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Research paper

First characterization of major histocompatibility complex class II *DRB3* diversity in cattle breeds raised in Egypt

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ABSTRACT

Genes encoding bovine leukocyte antigen (BoLA) enable the immune system to identify pathogens. Therefore, these genes have been used as genetic markers for infectious and autoimmune diseases as well as for immunological traits in cattle. Although BoLA polymorphisms have been reported in various cattle breeds worldwide, they have not been studied in cattle populations in Egypt. In this study, we characterized *BoLA-DRB3* in two local Egyptian populations and one foreign population using polymerase chain reaction-sequence-based typing (PCR-SBT) method. Fifty-four previously reported *BoLA-DRB3* alleles and eight new alleles (*BoLA-DRB3*005:08, *015:07*, *016:03, *017:04, **020:02:02*, **021:03*, **164:01*, and **165:01*) were identified. Alignment analysis of the eight new alleles revealed 90.7–98.9 %, and 83.1–97.8 % nucleotide and amino acid identities, respectively, with the *BoLA-DRB3* cDNA clone NR-1. Interestingly, *BoLA-DRB3* in Egyptian cattle showed a high degree of allelic diversity in native (na = 28, $h_E > 0.95$), mixed (na = 61, $h_E > 0.96$), and Holstein (na = 18, $h_E > 0.88$) populations. *BoLA-DRB3*002:01* (14.3 %), *BoLA-DRB3*001:01* (8.5 %), and *BoLA-DRB3*015:01* (20.2 %) were the most frequent alleles in native, mixed, and Holstein populations, respectively, indicating that the genetic profiles differed in each population. Based on the allele frequencies of *BoLA-DRB3*, genetic variation among Egyptian, Asian, African, and American breeds was examined using Nei's distances and principal component analysis. The results suggested that native and mixed cattle populations were most closely associated with African breeds in terms of their gene pool, whereas Holstein cattle were more distinct from the other breeds and were closely related to Holstein cattle populations from other countries.

Abbreviations: BoLA, bovine leukocyte antigen; PCR-SBT, polymerase chain reaction sequence-based typing; MHC, major histocompatibility complex; BLV, bovine leukemia virus; RCOE-SVU, Research Code of Ethics; SBT, sequenced-based typing; NJ, neighbor-joining; Na, number of alleles; h_O, observed heterozygosity; h_E, expected heterozygosity; HWE, Hardy Weinberg equilibrium; F_{ST}, F-statistics; PCA, Principal component analysis; F_{IS}, inbreeding coefficient; PCs, principal components.

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1. Introduction

The major histocompatibility complex (MHC) system in cattle is known as bovine leukocyte antigen (BoLA). A major rearrangement within the class II region has led to the division of the BoLA region into two distinct subregions, known as class IIa and class IIb, on chromosome 23 (Takeshima and Aida, 2006). MHC class II genes, which are highly polymorphic, have been linked to immune responses in various infectious diseases (Rothschild et al., 2000; Sharif et al., 1998; Takeshima and Aida, 2006). Among these genes, *BoLA-DRB3* is the most polymorphic locus (Aida et al., 1995); its polymorphisms, which are primarily in the second exon and encode the peptide-binding pocket of the molecule (Takeshima and Aida, 2006), have been extensively studied in cattle breeds from different geographical regions (Giovambattista et al., 2013; Miyasaka et al., 2012; Takeshima et al., 2003). However, *BoLA-DRB3* diversity in African cattle populations is not well-understoo. To date, 385 BoLA-DRB3 alleles have been listed in the IPD-MHC database (https://www.ebi.ac.uk/ipd/mhc/group/BoLA/; accessed on 3rd March 2024). These sequence variations have a major impact on variability in immune responsiveness and disease resistance (Baxter et al., 2008). Accordingly, numerous studies revealed that *BoLA-DRB3* polymorphisms are associated with the resistance or susceptibility to certain infectious diseases, such as bovine leukosis (Juliarena et al., 2008; Lo et al., 2020, 2021), mastitis (Rupp et al., 2007), foot and mouth disease (Othman et al., 2018), bovine herpesvirus 1 (Morales et al., 2020), bovine papillomavirus-induced bladder cancer (Longeri et al., 2021), dermatophilosis (Maillard et al., 2003), production traits (Sharif et al., 1999), and vaccine responses and immune system traits (Ballingall et al., 2004; Baxter et al., 2009).

Analysis of *BoLA-DRB3* polymorphism may provide a basis for inferring the evolutionary history of the MHC in ruminant species (Behl et al., 2012). Many studies showed that *BoLA-DRB3* allele frequencies differ among cattle breeds. For example, allele profiles vary among Jersey, Holstein, Latin American Creole, Japanese Shorthorn, Chinese Yellow, Iranian Sistani, Russian Ayrshire, and Japanese Black cattle (Behl et al., 2012; Giovambattista et al., 2013, 2020a; Miyasaka et al., 2011; Takeshima et al., 2003). Moreover, a high degree of genetic variability was reported for *BoLA-DRB3* polymorphisms among populations of indicine and taurine cattle (Takeshima et al., 2018). Thus, allelic data are essential for determining the link between MHC and disease, as well as for resolving issues pertaining to recording breed- and location-specific MHC allele frequencies in cattle (Takeshima et al., 2018). However, information regarding allelic polymorphism in *BoLA-DRB3* of various populations of Egyptian cattle appears to be lacking.

Egypt is a transcontinental country spanning north-east Africa and south-west Asia, connected via a land bridge formed by the Sinai Peninsula. Egypt has one of the longest histories of any country, and cattle were domesticated by farmers in ancient Egypt (Bateman, 2018). Various researchers have hypothesized that native Egyptian cattle descended as hybrids from humped cattle from Asia (Hamitic cattle) and short-horned humpless cattle introduced from Asia and Europe (Joshi et al., 1957). Other authors suggested that the cattle depicted in ancient Egyptian murals and engravings are descendants of African aurochs and Hamitic longhorns. Decker et al. (2014) asserted that domestication evidence for African taurines arose from the introduction of domesticated Fertile Crescent taurines and their hybridization with wild African aurochs (Decker et al., 2014). According to Felius et al. (2011), the ancestry of modern Egyptian cattle formed a transitional type between the breeds of North Africa (African aurochs) and Mediterranean Asia (taurine cattle domestication center) (Felius et al., 2011). Present-day Egyptian native cattle breeds are solely taurine cattle (Galal, 2007). Over the years, native Egyptian cattle have adapted to the Egyptian climate, such as by developing tolerance to endemic diseases and the ability to produce and reproduce under non-ideal feeding conditions. However, a well-coordinated national breeding strategy is currently lacking (Osman et al., 2016). Since the 1990 s, the Egyptian government has sought to develop well-organized national breeding programs to improve cattle production. Such programs aim to produce thousands of crossbred females (e.g., native × Holstein or native × Tarentaise) through selective breeding or artificial insemination to provide several economic advantages at the national level. These efforts would ensure the survival of local cattle populations as well as improve cattle production. With the growing demand for animal products, native cattle have been indiscriminately crossbred with exotic cattle to produce better-performing crossbreds, with neighborhood farmers neglecting the conservation of indigenous native cattle genetic resources (Delgado et al., 2012). This breeding has led to the scarcity of pure-bred local cattle in some regions of the country, resulting in a sizable, dispersed population maintained by neighborhood farmers in small- to medium-sized herds under the umbrella term “mixed,” Arabic nomenclature; khalit“ as described and characterized by the Ministry of Agriculture and Land Reclamation. The genetic resources of Egyptian native cattle have shown potentially alarming trends in genetic erosion because of declines in the proportion of local cattle following increases in the number of crossbred (mixed) cattle. Consequently, only a few Egyptian farmers rear local native breeds. Additionally, the importation of exotic breeds, such as Holstein, Fresian, Brown Swiss, Abondance, and Tarentaise and Simmental appears to be continuing (Faid-Allah et al., 2018; Galal, 2007; Osman et al., 2016). The FAOSTAT database estimates that Egypt owned 4.5 million cattle heads in 2018. However, large losses caused by infectious disease outbreaks led to a gradual decline in the number of livestock in the country, with 2.8 million cattle heads in 2021 (https://www.fao.org/faostat/en/#data/QCL, accessed 19 February 2023). Thus, it is vital to breed cattle capable of resisting infectious diseases. However, the bovine MHC gene, a marker of anti-disease properties, has not been adequately analyzed in Egyptian cattle.

