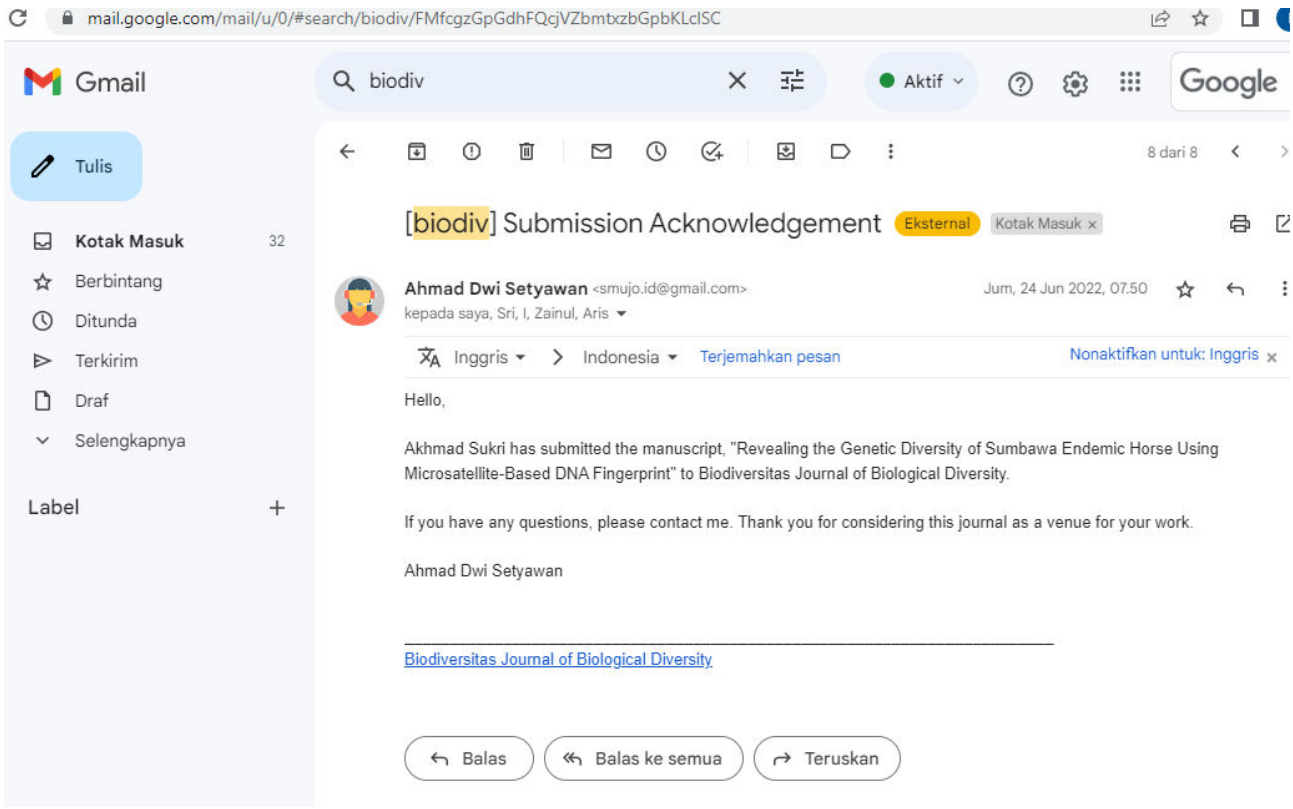
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Hello,
Akhmad Sukri has submitted the manuscript, "Revealing the Genetic Diversity of Sumbawa Endemic Horse Using Microsatellite-Based DNA Fingerprint" to Biodiversitas Journal of Biological Diversity.
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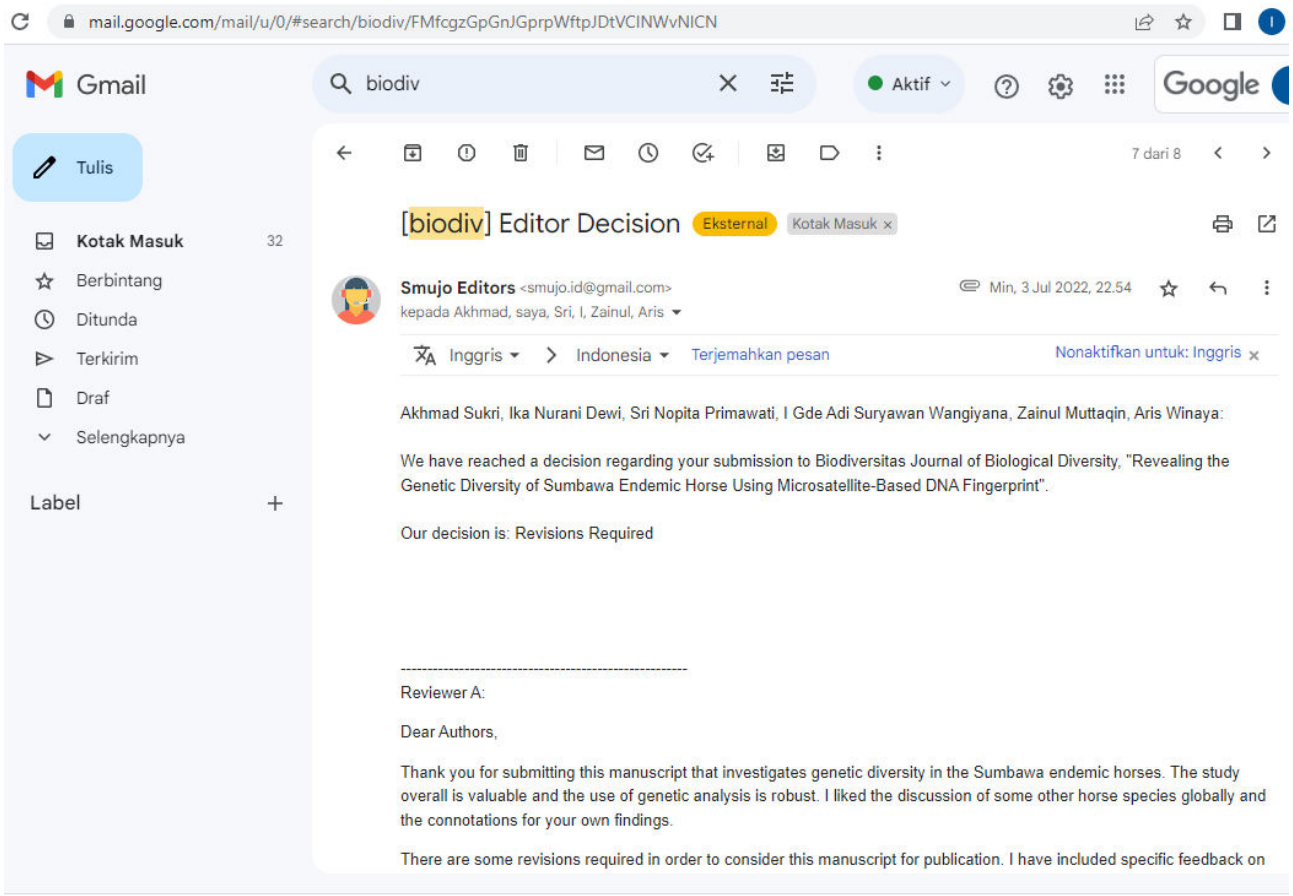
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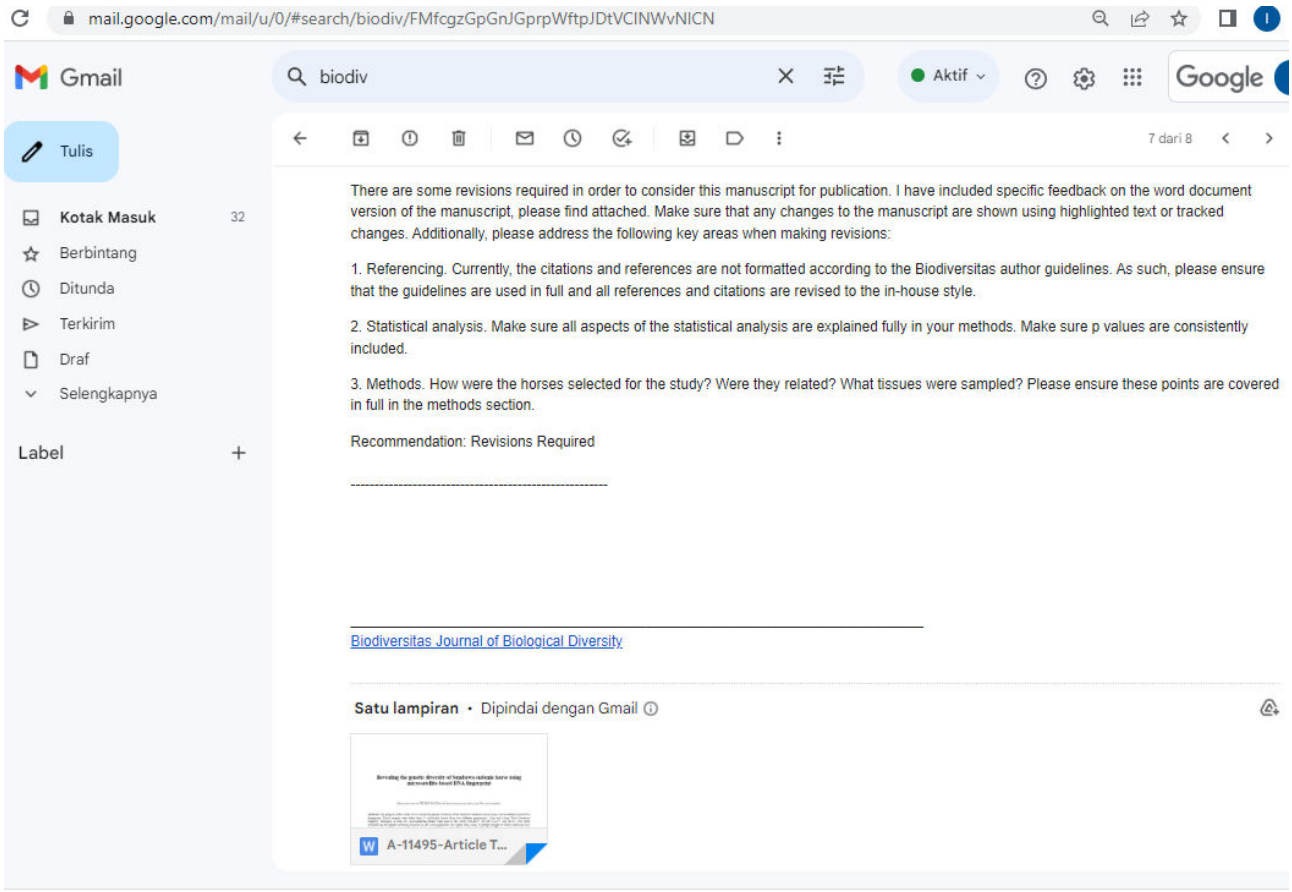
Akhmad Sukri, Ika Nurani Dewi, Sri Nopita Primawati, I Gde Adi Suryawan Wangiyana, Zainul Muttaqin, Aris Winaya:
We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Revealing the Genetic Diversity of Sumbawa Endemic Horse Using Microsatellite-Based DNA Fingerprint".
Our decision is: Revisions Required
Reviewer A:
Dear Authors,
