......
zebu cattle _ AY126697.1         ......A......AA..G...T.T...CGT...CC.....................T......
zebu cattle _ AF492350.1         ......A......AA..G...T.T...CGT...CC.....................T......
zebu cattle _ NC_005971.1        ......A......AA..G...T.T...CGT...CC.....................T......
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As seen from the table, individuals from each species or subspecies have 100% identical sequences. Twenty nine nucleotide variations are detected between the tested sequences. The percentage of similarity between water buffalo subspecies (river and swamp buffaloes) is 99.33% (only one nucleotide variation), whereas between cattle subspecies (domestic cattle and zebu cattle) it is 98.66% (2 nucleotides variation). A Bayesian phylogenetic tree, constructed based on the DNA sequence of the investigated segment (12bp of CYTB + MT-TT + MT-TP) of the 48 Egyptian river buffalo and the available corresponding sequences in Genbank for other river buffalo, swamp buffalo, zebu cattle, domestic cattle, sheep, goat and donkey (as an out group), is represented in figure 1.

Figure 1: A phylogenetic tree constructed from the 150 bp segment sequences from 48 Egyptian river buffalo and the available corresponding sequences in Genbank for river buffalo, swamp buffalo, zebu cattle, domestic cattle (cattle), sheep, goat and donkey (an out group). Accession numbers are given to Genbank selected samples.

The tree shows two distinct clades. The first clade includes all river buffalo samples and it has an internal branch for swamp buffalo. The second clade is re-branched into three branches; one branch has two sub-branches for cattle and zebu cattle whereas the other two branches are for sheep and goat.

The Bayesian barcode based- phylogenetic tree, constructed using the published 5' half of CO1 sequences in the same analyzed species or subspecies and using corresponding sequence in donkey as a root, and is presented in figure 2.