Understanding the allelic distribution of *BoLA-DRB3* among cattle breeds in various geographic regions and countries may reveal the effects of MHC polymorphisms on immune response efficacy and disease susceptibility, thereby improving livestock research (Takeshima and Aida, 2006). However, information on allele frequencies (obtained via sequence-based typing) is currently limited to a small number of the 1,050 breeds of cattle recognized globally (FAO, 2021; https://www.fao.org/3/ng620en/ng620en.pdf). Information pertaining to allelic polymorphisms of *BoLA-DRB3* in various Egyptian cattle populations is unavailable. Therefore, this study aimed to examine the allelic diversity of *BoLA-DRB3* in Egyptian cattle.

2. Materials and methods

2.1. Ethics statement

All animals were handled in accordance with good animal practices and complied with the rules of the Animal Ethics Committee at the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt, as well as with the RIKEN guidelines, Japan. The samples used in this study were reviewed and approved by the Research Code of Ethics (RCOE-SVU) at South Valley University and the RIKEN Animal Experiments Committee (approval number H29-2–104).

2.2. Animals and extraction of genomic DNA

Blood samples were collected from 343 cattle: 121 Holstein, 28 native Egyptian, and 194 mixed cattle. Samples were collected from 23 cattle farms and four abattoirs across six Egyptian provinces in southern (Qena and Luxor), middle (Cairo and Fayoum), and northern (Beheira and Damietta) Egypt. Animals of both sexes, ranging in age from 3 months to 12 years, were randomly selected from the herds and from purebred animals (Table 1). Genomic DNA was extracted from 300 μL of whole blood using a Wizard Genomic DNA Purification Kit (Promega; Madison, WI, USA), following the manufacturer’s instructions. The concentration, quality (A260/280), and purity (A260/230) of the

Table 1
Samples origin regarding the cattle populations analyzed in this study.

Province	Number farms/abattoirs	Number of investigated cattle		
		Native	Mixed	Holstein
Damietta	1/0	0	0	40
Beheira	15/0	1	83	42
Cairo	0/1	0	40	0
Fayoum	0/1	0	12	0
Qena	6/2	25	45	39
Luxor	1/0	2	14	0
Total	23/4	28	194	121

extracted DNA samples were assessed using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For polymerase chain reaction (PCR) assays, DNA samples were diluted in nuclease-free water to a final concentration of 30 ng/μL.

2.3. BoLA-DRB3 typing

BoLA-DRB3 alleles were genotyped using PCR sequence-based typing (SBT). Briefly, *DRB3* exon 2 was amplified via single PCR as described by Takeshima et al. (2011) using the primers DRB3FRW and DRB3REV. These primers were exon-spanning and corresponded to nucleotides 6345–6367 and 6648–6663 of bovine *DRB3* (GenBank Accession number NC_037350.1 REGION: 25723717..25734819) (Table 2). The PCR reactions contained 10 pmol/μL of each primer, 1.0 μL of template from the DNA samples, and r-Taq DNA Polymerase reaction mixture containing rTaq DNA polymerase (2.5U/μL), 10 × buffer, 25 mM $MgCl_2$, and 2 mM dNTPs (Toyobo; Tokyo, Japan) in a reaction volume specified by the manufacturer's instructions. The PCR conditions for thermocycling consisted of a pre-denaturation at 94 °C for 2 min; followed by 35 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min and a final extension step at 72 °C for 2 min. The amplified PCR fragments were purified using an ExoSAP-IT PCR product purification kit (USB Corp., Cleveland, OH, USA) and sequenced using an ABI PRISM BigDye1.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Raw sequence data were analyzed using Assign 400ATF ver. 1.0.2.41 software (Conexio Genomics, Fremantle, Australia) to determine the *BoLA-DRB3* genotype. When sequences could not be split into two known alleles, we predicted the existence of a new allele. SBT-PCR was performed repeatedly for each individual sample from animals thought to carry a new allele or alleles for multiple independent sequencing events (minimum of three replicates) to confirm the presence of real mutations. In heterozygous individuals, the novel allele was sequenced separately from the second allele using the T-A cloning method if either one or both alleles were novel.

2.4. Identification of new alleles by PCR cloning

Using the T-A cloning method, PCR products of the predicted new alleles were cloned into the pGEM-T Easy Vector (Promega; Madison, WI, USA), and transformed into *Escherichia coli* goldx10 competent cells. Several clones were selected via blue-white spot screening. Following *BoLA-DRB3* PCR typing of the extracted plasmid vector DNA from each clone, the PCR products were sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems; Foster City, CA, USA), and sequencing was performed in both directions. Using Assign 400ATF ver. 1.0.2.41, chromatograms of the sequence data from each clone were examined. To be considered novel, each allele required at least three identical clones sharing the same mismatched nucleotide(s) as the reference allele. The predicted novel sequences were submitted to the IPD database and identified as new alleles.

Table 2
Sequences of the used primer in this study.

Target gene	Primer ID	Primer sequences 5'-3'	Primer location [a]
DRB3 exon 2	DRB3FRW	CGCTCCTGTGAYCAGATCTATCC	NC_037350.1 (6345–6367)
	DRB3REV	CACCCCCGCGCTCACC	NC_037350.1 (6648–6663)

[a] Primer location described corresponding to nucleotide positions of the whole *DRB3* genomic sequence recorded in GenBank (accession No. NC_037350.1).

2.5. Sequence data analysis and phylogenetic tree construction

All chromatograms were manually adjusted using MEGA 7 software prior to analysis (Kumar et al., 2016). The ClustalW tool was used to perform numerous sequence alignments to reconstruct a 281 bp segment of exon 2 of *BoLA-DRB3*. A neighbor-joining (NJ) tree was drawn using the 234-bp nucleotide sequences of the β1 domain coding-regions of the *BoLA-DRB3* alleles identified in the native, mixed, and Holstein cattle. Evolutionary distances were calculated using the Jukes-Cantor with the gamma distribution model, and a tree was built based on the NJ method. We used 1000 replicates for bootstrapping and constructed the tree using MEGA 7.