Thank you for submitting this manuscript that investigates genetic diversity in the Sumbawa endemic horses. The study overall is valuable and the use of genetic analysis is robust. I liked the discussion of some other horse species globally and the connotations for your own findings.
There are some revisions required in order to consider this manuscript for publication. I have included specific feedback on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1. Referencing. Currently, the citations and references are not formatted according to the Biodiversitas author guidelines. As such, please ensure that the guidelines are used in full and all references and citations are revised to the in-house style.
2. Statistical analysis. Make sure all aspects of the statistical analysis are explained fully in your methods. Make sure p values are consistently included.
3. Methods. How were the horses selected for the study? Were they related? What tissues were sampled? Please ensure these points are covered in full in the methods section.
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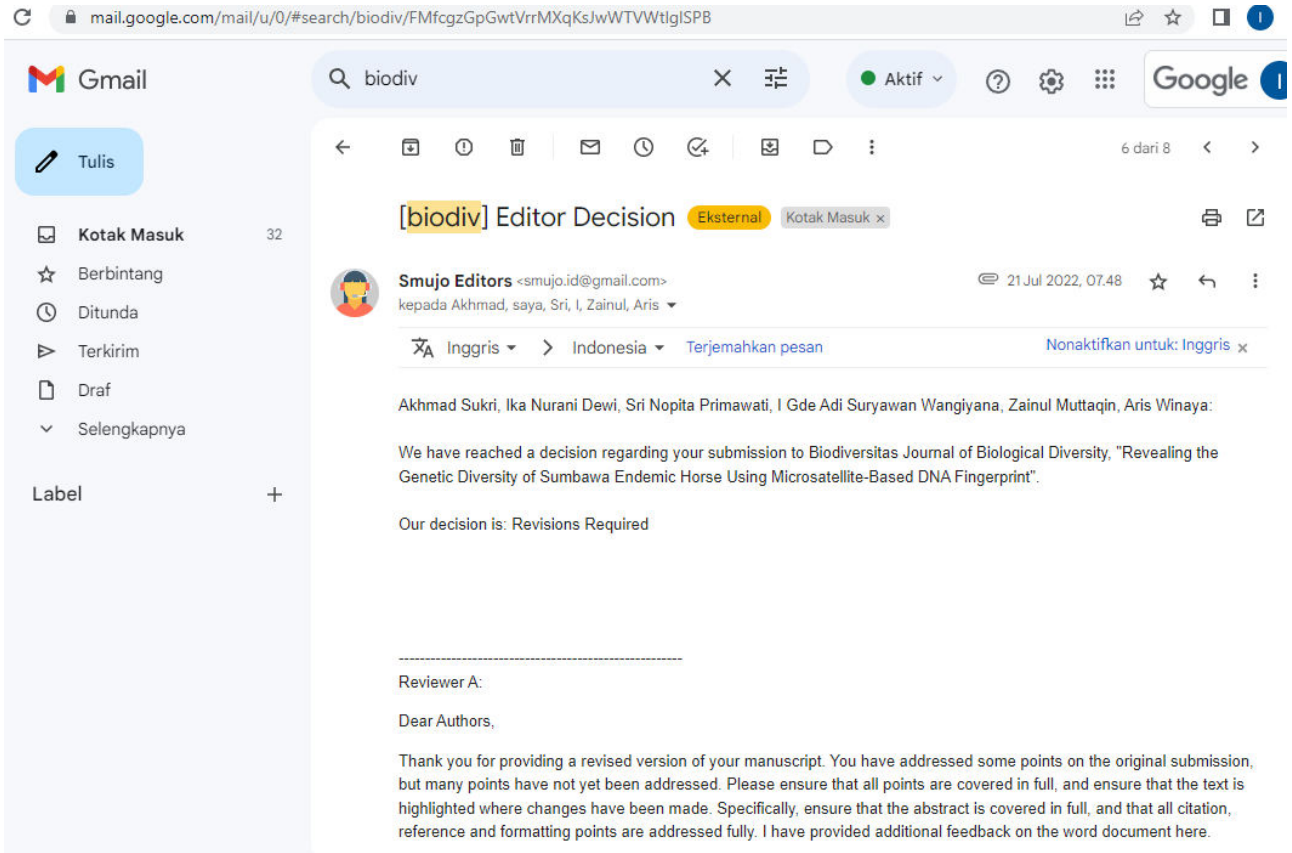
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Our decision is: Revisions Required
Reviewer A:
Dear Authors,
Thank you for providing a revised version of your manuscript. You have addressed some points on the original submission, but many points have not yet been addressed. Please ensure that all points are covered in full, and ensure that the text is highlighted where changes have been made. Specifically, ensure that the abstract is covered in full, and that all citation, reference and formatting points are addressed fully. I have provided additional feedback on the word document here.