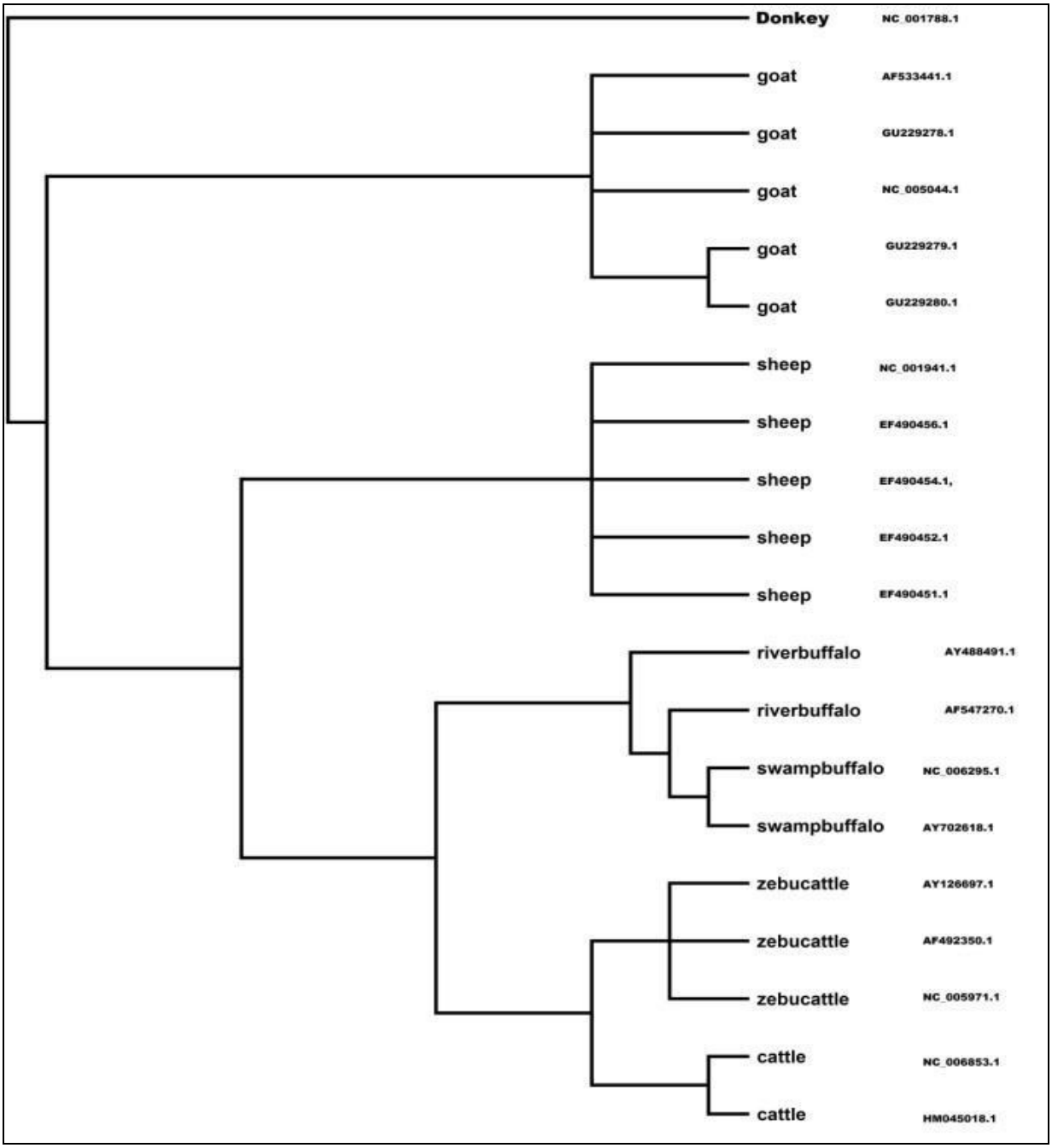

Figure 2: A phylogenetic tree assembled using the 5' half of CO1 gene sequence (650 bp) published in Genbank database for all the investigated species and donkey sequence as an out group. Accession numbers are given to all samples.

The tree shows two distinct clades. One clade includes all goat samples with an internal branch. The second clade has two sub-clades, one includes sheep samples and the other one is re-branched into two branches. One of these branches separates domestic cattle from zebu cattle in two sub-branches. The other branch includes river and swamp buffaloes. The tree illustrates that one of the two river buffalo samples (accession number: AF547270.1) clusters with swamp buffalo samples (accession numbers: NC_006295.1, AY702618.1) in one sub-branch whereas the second river buffalo sample (accession number: AY488491.1) clusters separately in the second sub-branch.

DISCUSSION

The use of short DNA sequences for the standardized identification of organisms has recently gained attention under the terms DNA bar-coding or DNA taxonomy (Floyd et al., 2002; Hebert et al., 2003a; Tautz et al., 2003). DNA bar-coding using the 5' half of cytochrome C oxidase 1 (CO1) has triggered a great debate between supporters who aim to identify all species (Hebert et al., 2003a,b; Hebert & Gregory 2005; Savolainen et al., 2005; Hajibabaei et al., 2007; Hübert et al., 2008) and opponents who highlight the pitfalls of the single-gene approach (Will & Rubinoff, 2004; Ebach & Holdrege, 2005; Wheeler, 2005; Will et al., 2005; Brower, 2006; Cameron et al., 2006; Rubinoff, 2006; Elias et al., 2007). It has been claimed that CO1 barcode achieves accuracy close to 100% in delimiting species in groups such as birds (Hebert et al., 2004a), fish (Hübert et al., 2008), lepidoptera (Hajibabaei et al., 2006) and skipper butterfly (Hebert et al., 2004b). Although in other groups like diptera (Meier et al., 2006) and amphibians (Vences et al., 2005), it has proved less successful where CO1 sequences were found to be divergent within their species.