2.6. Genetic variability, genetic distances, and population tree

Allele frequencies and the number of alleles (n_a) were determined by direct counting. The distribution of alleles across breeds was analyzed using a Venn plot created using the R package 'VennDiagram' (https://cran.r-project.org/). The observed (h_O) and unbiased expected heterozygosity (h_E) for the *BoLA-DRB3* locus were estimated as described by Nei (1978) using ARLEQUIN v.3.5, a software program used for population genetic analyses (Excoffier and Lischer, 2010). The Hardy–Weinberg equilibrium-p value (HWE-p) was measured using the exact test of HWE using ARLEQUIN v.3.5 software. In addition, Ewens–Watterson–Slatkin exact test of neutrality was performed as described by Slatkin (1996) and implemented in Arlequin v.3.5 software. Genetic differentiation of gene frequencies among populations was assessed using Wright's F-statistics (F_{ST}) and the variance-based method reported by Weir and Cockerham (1984) using ARLEQUIN v.3.5 software. To evaluate the relationships among breeds based on *BoLA-DRB3* polymorphisms, Nei's *DA* distances (Nei and Chesser, 1983) were calculated using allele frequencies, and cluster analysis was performed using NJ algorithms (Saitou and Nei, 1987). Confidence in the groupings was estimated through bootstrap resampling of data using 1000 replications. Genetic distances and phylogenetic trees were derived using POPTREE2 software (Takezaki et al., 2010). Principal component analysis (PCA), as described by Cavalli-Sforza (Cavalli-Sforza et al., 1994), was conducted using allele frequencies to condense genetic diversity at the *BoLA-DRB3* locus using Past software (Hammer et al., 2001). The NJ tree and PCA were performed using the *BoLA-DRB3* allele frequencies from the Egyptian native, mixed, and Holstein cattle obtained in this study as well as from nine previously reported breeds, Japanese Black and Japanese Holstein (Takeshima et al., 2003), Chilean Holstein (Takeshima et al., 2015), Bolivian Yacumeño creole (Giovambattista et al., 2013), Sudanese Baggara breed (Salim et al., 2020), Myanmar Pyer Sein (Giovambattista et al., 2020b), Philippine Brahman (Takeshima et al., 2014), and Bolivian Nellore and Peruvian Nellore-Brahman mixed cattle (Takeshima et al., 2018).

3. Results

3.1. Identification of BoLA-DRB3 alleles obtained from three different cattle populations in Egypt

We genotyped 343 individuals from three Egyptian populations (28 native, 194 mixed, and 121 Holstein cattle) for *BoLA-DRB3* using PCR-

SBT. This analysis revealed 62 *BoLA-DRB3* alleles, eight of which were novel variants and 54 were previously reported. The Venn diagram revealed that of the 62 *BoLA-DRB3* alleles identified in Egyptian cattle, 13 were unique to the mixed cattle population (Fig. 1**A**), while, 15 and four variants present in the mixed population were shared with native and Holstein populations, respectively, supporting that mixed cattle result from crossbreeding between native and Exotic animals. None of the variants were native, and only one variant was present in Holstein cattle.

The eight novel allele sequences were submitted to IPD-MHC (https://www.ebi.ac.uk/ipd/mhc/group/BoLA), where they were curated by experts and official names were assigned. Fig. 2 showed the alignment of the nucleotide sequences of the second exon of *BoLA-DRB3* for the eight novel alleles, showing a difference from the cDNA clone NR-1 (*BoLA-DRB3*016:01* allele; Aida et al., 1995). The new variants differed from the closer reference alleles as follows: *BoLA-DRB3*016:03* (detected in an individual from mixed cattle population from Cairo province) differed from *DRB3*016:01* by two non-synonymous changes at sites 206 (G>C) and 209–210 (AA>GC); *BoLA-DRB3*015:07* (present in one individual from a mixed population in Qena province) varied from *BoLA-DRB3*015:01* by one non-synonymous substitution at position 29 (T > G); *BoLA-DRB3*165:01* (found in two individuals from a mixed population in Beheira province) differed from *BoLA-DRB3*015:01* by one synonymous change at position 206–208 (CGG > GAC) and two non-synonymous changes at positions 217 (C > T) and 219–220 (AT > CG); the fourth was *BoLA-DRB3*021:03* (identified in one individual from a mixed population in Qena province) differed from *BoLA-DRB3*021:01* by a non-synonymous substitution at position 141 (G > A); *BoLA-DRB3*020:02:02* (detected in two heads of a mixed population from Beheira and Luxor provinces and one native breed from Qena province) diverged from *BoLA-DRB3*020:02* by a non-synonymous substitution at position 157 (G > A); *BoLA-DRB3*164:01* (detected in one animals from a mixed population in Luxor province) differed from *BoLA-DRB3*011:04* in five non-synonymous replacements at positions 27 (A > C), 72 (G > A), 86 (C > T), 138 (T > A), and 210 (A > G), as well as two synonymous substitutions at sites 124 (C > T) and 157 (G > A); *BoLA-DRB3*017:04* differed from *BoLA-DRB3*017:03* (found in one animal from a mixed population in Cairo) by a non-synonymous change at position 75 (A > T); and *BoLA-DRB3*005:08* (detected in an individual from a mixed population from Beheira) differed from *BoLA-DRB3*005:01* by a non-synonymous change at position 254 (G > T).

The nucleotide and deduced amino acid sequences of the eight novel alleles are shown in Fig. 2. These eight alleles were 90.7–98.9 % and 83.1–97.8 % identical to *BoLA-DRB3* cDNA clone NR-1, at the nucleotide level and predicted amino acid level, respectively (Aida et al., 1995).

3.2. NJ tree of BoLA-DRB3 alleles obtained from three distinct populations of cattle in Egypt

The 234 bp nucleotide sequences corresponding to the β1 domain were used to create a NJ tree (Fig. 3). The *BoLA-DRB3* NJ tree contained all previously reported alleles as well as eight novel variants found in Egyptian cattle populations. The 62 *BoLA-DRB3* alleles, including the eight new alleles, did not cluster in a specific clade and were dispersed along the tree. Although individuals of the mixed population studied here emerged from hybrid parents and of unknown initial breeds crossed with the native breed, mixed and native individuals both contained abundant mutual *BoLA-DRB3* alleles. Consistent with the hypothesis of the ancient origin of MHC alleles preceding domestication events and the establishment of bovine breeds, we predicted that prior to divergence of the main bovine types, the Auroch population of domestic cattle exhibited diversity in *BoLA-DRB3*. *BoLA-DRB3* alleles may have had an uneven distribution in the *Bos primigenius* population during domestication, retaining only a portion of the overall diversity in each cattle lineage, similar to the results reported for mitochondrial DNA haplogroups. Similarly, a later bottleneck during more recent breed formation and further adaptation to local environmental conditions may have enhanced the genetic differentiation of *BoLA-DRB3* alleles (Takeshima et al., 2018).

3.3. Distribution of BoLA-DRB3 alleles obtained from three distinct populations of cattle in Egypt

To differentiate between allelic variations in the three distinct Egyptian populations, we determined the frequencies of *BoLA-DRB3* alleles in each breed and compared them with those in other populations (Table 3). In native individuals, 28 alleles were identified. Seven of these 28 showed a gene frequency of ≥ 5 % (*BoLA-DRB3*001:01*, **002:01*, **011:01*, **014:01:01*, **022:01*, **028:02*, and **048:02*), with *BoLA-DRB3*002:01* as the most frequent (14.3 %). The gene frequency of the new variant, *DRB3*020:02:02*, in the native breed was 1.8 %. In the mixed population, only four of the 61 detected alleles (*BoLA-DRB3*001:01*, *002:01*, *011:01*, and *018:01*) exhibited a gene frequency ≥ 5 %, showing an even gene frequency distribution that was consistent with balancing selection in this population of mixed cattle. *BoLA-DRB3*001:01* was the most frequent variant (8.5 %); the eight new variants (*BoLA-DRB3*016:03*, **015:07*, **165:01*, **021:03*, **020:02:02*, **164:01*, **017:04*, and **005:08*) detected in this population exhibited gene frequencies between 0.3 % and 0.5 %. Of the 18 alleles detected in Holsteins, eight (*BoLA-DRB3*001:01*, *009:02*, *010:01*, *011:01*, *012:01*, *014:01:01*, *015:01*, and *027:03*) had gene frequencies ≥ 5 %, with *BoLA-DRB3*015:01* as the most frequent (20.2 %). Among the entire analyzed sample, 13 alleles showed gene frequencies ≥ 5.0 %, 10 of which were detected in only one population (Fig. 1**B**).