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Reviewer A:

Dear Authors,

Thank you for providing a revised version of your manuscript. You have addressed some points on the original submission, but many points have not yet been addressed. Please ensure that all points are covered in full, and ensure that the text is highlighted where changes have been made. Specifically, ensure that the abstract is covered in full, and that all citation, reference and formatting points are addressed fully. I have provided additional feedback on the word document here.

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[biodiv] Editor Decision Eksternal Kotak Masuk ×

Agustina Putri <smujo.id@gmail.com> Kam, 4 Agu 2022, 12.10
kepada AKHMAD, saya, SRI, I, ZAINUL, ARIS

Inggris Indonesia Terjemahkan pesan Nonaktifkan untuk: Inggris ×

AKHMAD SUKRI, IKA NURANI DEWI, SRI NOPITA PRIMAWATI, I GDE ADI SURYAWAN WANGIYANA, ZAINUL MUTTAQIN, ARIS WINAYA4:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Revealing the genetic diversity of Sumbawa endemic horse using microsatellite-based DNA fingerprint".

Our decision is to: Accept Submission

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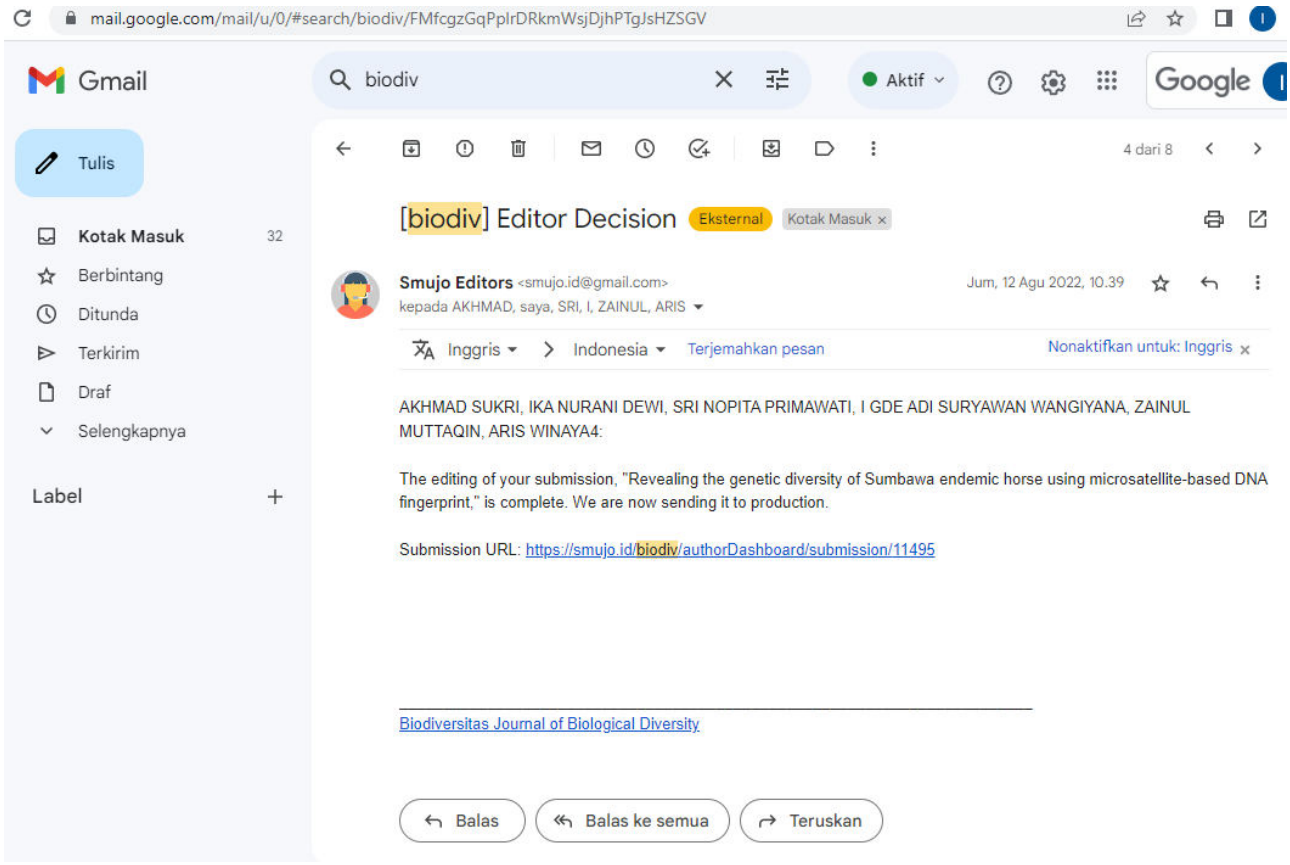
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AKHMAD SUKRI, IKA NURANI DEWI, SRI NOPITA PRIMAWATI, I GDE ADI SURYAWAN WANGIYANA, ZAINUL MUTTAQIN, ARIS WINAYA4:
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Lampiran:

Revealing the genetic diversity of Sumbawa endemic horse using microsatellite-based DNA fingerprint

Manuscript received: DD MM 2016 (Date of abstract/manuscript submission). Revision accepted:

Abstract. The purpose of this study was to reveal the genetic diversity of the Sumbawa endemic horse using a microsatellite-based DNA fingerprint. Blood samples were taken from 24 individual horses from two different populations, Lalar and Liang, West Sumbawa Regency, Indonesia. A total of 4 microsatellite primers were used in this study, INRA032, HEL09, CA425, and AHT4. This study revealed that the genetic diversity of horses in the Lalar population was higher than Liang. A greater number of alleles reinforces this; higher number and frequency of bands; and the presence of private bands that indicate unique alleles. This research shows that the Sumbawa horse is unique from other horse breeds in the world. This is evidenced by the lower number of alleles per locus (Na) with a maximum number of two alleles per locus. Sumbawa horses have higher observed heterozygosity (Ho) than expected heterozygosity (He), with a Ho value less than 0.5. Analysis of Molecular Variance result has shown that variation within the population was higher than among the population. This is presumably due to the high gene flow in both horse populations caused by inbreeding. Similarity analysis strengthens the hypothesis, which is indicated by mixing buffalo individuals from the two populations in one cluster. In general, AHT4 primers had the best ability to reveal the genetic diversity of Sumbawa horses with the highest Shannon's information index compared to other markers.

Keywords: Genetic diversity, Sumbawa horse, DNA fingerprint, microsatellite

Running title: Sumbawa horse microsatellite DNA fingerprint

Kommentar [JB1]: Describe the background. Is this well studied?

Kommentar [JB2]: Include scientific name on first mention

Kommentar [JB3]: How and where were samples collected from?

Kommentar [JB4]: What do you mean here?

Kommentar [JB5]: This doesn't make sense.

Kommentar [JB6]: Some of the key words are already included in the title. Remove any key words that are in the title and use new terms to increase paper discoverability

INTRODUCTION

DNA fingerprinting is an individual identification technique using individual DNA profiles (Singla et al., 2017). This identification process is carried out by comparing DNA sequences that are unique to each individual (Choi et al., 2008). DNA fingerprinting is widely used in forensics and paternity identification (Krishnamurthy et al., 2011). In addition, DNA fingerprints are also widely used to identify genetic diversity and relationships in plants (Selvakumari et al., 2017; Jamil et al., 2021), poultry genetic diversity (Farrag et al., 2010), livestock (Fadhil et al., 2013), even widely used for the identification of fish species (Al-Faisal et al., 2019). DNA fingerprinting includes techniques such as Restriction Fragment Length Polymorphism (Nishikaku et al., 2019), randomly amplified polymorphic DNA (El-Mouhamady et al., 2019), and Amplified Fragment Length Polymorphism (Vigneshwaran et al., 2017; Malik et al., 2022). Variable Number Tandem Repeat (VNTR) or Short Tandem Repeat (STR) based PCR is most often used in DNA fingerprinting because it has high sensitivity and the procedure takes less time (Choi et al., 2008). One of the molecular markers, a Short Tandem Repeat often used for genetic analysis of livestock, is a microsatellite (Teneva et al., 2018).

Microsatellites are single locus DNA sequences with very high polymorphisms that are spread throughout the genome (Heryani et al., 2019). Microsatellites have repeating copies, usually 1 to 6 nucleotides long (Garkovenko et al., 2018). The repetition of this DNA unit can be in the form of mononucleotides, dinucleotides, trinucleotides, tetranucleotides, and so on (Mason, 2015; Donnik et al., 2017). Microsatellite DNA markers have been widely used to study genetic diversity because they are randomly distributed throughout the genome, codominant, and have high polymorphism (Putman & Carbone, 2014). Microsatellite markers have been widely used for genetic diversity analysis in plants (Saptadi et al., 2020; Parmar et al., 2022), poultry (Luis-Chincoya et al., 2021), cattle (Agung et al., 2019), buffalo (Vohra et al., 2021), and horses (Kim et al., 2021). In Indonesia, research on livestock genetic diversity still needs to be done because some of the genetic resources of native Indonesian livestock are threatened with extinction due to a small population and limited distribution (Sutarno et al., 2015). One of the leading local livestock owned by Indonesia is the Sumbawa horse (Wibisono et al., 2017; Mujahid et al., 2019).