Intra-specific divergences were also observed within water buffalo species from family bovidae using CO1 sequence as DNA bar-coding (Cai et al., 2010). In the present study we explored the use of a mitochondrial locus other than the 5' half of the mitochondrial gene CO1 for identifying water buffalo and three other species from family bovidae. A 150 bp mitochondrial DNA fragment covering a partial sequence of CYTB gene and two successive transfer RNA genes; MT-TT and MT-TP (12bp of CYTB + MT-TT + MT-TP) has been investigated in water buffalo (river buffalo and swamp buffalo), cattle (domestic cattle and zebu cattle), sheep and goat. The DNA sequence of the 150 bp fragment investigated in the present study was determined in 48 Egyptian river buffalo samples representing buffalo population in Egypt. The corresponding fragment sequences in other river buffalo counterparts, swamp buffalo, domestic cattle, zebu cattle, sheep and goat were retrieved from Genbank database. Sequence analysis indicated that the sequence of the fragment was 100% conserved in the river buffalo whether Egyptian or others. It was also 100% conserved within the other species or subspecies investigated (domestic and zebo cattle, sheep and goat). Twenty nine variable sites were detected between the fragment sequences in river buffalo, swamp buffalo, domestic cattle, zebu cattle, sheep and goat. However this fragment has a unique sequence for each of the investigated species or subspecies suggesting that it has a barcode character and could be used for identification of the investigated groups. A genetic marker suitable for DNA bar-coding needs to be sufficiently variable to discriminate among most species, but sufficiently conserved to be less variable within than between species (Hebert et al., 2003a, b; Hajibabaei et al., 2007).

The performance of the investigated segment (12bp of CYTB + MT-TT + MT-TP) in delineation of the four bovidae species was compared with the performance of CO1 barcode in delineation of the same investigated species by constructing two phylogenetic trees; one of them based on the sequence of the 150 bp fragment (12bp of CYTB + MT-TT + MT-TP) sequence whereas the other tree based on the 5' half sequence of CO1 (650 bp). The results obtained from the phylogenetic tree constructed using the150 bp mitochondrial DNA fragment (12bp of CYTB + MT-TT + MT-TP) sequence showed 100% success in delineating all the investigated species. Moreover it was able to discriminate between the two subspecies of water buffalo (river and swamp buffaloes) as it gathered the swamp buffalo sequences in one separate internal branch of the clade which includes river and swamp buffaloes sequence samples. It also was able to discriminate between the two subspecies of cattle (domestic cattle and zebu cattle) as they were separated in two separate sub-branches.

The barcode-based phylogenetic tree assembled using the 5' half of CO1 gene sequence (650 bp) in the investigated species successfully delineated the investigated species and discriminated between the two subspecies of cattle (domestic cattle and zebu cattle).

The only exception in the present study was in the case of water buffalo species were the CO1 barcode-based tree was not able to discriminate between river buffalo and swamp buffalo subspecies. These results supported the results previously reported by Cai et al. (2010). Results obtained from the CO1-based tree indicated that the only two available sequences in Genbank database for river buffalo CO1 are divergent as one of these sequences clustered with swamp buffalo samples and the other one clustered separately. This finding suggests the necessity of submitting more sequence data for the 5' half of CO1 gene of the two subspecies representing water buffalo species to Genbank database.

Also, the results obtained from the two phylogenetic trees demonstrated that the grouping above the species level does not match the accepted phylogeny of the investigated species. These findings agree with previous studies which demonstrated that short DNA sequences may delineate individual species with a high degree of confidence but they do not reliably uncover many of the phylogenetic relationships (Hajibabaei 2006; Hajibabaei 2007).

CONCLUSION

This study revealed that a short fragment (150 bp) of the mitochondrial DNA consisting of: a partial sequence of CYTB gene and the two successive transfer RNA genes; MT-TT and MT-TP showed 100% success in the identification and delineation of the investigated species or subspecies and that it could be used as a complementary locus for CO1 barcode in identifying and discriminating between the two subspecies of water buffalo species.

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REFERENCES

Baker, C.S. (2008) A truer measure of the market: the molecular ecology of fisheries and wildlife trade. Mol. Ecol. 17: 3985-3998.

Blaxter, M.L. (2004) The promise of a DNA taxonomy. Philos. Trans. R. Soc. Lond. B Biol. Sci. 359: 669–679.