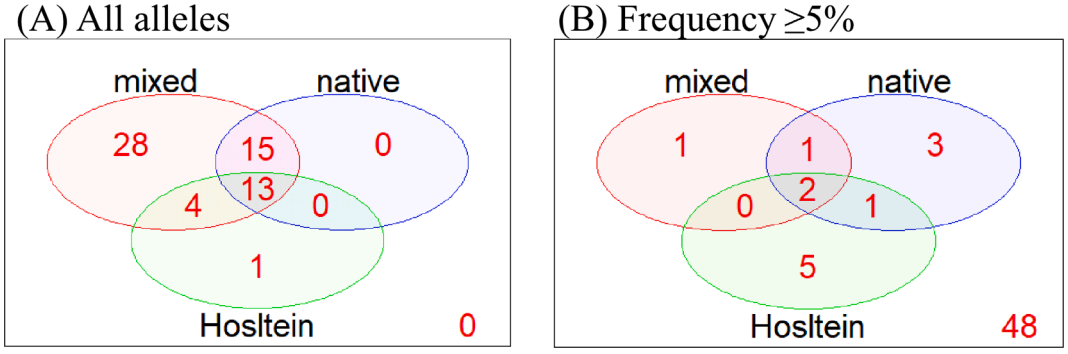

Fig. 1. Venn plot of *BoLA-DRB3* alleles shared by native, mixed, and Holstein populations. (A) Including all alleles and (B) including only alleles with gene frequency ≥ 5.0 %.

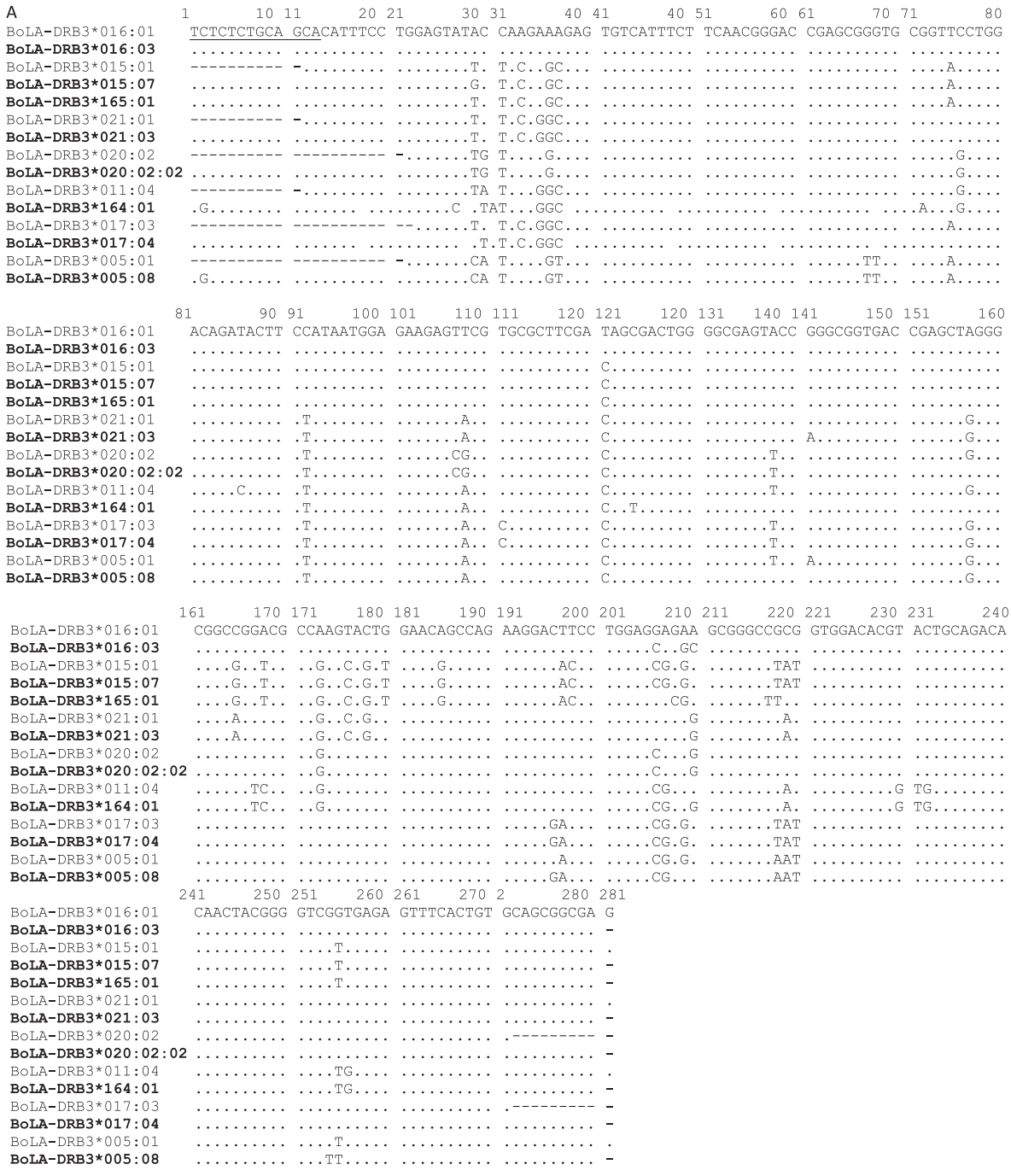

Fig. 2. Alignment of the nucleotide (A) and predicted amino acid (B) sequences of the ß1 domains encoded by eight novel BoLA-DRB3 alleles derived from 343 animals belonging to three distinct cattle populations: Holstein, mixed, and native, showing the difference in comparison with the cDNA clone NR-1 (BoLA-DRB3*016:01 allele; Aida et al., 1995). The closer BoLA-DRB3 reported alleles to the new variants were also included in the alignment. Novel alleles are shown in boldface. The nucleotide sequences reported were submitted to the International Nucleotide Sequence Database and assigned the following accession numbers: LC536590 for BoLA-DRB3*016:03, LC517534 for BoLA-DRB3*015:07, LC536592 for BoLA-DRB3*165:01, LC517535 for BoLA-DRB3*021:03, LC517536 for BoLA-DRB3*020:02:02, LC536589 for BoLA-DRB3*164:01, LC536591 for BoLA-DRB3*017:04, and LC536593 for BoLA-DRB3*005:08. The numbers indicate the positions of the amino acids in the mature protein. The amino acid residues are identical to those encoded by the BoLA-DRB3*016:01 cDNA clone.