Sumbawa horse is one of Indonesia's local horse families, which is a native Indonesian genetic resource that needs to be protected and conserved with a geographical distribution on Sumbawa Island, West Nusa Tenggara (Keputusan Menteri Pertanian, 2011). The Sumbawa horse has an essential meaning for the Sumbawa people, both from an economic and socio-cultural perspective. From the economic aspect, Sumbawa horse milk is used for consumption needs in the form of wild horse milk which is beneficial for health (Prastyowati, 2021), while from the socio-cultural aspect, Sumbawa horses

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(Choi et al. 2008)

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Kommentar [JB10]: Sequences?

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Kommentar [JB12]: Please include scientific name on first mention

are used as a horse racing vehicle called "Main Jaran". This horse racing culture has a high philosophy for one's social status. In addition, horse racing culture can strengthen brotherhood, preserve culture and serve as people's entertainment (Asidah, 2020). Seeing the importance of the existence of the Sumbawa horse, it is necessary to preserve the genetic resources of the Sumbawa horse. Information on the genetic status of the Sumbawa Horse as one of Indonesia's native livestock is very important as a step in developing a conservation strategy in the long term (Sutarno et al., 2015).

Research that reveals the genetic diversity of Sumbawa horses has never been done. So far, research related to Sumbawa horses has focused on milk quality (Saragih et al., 2013). The latest research on the genetic diversity of horses was reported by Lapian (2021). The study is only a review of the results of research that has been carried out by people in several countries and has not revealed the genetic diversity of the Sumbawa Horse. Another study by Wibisono et al., (2017) only revealed the morphological diversity of horses and had not used genetic markers. Therefore, this research is critical because it is one of the pioneers in revealing the genetic diversity of the Sumbawa Horse based on DNA fingerprints. The purpose of this study was to reveal the genetic diversity of Sumbawa horses based on DNA fingerprints using microsatellite DNA markers.

MATERIALS AND METHODS

Sampling and DNA purification

Blood samples were taken from 24 individual horses from two different populations, namely Lalar and Liang, West Sumbawa Regency, West Nusa Tenggara, Indonesia (Figure 1). Blood was taken from the jugular vein (Sikka & Sethi, 2008), put into a tube containing EDTA solution, and brought to the laboratory for DNA extraction. DNA was extracted from whole blood samples (Griffiths & Chacon-Cortes, 2014) using the NucleoSpin Blood QuickPure kit (Macherey-Nagel, Germany) following a predetermined procedure. The extracted DNA was then separated using Agarose Gel Electrophoresis (Lee et al., 2012) with a concentration of 0.5%.

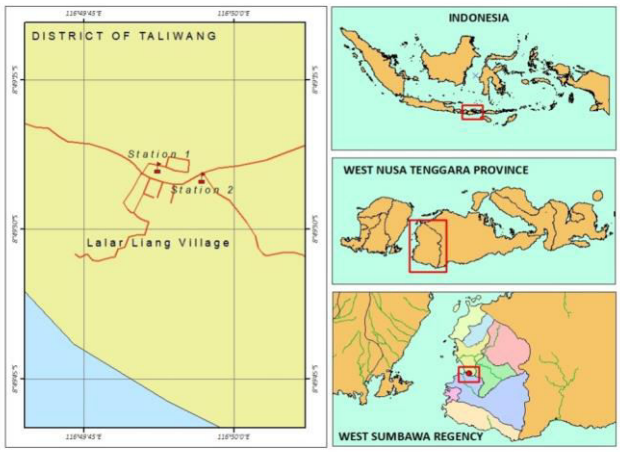

Figure 1. Sumbawa horse sampling location, station 1 = Liang (8°49'23.8"S 116°49'52.4"E), station 2 = Lalar (8°49'24.8"S 116°49'56.8"E)

PCR amplification

A total of 4 microsatellite primers were used in this study. Two microsatellite primers were adopted from buffalo microsatellite primers, namely INRA032 (Navani et al., 2002) and HEL09 (Uffo et al., 2017). Meanwhile, two primers were adopted from horse microsatellite primers recommended by the International Society for Animal Genetics, namely CA425 and AHT4 (ISAG, 2016). The primer sequences are shown in Table 1. PCR was carried out with a total volume of 25 µL with a mixture of: 2.5 µL DNA template, 2.5 µL forward primer, 2.5 µL reverse primer, 12.5 µL PCR mix, and 5 µL dH2O (Sukri, 2014). The PCR process was carried out in several stages, namely initial denaturation at 95^0C for 10 minutes, 30 cycles of 30 seconds at 95^0C (Denaturation), 30 seconds at 60^0C (Annealing) , 1 minute at 72^0C (Extention), and finally, final elongation at 72^0C for 10 minutes (Moshkelani et al., 2011).

Microsatellite analysis

Genotyping of microsatellite DNA polymorphisms was carried out through agarose gel electrophoresis (Asif et al., 2008), then DNA fragments were analyzed using GeneScan and Genotyper® software (Cozzi et al., 2022). Genetic diversity measures include allele frequency, the observed number of alleles, banding patterns and expected heterozygosity,

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1. Where were horses kept? Wild or captive?
2. Where on the body were samples taken? E.g. hair, skin, muscle?
3. How were the horses selected? Were any related to one another?
4. Was the study approved by any ethics committees?

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molecular variance, and similarity relationship based on the dendrogram. Allele frequencies, observed number of alleles, and expected heterozygosity were analyzed using POPGENE v.1.32 and GenAlEx 6.5 software (Peakall & Smouse, 2012), while molecular variance analysis used Arlequin v 3.0 software (Excoffier et al., 2005). Cophenetic correlation analysis was performed on the similarity matrix based on DNA fingerprinting data from microsatellite primers using Co-Stat for Windows. Furthermore, the dendrogram was constructed using the clustering method based on the optimum R value cophenetic correlation results using the Multivariate Statistical Package Ver. 3 (Kasiamdari et al., 2019).