BOLD; http//www.barcodeinglife.com

Brower, A.V.Z. (2006) Problems with DNA barcodes for species delimitation: 'ten species' of Astraptes fulgerator reassessed (Lepidoptera: Hesperiidae). Syst. Biodiv. 4: 127–132. (doi:10.1017/S147720000500191X)

Cai, Y.S., Zhang, L., Shen, F.J., Zhang, W.P., Hou, R., Yue, B.S., Li, J., Zhang, Z.H. (2010) DNA bar-coding of 18 species of bovidae. Chinese Science Bulletin, 56 (2): 164-168. (doi: 10.1007/s11434-010-4302-1)

Cameron, S., Rubinoff, D., Will, K. (2006) Who will actually use DNA bar-coding and what will it cost? Syst. Biol. 55: 844–847. (doi:10.1080/10635150600960079)

Ebach, M.C., Holdrege, C. (2005) DNA bar-coding is no substitute for taxonomy. Nature 434: 697–697. (doi:10.1038/434697b)

Elbeltagy, A.R., Galal, S., Abdelsalam, A.Z., El Keraby, F.E., Blasi, M., Mohamed, M.M. (2008) Biodiversity in Mediterranean buffalo using two microsatellite multiplexes. Livest Sci. 114: 341–346.

Elias M., Hill, R.I., Willmott, K.R., Dasmahapatra, K.K., Brower, A.V.Z., Mallet, J., Jiggins, C.D. (2007) Limited performance of DNA bar-coding in a diverse community of tropical butterflies. Proc. Soc. B. 274: 2881-2889.

Floyd, R., Eyualem, A., Papert, A., Blaxter, M.L. (2002) Molecular barcodes for soil nematode identification. Mol. Ecol. 11: 839-850.

Galal, S., Elbeltagy, A. (2006) Achievement of research in the field of buffalo production in Egypt. WAAP Book of the Year, pp. 177–188.

Hajibabaei, M., Singer, G.A.C., Hickey, D.A. (2006) Benchmarking DNA barcodes: an assessment using available primate sequences. Genome 49: 851-854

Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N., Hickey, D.A. (2007) DNA bar-coding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet. 23: 167–172. (doi:10.1016/j.tig.2007.02.001)

Hassan, A.A., El Nahas, S.M., Kumar, S., Godithala, P.S., Roushdy, K.H. (2009) Mitochondrial D-

loop nucleotide sequences of Egyptian river buffalo: Variation and phylogeny studies. Livestock Science 125: 37-42.

Hebert, P.D.N., Gregory, T.R. (2005) The promise of DNA bar-coding for taxonomy. Syst. Biol. 54: 852–859. (doi:10.1080/10635150500354886)

Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R (2003a) Biological identifications through DNA barcodes. Proc. R. Soc. B 270: 313–321. (doi:10.1098/rspb.2002.2218)

Hebert, P. D. N., Ratnasingham, S., deWaard, J. R. (2003b) Bar-coding animal life: cytochrome c oxidase subunit1 divergences among closely related species. Proc. R. Soc. B. 270: S96–S99. (doi:10.1098/rsbl.2003.0025)

Hebert, P. D. N., Stoeckle, M. Y., Zemlak, T. S., Francis, C. M. (2004a) Identification of birds through DNA barcodes. PLoS Biol. 2: 1657–1663. (doi:10.1371/journal.pbio.0020312)

Hebert, P.D.N., Penton, E. H., Burns, J. M., Janzen, D.H., Hallwachs, W. (2004b) Ten species in one: DNA bar-coding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. Proc. Natl Acad. Sci. USA 101: 14 812–14 817. (doi:10.1073/pnas.0406166101)

Hübert, N., Hanner, R., Mandrak, N.E., Taylor, E., Burridge, M., Watkinson, D., Dumont, P., Curry, A., Bentzen, P., Zhang, J., April, J., Bernatchez, L. (2008) Identifying Canadian freshwater fishes through DNA Barcodes. PLOS ONE 3(6): e2490. (Doi:10.1371/journal.pone.0002490)