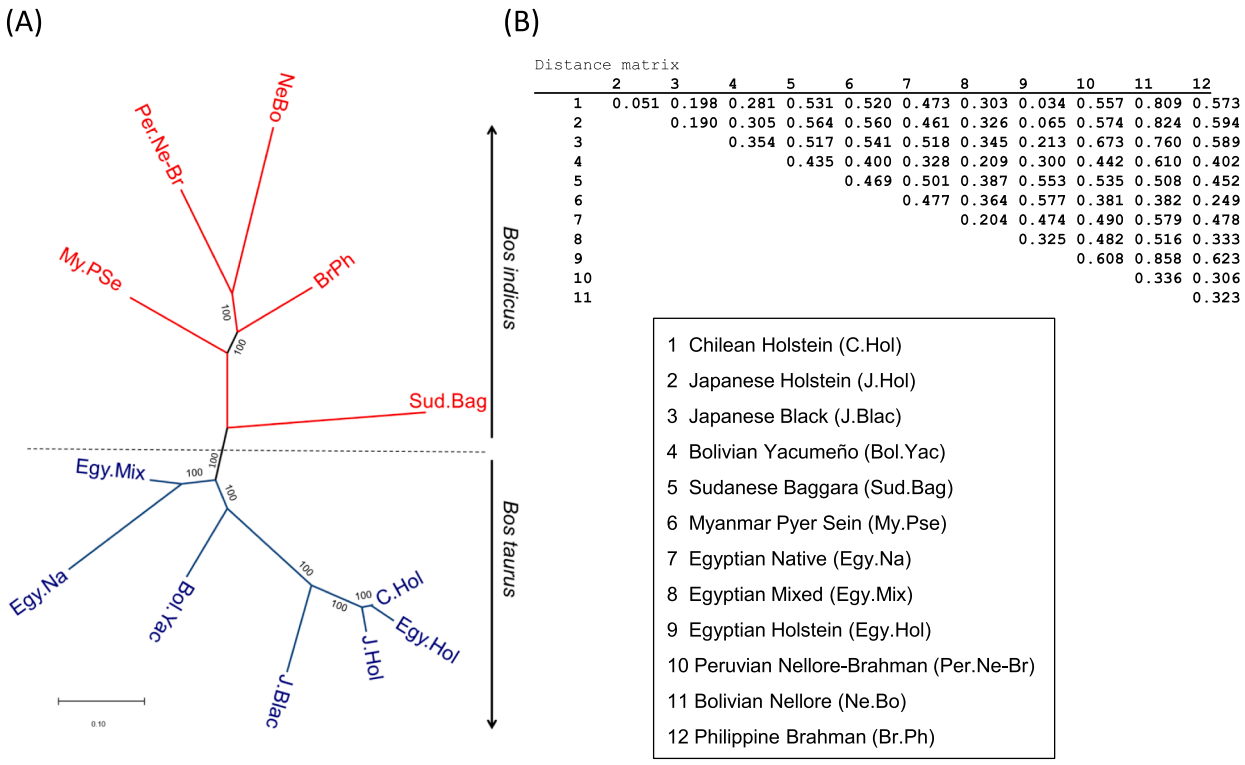

Fig. 3. Neighbor-joining (NJ) tree constructed using 234-bp nucleotide sequences of the ß1 domain coding-regions of *BoLA-DRB3* alleles derived from three distinct Egyptian cattle populations; Holstein (H), native (N), and mixed (M). The novel alleles identified in this study are marked by filled circles (•). The nucleotide sequences were submitted to the International Nucleotide Sequence Database and assigned the following accession numbers: LC536590 through *BoLA-DRB3*016:03*, LC517534 through *BoLA-DRB3*015:07*, LC536592 through *BoLA-DRB3*165:01*, LC517535 through *BoLA-DRB3*021:03*, LC517536 through *BoLA-DRB3*020:02:02*, LC536589 through *BoLA-DRB3*164:01*, LC536591 through *BoLA-DRB3*017:04*, and LC536593 through *BoLA-DRB3*005:08*.

3.4. Genetic diversity of BoLA-DRB3 in cattle breeds at the allele level

The number of alleles (na), expected heterozygosity (h_E) observed heterozygosity (h_O), HWE, and calculated inbreeding coefficient (F_{IS}) were used to determine the genetic diversity within the three Egyptian cattle breeds (Table 3). As previously mentioned, the na was 62 for the entire sample. We identified 18 previously described alleles in Holstein cattle, 28 in native Egyptian cattle (including one novel variant), and 61 in mixed-population cattle (53 previously reported and eight novel alleles). In contrast to in native cattle (28/56; 50.0 %) and mixed cattle (61/388; 15.7 %), Holstein cattle showed lower allele diversity (18/242; 7.4 %). Comparison of the h_O, h_E, and calculated inbreeding coefficient indicated that *BoLA-DRB3* had extremely high genetic diversity in all studied breeds, with an h_O of 86.8–96.4 % and h_E of 88.1–96.7 %. Deviations from HWE analysis did not reveal significant differences from the theoretical proportions based on the HWE-p values in any of the studied populations (Table 3). Analysis of the gene frequency distribution within a population using a neutrality test indicated the presence of selection (balancing, neutral, or positive). Slatkin's exact neutrality test showed that the gene frequency distributions from the three studied populations were consistent with the theoretical proportion expected under neutral selection ($0.0975 > p > 0.025$) (Table 3). However, although not significant, the mixed population trended towards balanced selection (0.046). The F_{ST} did not significantly differ for the gene frequencies between native and mixed populations ($F_{ST} = 0.003$; $p = 0.126$), whereas the Holstein breed presented significant pairwise F_{ST} values when the gene frequency profile was compared with that of the native ($F_{ST} = 0.033$; p value < 0.001) and mixed populations ($F_{ST} = 0.049$; $p < 0.001$).

3.5. Population relationships based on frequencies of BoLA-DRB3 alleles

To examine the genetic relationships between Egyptian cattle breeds and global cattle populations, we constructed NJ dendrograms and performed PCA. First, based on the frequencies of *BoLA-DRB3* alleles estimated for Egyptian cattle populations and previously examined taurine and indicine breeds, a *DA* genetic distance matrix and NJ dendrogram were drawn. The topology was consistent with the historical and geographical origins of the breeds (Fig. 4). Two distinct clusters were evident in this evolutionary tree: cluster 1 (*Bos indicus*) included populations of Myanmar Pyer Sein, Sudanese Baggara, Philippine Brahman, Bolivian Nellore, and Peruvian Nellore-Brahman cattle breeds, whereas cluster 2 (*Bos taurus*) comprised Holstein cattle (Chilean, Egyptian, and Japanese Holstein), Japanese Black, Egyptian native, Egyptian mixed, and Yacumeño Creole cattle breeds. Egyptian Holstein was grouped with Chilean Holstein and Japanese Holstein and was at one end of the NJ tree. Indicine breeds were at the opposite extremes (Myanmar Pyer Sein, Philippine Brahman, Bolivian Nellore, and Peruvian Nellore-Brahman). Egyptian native cattle and Egyptian mixed cattle, which originated via crossing between native and other exotic foreign breeds imported to Egypt, were placed within the same cluster in the middle of the un-rooted tree, with Yacumeño creole from Latin America (toward the taurine end) and Sudanese Baggara (toward the indicine end) in close proximity to the Egyptian cluster. Interestingly, these breeds influenced the genetic pool in Africa. Second, using allele frequencies, PCA was used to condense genetic variation and evaluate correlations between the analyzed breeds. The results were consistent with that for the NJ tree topology. The first and second principal components (PCs) estimated based on the gene frequency of *BoLA-DRB3* are shown (Fig. 5), as a summary of the PCA results. The first PC showed a clear pattern of divergence between indicine (positive values) and taurine breeds (negative values), whereas Egyptian native and Egyptian

Table 3
BoLA-DRB3 alleles count (n) and frequencies (%), genetic variation of observed and expected heterozygosity and Hardy Weinberg Equilibrium for the studied cattle populations in Egypt.