Kommentar [JB19]: Shannon is not explained here. Please explained this.

Table 1. Characteristics of the microsatellite markers used

No	Name	Sequence	Number of bases
1	CA425	F: AGCTGCCTCGTTAATTCA	18
		R: CTCATGTCCGCTTGTCTC	18
2	AHT4	F: AACCGCCTGAGCAAGGAAGT	20
		R: CCCAGAGAGTTTACCCT	17
3	INRA032	F: AAACTGTATTCTCTAATAGCTAC	23
		R: GCAAGACATATCTCCATTCCTTT	23
4	HEL09	F: GGAAGCAATGAAATCTATAGCC	22
		R: TGTTCTGTGAGTTTGTAAGC	20

RESULTS AND DISCUSSION

A total of 49 alleles were detected in 24 individuals of the two tested populations (Lalar and Liang). Each microsatellite marker produced various alleles on the two tested populations. On average, the Lalar population had more alleles than the Liang population. This result was an early indication of the genetic variation between these populations since a different number of the allele was an essential parameter of genetic variation based on microsatellite marker (Ustyantseva et al., 2019)

Kommentar [JB20]: marker revealed various alleles in the two

Table 2. Allele frequencies and estimated diversity each primer

Marker	Population	Na	Ne	I	Ho	He
HELL09	Lalar	1.750	1.342	0.366	0.233	0.254
	Liang	2.000	1.491	0.484	0.313	0.341
	Mean	1.875	1.417	0.425	0.273	0.297
INRA032	Lalar	2.000	1.900	0.665	0.472	0.515
	Liang	1.000	1.400	0.318	0.222	0.242
	Mean	1.500	1.650	0.492	0.347	0.379
CA415	Lalar	2.000	1.552	0.493	0.325	0.355
	Liang	1.600	1.476	0.424	0.283	0.309
	Mean	1.800	1.514	0.459	0.304	0.332
AHT4	Lalar	2.000	1.432	0.452	0.286	0.312
	Liang	2.000	1.557	0.540	0.356	0.388
	Mean	2.000	1.495	0.496	0.321	0.350

Na : Number of alleles per locus
Ne : Number of effective alleles per locus
I : Shannon's information index
Ho : Observed heterozygosity
He : Expected heterozygosity

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Kommentar [JB22]: How was this generated? Make sure this is explained fully

Several parameters were computed to measure genetic variability between the Lalar and Liang populations (Table 2). The number of alleles per locus (Na) in the Lalar population and Liang population was always higher than the number of effective alleles per locus (Ne) on all markers (except for the INRA032 marker). A genetic population study on horse breeds based on horse microsatellite markers commonly resulted in higher Na than Ne (Fornal et al., 2020). Higher Ne than Na on the INRA032 marker may be due to this marker was not a specific microsatellite marker for horse breed.

The number of different alleles per locus (Na) was a common genetic variation in horse breed based on microsatellite markers. Western Arabian Horse Na varied from 3–5 (Khanshour et al., 2013), Iranian horse has Na from 3–4 (Moshkelani et al., 2011), Polish Konik Horse has Na varied from 5–6 (Fornal et al., 2020), Akhal–Teke horse has Na varied from 3–4 (Ustyantseva et al., 2019). Sumbawa–Indonesian horse in this study has a lower number of Na than horse breeds from

other regions of the world since these horse breeds only have a maximum of two numbers of alleles per locus. The lalar population has an average Na value higher than the Na value of the Liang population.

The number of observed heterozygosity (Ho) in the Sumbawa-Indonesia horse population was lower than the number of expected heterozygosity (He). The low value of Ho (below 0.5) indicates that this horse population has suffered a loss of heterozygosity due to increased inbreeding. However, this result also indicates the unique genetic characteristic of the Sumbawa-Indonesian breed compared to the genetic characteristic of horse breeds from Asia, Africa, and Europe. Most horse populations from Asia, Europe, and Africa have Ho values higher than 0.5 (Benahamadi et al., 2020; Dorji et al., 2018; Gómez et al., 2017). Sumbawa-Indonesian horse population has heterozygosity characteristics similar to the American (Brazilian) horse breed with a Ho value less than 0.5 (Reis et al., 2008). Moreover, the Brazilian American horse mostly has a Ho value lower than the He value, just like Indonesia –Sumbawa horse (Silva et al., 2012).

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Molecular variation based on microsatellites (Single Sequence Repeats) has become a typical analysis for population genetic study (Vieira et al., 2016). Microsatellite marker specified for horse breed has been developed to reveal genetic variation in different horse breed (Mahrous et al., 2011). AHT4 in this study was a specified microsatellite marker for a horse that could reveal genetic diversity among the Sumbawa-Indonesia horse population compared to other markers. AHT4 marker could produce the highest Shannon's information index among other markers. This marker also produces a consistent number of alleles per locus on the Lalar and Liang populations. This result implicated that AHT4 was a useful microsatellite marker for the genetic population study of the Indonesian horse breed.

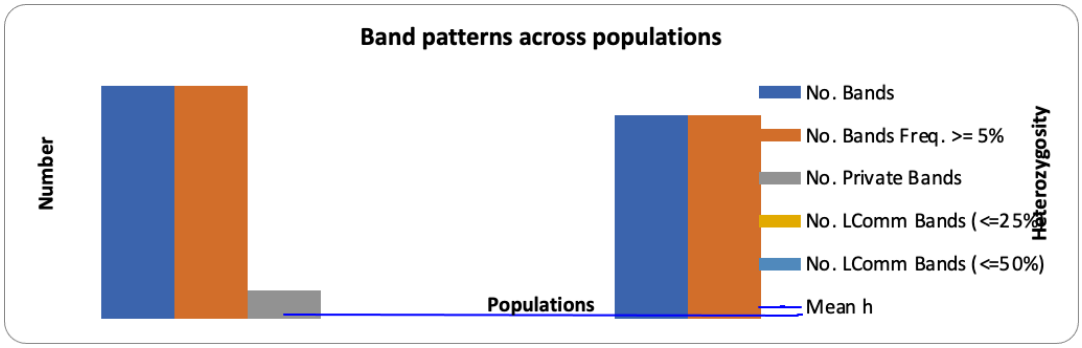

Figure 2. Band pattern analysis using all primers

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X and Y axis labels.
An axis for the y – what are the numbers here?
Error bars where appropriate.