Jerome, M., Lemaire, C., Verrez-Bagnis, V., Etienne, M. (2003) Direct sequencing method for species identification of canned sardine and sardine-type products. J.Agric. Food chem. 51: 7326-7332. (DOI: 10.1021/jf034652t)

Kerr, K.C.R., Stoeckel, M.Y., Dove, C.J., Weigt, L.A., Francis, C.M., Hebert, P.D.M. (2007) Comprehensive DNA barcode coverage of North American birds. Molecular Ecology Notes 7(4): 535-543

Kress, W.J., Wurdack K.J., Zimmer E.A., Janzen D.H. (2005) Use of DNA barcodes to identify flowering plants. Proc. Natl. Acad. Sci. U. S. A. 102: 8369–8374.

Meier, R., Shiyang, K., Vaidya, G., Ng, P.K.L. (2006) DNA bar-coding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. Syst. Biol. 55: 715–728. (doi:10.1080/10635150600969864).

Parma, P., Erra-Pujada, M., Feligini, M., Greppi G., Anne, G. (2004) Water buffalo (Bubalus bubalis): Complete nucleotide mitochondrial genome sequence. DNA Sequence 15(5/6): 369-373.

Roth, J. and P. Myers. (2004). "Bubalus bubalis" (On-line), Animal Diversity Web(http://animaldiversity.ummz.umich.edu/site/accounts/information/Bubalus_bubalis.html

Ronquist, F., Huelsenbeck, J.P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Rubinoff, D., 2006. Utility of mitochondrial DNA barcodes in species conservation. Conserv. Biol. 20: 1026–1033. (doi:10.1111/j.1523-1739.2006.00542.x)

Sambrook, J., Russell, D. (2001) Molecular cloning: A laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press.

Savolainen, V., Cowan, R.S., Vogler, A.P., Roderick, G.K., Lane, R. (2005) Towards writing the encyclopaedia of life: an introduction to DNA bar-coding. Phil. Trans. R. Soc. B. 360: 1805–1811. (doi:10.1098/rstb.2005.1730)

Smith, M.A., Woodley, N.E., Janzen, D.H., Hallwachs, W., Hebert, P.D.N. (2006) DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). Proc. Natl. Acad. Sci. U. S. A. 103: 3657–3662.

Tautz, D., Arctander, P., Minelli, A., Thomas, R.H., Vogler, A.P. (2003) A plea for DNA taxonomy. Trends Ecol Evol. 18: 70-74.

Toffoli, D., Hrbek, T., de Araujo, M.L.G., de Almeida, M.P., Chavert-Almeida, P., Farias, I.P. (2008) Atest of the utility of DNA bar-coding in the radiation of the freshwater stingray genus Potamotrygon (Potamotrygonidae, Myliobatiformes). Genetics and Molecular Biology, 31 (1): 324-336.

Vences, M., Thomas, M., Bonett, R.M., Vieites, D.R. (2005) Deciphering amphibian diversity through DNA bar-coding: chances and challenges. Philosophical Transactions of the Royal Society, London, Ser. B, 360: 1859-1868.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., Hebert, P.D.N. (2005) DNA bar-coding Australia's fish species. Phil.Trans. R. Soc. Lond. B, Biol Sci. 360:1-11.

Wheeler, Q.D. (2005) Losing the plot: DNA "barcodes" and taxonomy. Cladistics 21: 405–407. (doi:10.1111/j.1096-0031.2005.00075.x)

Will, K.W. & Rubinoff, D. (2004) Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics 20: 47–55. (doi:10.1111/j.1096-0031.2003.00008.x)

Will, K.W., Mishler, B.D., Wheeler, Q.D. (2005) The perils of DNA barcoding and the need for integrative taxonomy. Syst. Biol. 54: 844–851. (doi:10.1080/10635150500354878)

Yan P., Wu X.B., Shi Y., Gu C.M., Wang R.P., Wang C.L. (2005) Identification of Chinese alligators (Alligator sinensis) meat by diagnostic PCR of the mitochondrial cytochrome b. Biological Conservation, 121(1): 45-51.