	BoLA-DRB3 allele	Native		Mixed		Holstein	
		N = 28		N = 194		N = 121	
		n	%	n	%	n	%
*	001:01	3	**5.4**	33	**8.5**	38	**15.7**
*	002:01	8	**14.3**	24	**6.2**	7	2.9
*	003:02:01	0	0.0	3	0.8	0	0.0
*	005:01	1	1.8	11	2.8	1	0.4
*	005:02	1	1.8	3	0.8	0	0.0
*	005:03	0	0.0	8	2.1	0	0.0
*	005:04	0	0.0	4	1.0	0	0.0
*	005:08	0	0.0	1	0.3	0	0.0
*	006:01	1	1.8	5	1.3	2	0.8
*	007:01	2	3.6	19	4.9	4	1.7
*	007:04	0	0.0	0	0.0	2	0.8
*	008:01	1	1.8	7	1.8	2	0.8
*	009:02	1	1.8	8	2.1	18	**7.4**
*	010:01	0	0.0	11	2.8	18	**7.4**
*	010:02	0	0.0	1	0.3	0	0.0
*	011:01	4	**7.1**	24	**6.2**	41	**16.9**
*	011:03	1	1.8	5	1.3	0	0.0
*	011:04	2	3.6	8	2.1	0	0.0
*	012:01	0	0.0	17	4.4	16	**6.6**
*	013:01	2	3.6	3	0.8	1	0.4
*	014:01:01	3	**5.4**	17	4.4	21	**8.7**
*	015:01	1	1.8	10	2.6	49	**20.2**
*	015:07	0	0.0	1	0.3	0	0.0
*	016:01	1	1.8	11	2.8	3	1.2
*	016:03	0	0.0	1	0.3	0	0.0
*	017:01	1	1.8	11	2.8	2	0.8
*	017:04	0	0.0	1	0.3	0	0.0
*	018:01	0	0.0	20	**5.2**	2	0.8
*	020:01:02	1	1.8	5	1.3	0	0.0
*	020:02	0	0.0	5	1.3	0	0.0
*	020:02:02	1	1.8	2	0.5	0	0.0
*	020:03	0	0.0	1	0.3	0	0.0
*	020:05	0	0.0	1	0.3	0	0.0
*	020:06	0	0.0	1	0.3	0	0.0
*	021:03	0	0.0	1	0.3	0	0.0
*	022:01	5	**8.9**	9	2.3	0	0.0
*	024:01	0	0.0	1	0.3	0	0.0
*	024:03	0	0.0	2	0.5	0	0.0
*	025:01:01	1	1.8	1	0.3	0	0.0
*	026:01	0	0.0	3	0.8	0	0.0
*	027:03	0	0.0	3	0.8	15	**6.2**
*	027:04	0	0.0	2	0.5	0	0.0
*	027:10	0	0.0	1	0.3	0	0.0
*	028:01	0	0.0	4	1.0	0	0.0
*	028:02	4	**7.1**	13	3.4	0	0.0
*	030:01	1	1.8	4	1.0	0	0.0
*	031:01	0	0.0	2	0.5	0	0.0
*	032:01	0	0.0	2	0.5	0	0.0
*	032:02	1	1.8	12	3.1	0	0.0
*	033:01	0	0.0	1	0.3	0	0.0
*	035:01	1	1.8	6	1.5	0	0.0
*	036:01	0	0.0	6	1.5	0	0.0
*	039:01	0	0.0	3	0.8	0	0.0
*	041:01	2	3.6	3	0.8	0	0.0
*	042:01	1	1.8	3	0.8	0	0.0
*	043:01	0	0.0	2	0.5	0	0.0
*	044:01	0	0.0	1	0.3	0	0.0
*	048:02	4	**7.1**	14	3.6	0	0.0
*	057:02	1	1.8	1	0.3	0	0.0
*	072:01	0	0.0	3	0.8	0	0.0
*	164:01	0	0.0	1	0.3	0	0.0
*	165:01	0	0.0	2	0.5	0	0.0
	H observed (h_O)	*0.96429*		*0.93814*		*0.86777*	
	H expected (h_E)	*0.95714*		*0.96733*		*0.88083*	
	No. alleles (*na*)	*28*		*61*		*18*	
	HWE-p	0.16184		0.07958		0.52754	
	Fis	−0.0074		0.0301		0.0148	

N, Number of animals analyzed; Frequent alleles in each breed are indicated in bold (>5%); Novel alleles identified in this study are underlined; h_O. Observed heterozygosity; h_E. Expected heterozygosity; n, *BoLA-DRB3* allele number, HWE-p. Hardy-Weinberg Equilibrium, Fis. Wright's Fis.

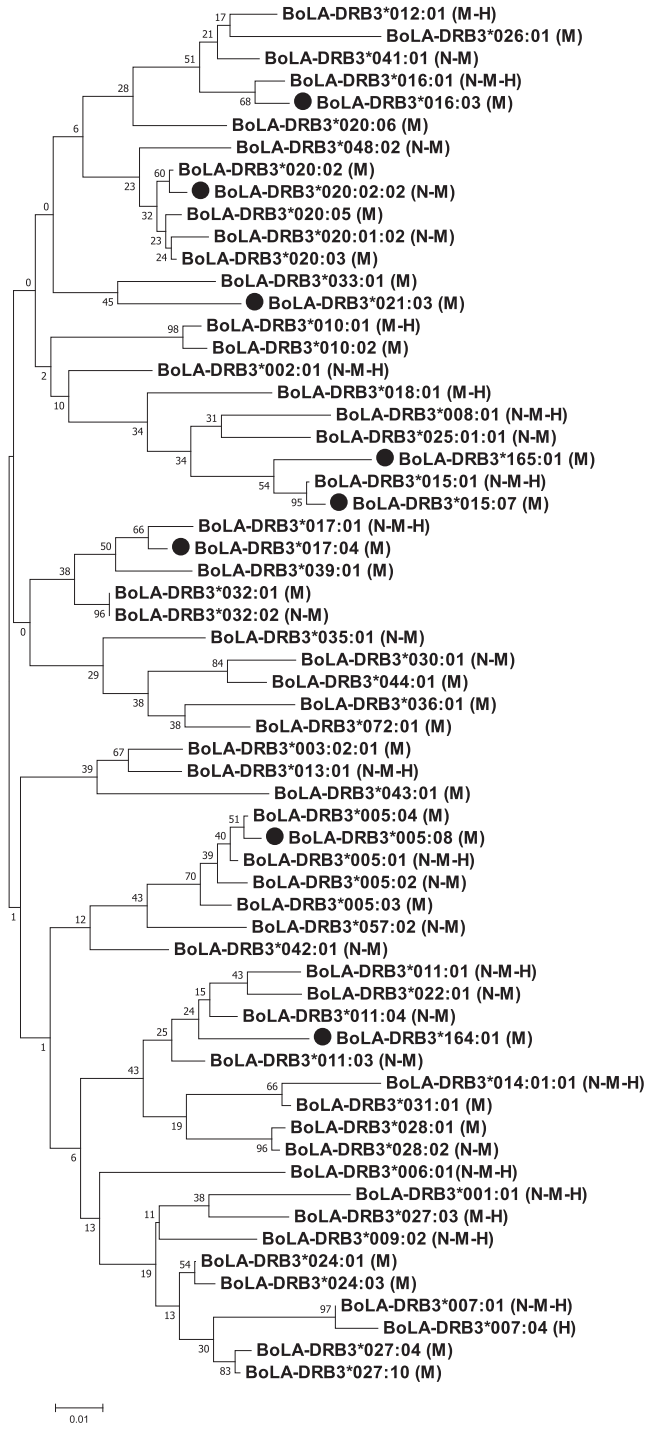

Fig. 4. Un-rooted neighbor-joining (NJ) tree (A) constructed from a matrix of Nei's D_A genetic distances (B), which were based on the frequencies of alleles of BoLA-DRB3 in the three Egyptian populations studied and nine other global breeds: Japanese Black and Japanese Holstein (Takeshima et al., 2003), Chilean Holstein (Takeshima et al., 2015), Bolivian Yacumeño (Giovambattista et al., 2013), Sudanese Baggara breed (Salim et al., 2020), Myanmar Pyer Sein (Giovambattista et al., 2020b), Philippine Brahman (Takeshima et al., 2014), and some South American populations (Bolivian Nellore and Peruvian Nellore-Brahman mixed cattle) (Takeshima et al., 2018).

mixed populations occupied the middle region, close to Yacumeño Creole, Sudanese Baggara, Myanmar Pyer Sein, and Philippine Brahman. The first PC accounted for 48.88 % of the overall variation. The second PC separated the Holstein breed from the other taurine populations (Japanese Black, Egyptian mixed, and Bolivian Yacumeno) and explained 15.05 % of the overall variation. Furthermore, the second PC showed a gradient among indicine breeds and discriminated between Nellore populations (Bolivian Nellore and Peruvian Nellore-Brahman) (positive values) and other indicine breeds (Sudanese Baggara, Myanmar Pyer Sein, and Philippine Brahman (negative values).