The banding pattern across the population graphic at Figure 2 has shown that the Lalar population has a higher number of bands and band frequency than that of the Liang population. The lalar population also has several private bands representing unique allele loci to a single population. Banding patterns and heterozygosity graphics were standard analyses for horse genetic population diversity studies (Mahrous et al., 2011; Seo et al., 2016). This result indicates that the genetic diversity of the Lalar population was higher than the genetic diversity of the Liang population.

Kommentar [JB25]: Make sure place names are consistently capitalised.

Table 3. Analisis of molecular variance result of lalar population and liang population

Source	df	SS	MS	Est. variance	Variance percentage
Among Population	1	6.125	6.125	0.287	10%
Within Population	22	58.917	2.678	2.678	90%
Total	23	65.042		2.965	100%

Analysis of Molecular Variance (AMOVA) based on F-statistics calculation is one of the most frequent methods to determine a population's genetic structure (Meirmans, 2012). AMOVA result has shown that variation within the population was higher than variation among the population (Table 3). This genetic structure indicated the high level of gene flow between the Lalar population and Liang population. The same result was found in the AMOVA result of the Bosnian mountain horse population which variation within the population was much higher than variation among the population. It is suggested that the high gene flow in several horse populations is due to a high level of inbreeding between populations (Rukavina et al., 2021).

Kommentar [JB26]: This is the first time AMOVA is discussed. Please introduce this in your methods section, data analysis subheading.

Table 4. Comparison different methods for constructing similarity matrices and dendrogram

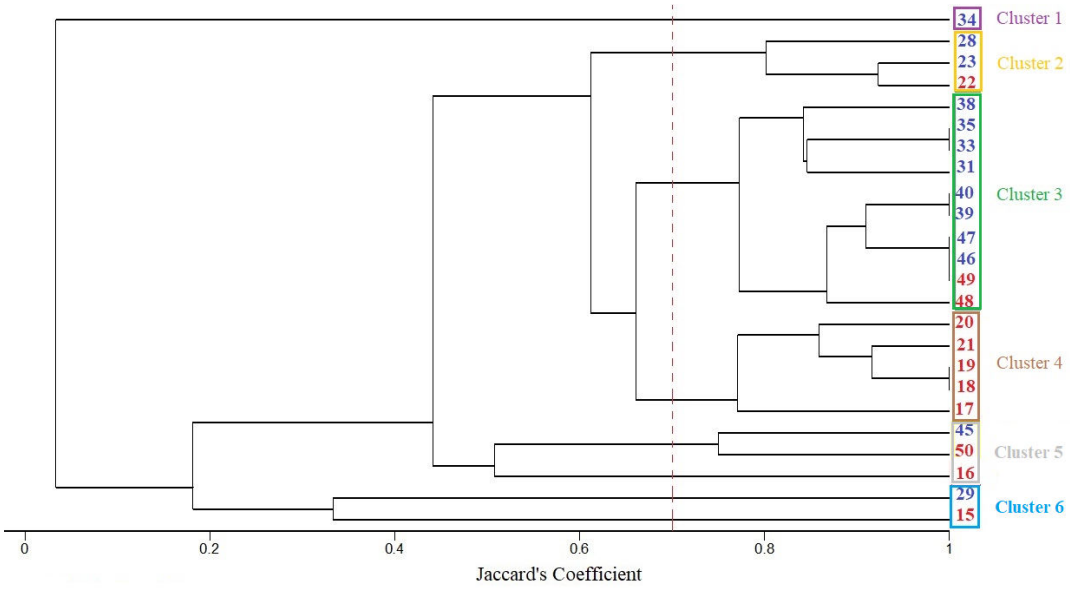
Similarity matrices	Algorithm	Co-phenetic coefficient	S.E. of r
Simple Matching	UPGMA	0.827	0.034
Simple Matching	Single Linkage	0.697	0.043
Simple Matching	Complete Linkage	0.827	0.034
Jaccard	UPGMA	0.940	0.021
Jaccard	Single Linkage	0.925	0.023
Jaccard	Complete Linkage	0.900	0.026
Nei and Li (Dice)	UPGMA	0.889	0.028
Nei and Li (Dice)	Single Linkage	0.929	0.022
Nei and Li (Dice)	Complete Linkage	0.893	0.027

UPGMA = un-weighted pair-group method using arithmetic average

Kommentar [JB27]: Please provide the P values

Various banding patterns of all microsatellite primers were tabulated and sorted based on their molecular weight. These bands were treated as DNA fingerprinting characters for similarity value calculation. Similarity value was essential for clustering analysis for dendrogram construction (Wangiyana et al., 2022). This systematic step has been used recently for genetic diversity studies based on microsatellite DNA fingerprinting (Driskill et al., 2022; Fiore et al., 2022). This research also applied microsatellite DNA fingerprinting to study horse population genetic diversity. It could update different approaching methods for genetic diversity study on horse populations based on microsatellite markers that commonly only focus on allele frequency, heterozygosity, and polymorphism information (Mahrous et al., 2011).

Similarity matrices and dendrograms based on microsatellite DNA fingerprinting were constructed using different methods (Table 4). Co-phenetic correlation analysis was used to examine the correlation between the similarity value represented on the dendrograms (sorted similarity matrix) and the actual similarity value calculated for each dendrogram (unsorted similarity matrix). Co-phenetic correlation coefficient (r) could determine the distortion between the unsorted similarity matrix and the sorted similarity matrix (Carvalho et al., 2019). UPGMA algorithm and Jaccard's similarity coefficients have the highest r-value (0.940) among the other methods. This result is similar to the co-phenetic correlation analysis based on microsatellite DNA fingerprinting that used different similarity matrices (Jaccard, Dice, and simple matching) and algorithms (UGPMA, complete linkage, single linkage) on flue-cured tobacco genotypes (Gholizadeh et al., 2012). However, this result could be considered a novel finding on microsatellite DNA fingerprinting on horse samples since no co-phenetic correlation analysis has been reported in horse population genetic study.