4. Discussion

Studies are necessary to gain an in-depth understanding of the genetic diversity of the bovine MHC complex, which contains a large number of loci implicated in immunological responses and defense against invasive pathogens. Of these loci, exon 2 of *BoLA-DRB3* was the most polymorphic region within the genome. Although numerous studies have focused on allelic characterization of *BoLA-DRB3* and its genetic diversity in cattle breeds in various geographical regions, information regarding *BoLA-DRB3* polymorphisms in cattle populations in Egypt is unavailable. To the best of our knowledge, this is the first study to use PCR-SBT to determine the allelic distribution of *BoLA-DRB3* in different cattle populations in Egypt, including two local populations (native and mixed) and one exotic population (Holstein). Sample sites were distributed in the northern, middle, and southern regions of Egypt, and hence were representative of the cattle diversity in this country. First, the allelic diversity of exon 2 of *BoLA-DRB3* in Holstein, mixed, and native cattle was investigated. Second, the allelic diversity of this gene in Egyptian populations was estimated. Third, genetic distances based on the breed gene frequency and population relationships were assessed.

We detected 62 alleles in the studied cattle populations, including eight novel variants of exon 2 of *BoLA-DRB3* detected in ten mixed animals and one native animal. These novel alleles were submitted to the IPD-MHC database and assigned the following allele names: *BoLA-DRB3* *016:03*, **015:07*, *017:04, **165:01*, **021:03*, **020:02:02*, **164:01*, and **005:08*. These results are consistent with those of other studies that reported novel alleles in various native and/or cross-bred cattle, including Japanese black cattle, Jersey cattle (Takeshima et al., 2003), Chinese yellow cattle (Wang et al., 2008), Philippian native, Philippian Holstein × Sahiwal (Takeshima et al., 2014), native cattle and Holstein-Friesian crossbreed in Myanmar (Giovambattista et al., 2020b), Creole cattle (Giovambattista et al., 2013, 2020b), and Sudanese native cattle (Salim et al., 2020). Unexpectedly, the novel variant was detected mainly in a population of mixed cattle; however, only one new allele was identified in native individuals. This result may be because of the limited number of native Egyptian cattle studied or because individuals belonging to the mixed population have a pool genome comprised of an indiscriminate admixture of native and exotic breeds formed through generations, resulting in higher allelic richness. To date, *BoLA-DRB3* diversity has only been reported in approximately 1,050 cattle breeds recognized by the Food and Agriculture Organization (FAO, 2021). Therefore, further studies of other breeds, mainly native breed adapted to local environmental conditions and considered as important reservoirs of global cattle diversity, may be required. In contrast, the alleles observed in Egyptian Holstein cattle were not new. This result was not unexpected, considering that *BoLA-DRB3* diversity in this breed has been studied in different countries worldwide and found to show a high level of genetic homogeneity (Takeshima et al., 2003, 2015).

The frequency distribution of *BoLA-DRB3* alleles in the studied populations was characterized. The allele frequencies showed a conserved pattern, which is generally consistent with those of other published studies. Most of the more abundant alleles in Holsteins were *BoLA-DRB3*015:01*, **011:01*, and **001:01*, which is concordant with the results of previous studies of the Holstein breed (Takeshima et al., 2003; Miyasaka et al., 2012; Nikbakht Brujeni et al., 2016; **Takeshima *et al.*, 2019**). In contrast, 61 alleles, including the eight new variants, in the mixed population showed an even distribution frequency, which is attributable to native admixture with some exotic breeds, resulting in a

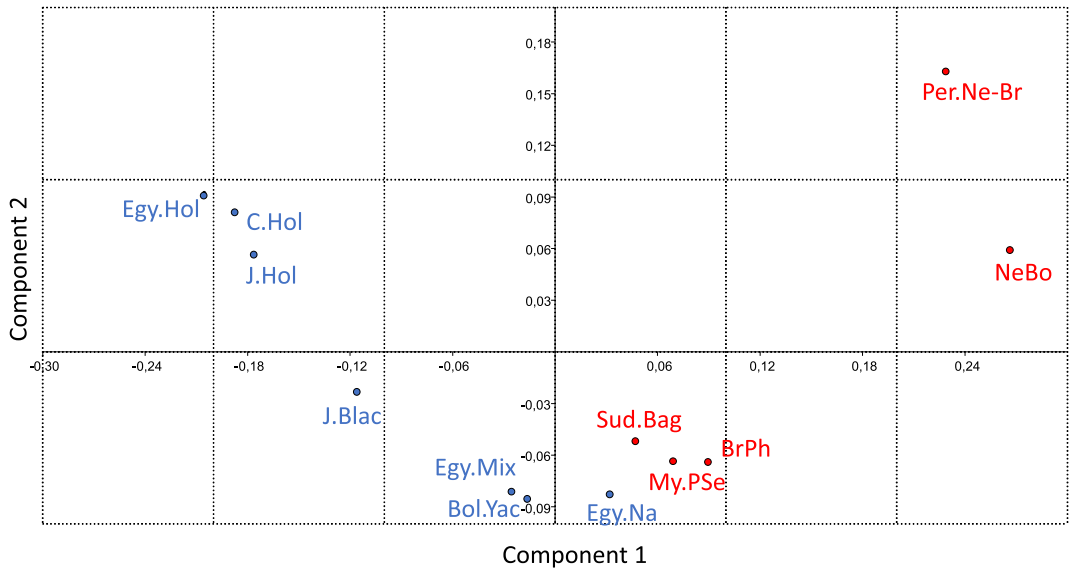

Fig. 5. Principal component analysis (PCA) of 12 cattle breeds based on *BoLA-DRB3* allele frequencies. C.Hol = Chilean Holstein, J.Hol = Japanese Holstein, J.Blac = Japanese Black, Bol.Yac = Bolivian Yacumeño, Sud.Bag = Sudanese Baggara, My.Pse = Myanmar Pyer Sein, Egy.Na = Egyptian native, Egy.Mix = Egyptian mixed, Egy.Hol = Egyptian Holstein, Per.Ne-Br = Peruvian Nellore-Brahman, Ne.Bo = Bolivian Nellore, Br.Ph = Philippine Brahman.

high degree of genetic polymorphism maintained by balancing selection. The neutrality test showed a tendency toward this type of natural selection in the population. A wide range of *BoLA-DRB3* alleles increases the survival of the population under infectious disease conditions. This result is consistent with previously published data for crossbred populations, as exemplified by the 46, 57, 33, and 33 *BoLA-DRB3* alleles identified in Holstein × Sahiwal, Philippine native × Brahman (Takeshima et al., 2014), Nellore × Brahman (Takeshima et al., 2018), and Holstein-Friesian (Giovambattista et al., 2020b), respectively. *BoLA-DRB3*001:01*, **002:01* and **011:01* were the most frequent alleles in mixed individuals. Similarly, *BoLA-DRB3*002:01*, **011:01*, **022:01*, **028:02*, **048:02*, **001:01*, and **014:01:01* were the most frequent alleles in samples obtained from native cattle. As expected, *BoLA-DRB3*001:01*, **002:01*, and **011:01*, the most abundant alleles between mixed and native cattle samples in this study, were also identified in European taurine cattle populations (Swedish Red and White breeds and American Angus breed) (Mikko and Anderson, 1995). Moreover, high frequencies of *BoLA-DRB3*001:01* and **011:01* have been found in Muturuan cattle, an African taurine-type cattle (Ahmed et al., 2020).

We examined the genetic diversity of *BoLA-DRB3* at the allelic level in the populations. The large number of alleles found indicates a high degree of gene diversity at the allelic level in the native (na = 28, h_E > 0.95), mixed (na = 61, h_E > 0.96), and Holstein (na = 18, h_E > 0.88) populations. These levels are similar to those reported for South American Holstein populations (na > 20 and h_E > 0.87) (Takeshima et al., 2015), as well as for Japanese Holstein (na = 18, h_E > 0.90) and Jersey populations (na = 14, h_E > 0.89) (Takeshima et al., 2003). The extremely high h_E (≥95 %) values observed in native and mixed cattle populations were also reported in Yacumeño creole cattle (Giovambattista et al., 2013), as well as in other cattle breeds such as Philippine native, Philippine Brahman (Takeshima et al., 2014), Holstein-Friesian crossbreed, Myanmar native cattle (Giovambattista et al., 2020b), Colombian Normande (Bohórquez et al., 2020), and Sudanese native (Salim et al., 2020). These results indicate large breed-specific differences between the polymorphism levels of *BoLA-DRB3*. Other diversity analyses, such as those focused on clarifying nucleotide and codon variability in the peptide-binding region and peptide-binding region-logos matrix, must be examined in further proscriptive studies because sequence similarity and covariation in the *BoLA-DRB3* peptide-binding region between populations were previously assumed to influence the functional diversity of MHCs in cattle based on the *BoLA-DRB3* peptide repertoire size (Bohórquez et al., 2020).

Putative evolutionary relationships among different populations can be determined based on genetic distances derived from allele frequencies (Mizuki et al., 1997). The constructed population tree indicated that based on the *BoLA-DRB3* frequency, the smallest genetic distance among Egyptian cattle existed between native and mixed populations (*DA* = 0.204), whereas the largest distance was between native and Holstein cattle (*DA* = 0.474). This result suggests that the mixed population, resulting from crossing local Egyptian cattle with exotic breeds to improve production traits, maintained a large proportion of native genes associated with adaptation to the natural environment of Egypt in their genome. Interestingly, this tree also indicated that the Egyptian, Chilean, and Japanese Holsteins were the closest breeds. Similarly, Takeshima et al. (2015) found that populations belonging to the Holstein group from different countries (Japanese Holstein, Peruvian Holstein, Chilean Holstein, and Argentinian Holstein) were closely related on the dendrogram (Takeshima et al., 2015), supporting the extreme genetic homogeneity of the Holstein breed worldwide. Additionally, the highest distribution was between the native, mixed, and Holstein populations and the indicine populations, as indicated by the taurine phenotype of these populations. Furthermore, the three African cattle populations influencing their genome composition (native populations from Egypt and Sudan and Creole cattle from Latin America) were close to each other in the middle of the unrooted tree (and in PCA, as expected).

The PCA results were consistent with the overall grouping observed in the NJ tree, demonstrating divergence mainly between the taurine and indicine populations. The clearest differentiation between the taurine and indicine breeds was observed in the first PC. The grouping of Holstein cattle from the other taurine groups was clearly explained in the second PC. The indigenous Egyptian populations (native and mixed) were close to the Yacumeño creole populations. Furthermore, PCA indicated that some Egyptian native cattle were closer to the indicine breeds (Sudanese Baggara, Myanmar Pyer Sein, and Philippine Brahman). Similar results were observed for other indigenous native breeds from other geographical regions (Takeshima et al., 2015; Giovambattista et al., 2020b). Interestingly, among the indicine populations, those of Nellore from South America diverged greatly from those of other indicine breeds, including the Philippine Brahman, Sudanese Baggara, and Pyer Sein from Myanmar. As expected, the two Asian indicine breeds (Myanmar Pyer Sein and Philippine Brahman)

appeared to be closely related on the PCA plot; however, the Sudanese Baggara diverged from their African ancestry.

Several relevant *BoLA-DRB3* alleles identified in this study were previously reported as indicators of resistance or susceptibility to infectious diseases. The most prevalent allele in Egyptian Holstein cattle was *BoLA-DRB3*015:01*, as observed in all previously examined Holstein cattle populations (Lo and Aida, 2022). *BoLA-DRB3*015:01* has been linked to the susceptibility to bovine leukemia virus (BLV)-high proviral load in the circulation of Holsteins (Juliarena et al., 2008; Takeshima et al., 2019; Farias et al., 2017). Additionally, this allele is closely associated with the development of mastitis and mastitis pathogens such as *Streptococci*, coagulase-negative *Staphylococci*, *E. coli*, and *Staphylococcus aureus* in Holstein cows (Yoshida et al., 2012, 2009). *BoLA-DRB3*002:01*, which is the most prevalent allele in Egyptian native cattle, has been linked to resistance to BLV-proviral load elevation (Lo et al., 2020; Takeshima et al., 2019; Farias et al., 2017). Among the alleles found in the mixed-population cattle, *BoLA-DRB3*001:01* was the most abundant. This allele has been reported to be both susceptible and resistant in disease interactions. For example, studies of the relationship between *BoLA-DRB3* and BLV disease showed that *DRB3*001:01* is associated with both lymphoma resistance and susceptibility to persistent lymphocytosis (Nikbakht Brujeni et al., 2016). In addition, neither sensitivity to high or low BLV-proviral load profiles nor resistance to either of these profiles was associated with this allele (Lo and Aida, 2022; Lo et al., 2021). And, cows carrying *DRB3*001:01* are highly susceptible to pathogens linked to mastitis (Yoshida et al., 2009) but was also associated with resistance to mastitis in cows from other dairy herds (Yoshida et al., 2012). Further association studies of endemic diseases are necessary to determine the significance of *BoLA-DRB3* diversity in the adaptation of native Egyptian cattle.

BoLA genes are primarily involved in immune system-based identification of pathogens and are therefore used as markers of diseases and immunological traits in cattle. More studies focusing on the *BoLA-DRB3* allelic distribution in different cattle breeds adapted to different geographical regions are needed. An in-depth knowledge of the effects of *BoLA* polymorphisms on MHC function in relation to pathogen identification is important (Bohórquez et al., 2020). Given the variable impacts exerted by this gene on a wide range of diseases and production traits, genetic selection based on the selected *BoLA-DRB3* alleles should be further explored. This study provides a preliminary assessment of the genetic diversity of *BoLA-DRB3* in Egyptian cattle. Further studies of larger sample sizes are needed.

5. Conclusion

We used an SBT assay to explore the allelic diversity of *BoLA-DRB3* in Egyptian cattle. Our findings revealed the unique genetic diversity of native breeds and their hybrid populations. This genetic information provides basic data on the genetic diversity of *BoLA-DRB3* in Egyptian cattle, which can be used to improve the design of breeding schemes and perform further studies aimed at determining the association between *BoLA-DRB3* alleles and infectious diseases in cattle.

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Authorship contribution statement

Y.A. conceived of and designed the study. S.M., A.A., H.M., A.M. collected the samples. R.H., G.G., S.M., L.B., M.P.Y., R.M., K.K.M., S-N. T., S.W., Y.A. acquired, analysed, and interpreted the data. Y.A. and S.M. contributed reagents, materials, and analysis tools. R.H., G.G., Y.A. drafted and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

CRediT authorship contribution statement

Rania Hamada: Writing – original draft, Formal analysis, Data curation, Investigation. **Guillermo Giovambattista:** Writing – original draft, Formal analysis. **Samy Metwally:** Resources, Formal analysis, Data curation. **Liushiqi Borjigin:** Investigation. **Meripet Polat Yamanaka:** Investigation. **Ryosuke Matsuura:** Formal analysis. **Alsagher O. Ali:** Resources. **Hassan Y.A.H. Mahmoud:** Resources. **Adel E.A. Mohamed:** Resources. **Kyaw Kyaw Moe:** Investigation. **Shin-nosuke Takeshima:** Formal analysis. **Satoshi Wada:** Data curation. **Yoko Aida:** Writing – original draft, Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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