15, 16, 17, 18, 19, 20, 21, 22, 48, 49, 50 = Lalar Population
23, 28, 29, 31, 33, 35, 38, 39, 40, 45, 46, 47 = Liang Population

Figure 3. Dendrogram based on UPGMA algorithm and Jaccard coefficient

Kommentar [JB28]: The numbers don't make sense. Why start at 15?

Dendrograms were constructed using the UPGMA algorithm and Jaccard Similarity coefficient based on co-phenetic correlation analysis (Figure 3). Cluster member on this dendrogram could be determined by the cut of value on the Jaccard coefficient, recommended at 0.7 (Wangiyana et al., 2021). Based on the cut of value, there were 6 clusters on this dendrogram with various members. The uniqueness of this dendrogram is that most cluster has both Lalar population individuals and Liang population individual. Cluster 5 is the only cluster with Lalar Population individuals without mixing with Liang population individuals. This result has emphasized the high gene flow between Lalar Population and Liang Population that was previously described in the AMOVA result.

Similarity relationship on the horse by dendrogram construction based on microsatellite marker could be tested its validity compared with other markers. RAPD marker could be chosen as a comparison since this marker was commonly used in the similarity relationship study of the horse with dendrogram construction (Abdulrazaq et al., 2019). Dendrogram based on microsatellite marker has lower similarity value than dendrogram based on RAPD marker (Hassan et al., 2019). This result indicated that the microsatellite marker could differentiate Organism Taxonomical Unit (OTU) on particular similarity values that could not differentiate by the RAPD marker.

Kommentar [JB29]: Provide a conclusion here

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Kommentar [JB30]: Currently, the references are not formatted to the style of Biodiversitas. Please review the author guidelines and reformat all references. Specifically, pay attention to:

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Revealing the genetic diversity of Sumbawa endemic horse using microsatellite-based DNA fingerprint

Manuscript received: DD MM 2016 (Date of abstract/manuscript submission). Revision accepted:

Abstract. The purpose of this study was to reveal the genetic diversity of the Sumbawa endemic horse (*Equus caballus*) using a microsatellite-based DNA fingerprint. Blood samples were taken from 24 individual horses from two different populations, Lalar and Liang, West Sumbawa Regency, Indonesia. A total of 4 microsatellite primers were used in this study, INRA032, HEL09, CA425, and AHT4. This study revealed that the genetic diversity of horses in the Lalar population was higher than Liang. A greater number of alleles reinforces this; higher number and frequency of bands; and the presence of ~~private~~ specific bands that indicate unique alleles. This research shows that the Sumbawa horse is unique from other horse breeds in the world. This is evidenced by the lower number of alleles per locus (Na) with a maximum number of two alleles per locus. Sumbawa horses have higher observed heterozygosity (Ho) than expected heterozygosity (He), with a Ho value less than 0.5. Analysis of Molecular Variance result has shown that variation within the population was higher than among the population. This is presumably due to the high gene flow in both horse populations caused by inbreeding. Similarity analysis strengthens the hypothesis, which shows that there is a cluster consisting of individual horses from the two observed populations. In general, AHT4 primers had the best ability to reveal the genetic diversity of Sumbawa horses with the highest Shannon's information index compared to other markers.

Keywords: Genetic diversity, Sumbawa horse, DNA fingerprint, microsatellite

Running title: Sumbawa horse microsatellite DNA fingerprint

INTRODUCTION

DNA fingerprinting is an individual identification technique using individual DNA profiles (Singla et al. 2017). This identification process is carried out by comparing DNA sequences that are unique to each individual (Choi et al. 2008). DNA fingerprinting is widely used in forensics and paternity identification (Krishnamurthy et al. 2011). In addition, DNA fingerprints are also widely used to identify genetic diversity and relationships in plants (Selvakumari et al. 2017; Jamil et al. 2021), poultry genetic diversity (Farrag et al. 2010), livestock (Fadhil et al. 2013), even widely used for the identification of fish species (Al-Faisal et al. 2019). DNA fingerprinting includes techniques such as Restriction Fragment Length Polymorphism (Nishikaku et al. 2019), randomly amplified polymorphic DNA (El-Mouhamady et al. 2019), and Amplified Fragment Length Polymorphism (Vigneshwaran et al. 2017; Malik et al. 2022). Variable Number Tandem Repeat (VNTR) or Short Tandem Repeat (STR) based PCR (Polymerase chain reaction) is most often used in DNA fingerprinting because it has high sensitivity and the procedure takes less time compare to other DNA fingerprint method (Choi et al. 2008). One of the molecular markers, a Short Tandem Repeat often used for genetic analysis of livestock, is a microsatellite (Teneva et al. 2018).

Microsatellites are single locus DNA sequences with very high polymorphisms that are spread throughout the genome (Heryani et al. 2019). Microsatellites have repeating ~~copies~~ sequences usually 1 to 6 nucleotides long (Garkovenko et al. 2018). The repetition of this DNA unit can be in the form of mononucleotides, dinucleotides, trinucleotides, tetranucleotides, and so on (Mason 2015; Donnik et al. 2017). Microsatellite DNA markers have been widely used to study genetic diversity because they are randomly distributed throughout the genome, codominant, and have high polymorphism (Putman and Carbone 2014). Microsatellite markers have been widely used for genetic diversity analysis in plants (Saptadi et al. 2020; Parmar et al. 2022), poultry (Luis-Chincoya et al. 2021), cattle (Agung et al. 2019), buffalo (Vohra et al. 2021), and horses (Kim et al. 2021). In Indonesia, research on livestock genetic diversity still needs to be done because some of the genetic resources of native Indonesian livestock are threatened with extinction due to a small population and limited distribution (Sutarno et al. 2015). One of the leading local livestock owned by Indonesia is the Sumbawa horse (*Equus caballus*) (Wibisono et al. 2017; Mujahid et al. 2019).

Sumbawa horse is one of Indonesia's local horse families, which is a native Indonesian genetic resource that needs to be protected and conserved with a geographical distribution on Sumbawa Island, West Nusa Tenggara (Keputusan Menteri Pertanian 2011). The Sumbawa horse has an essential meaning for the Sumbawa people, both from an economic and socio-cultural perspective. From the economic aspect, Sumbawa horse milk is used for consumption needs in the form of

Kommentar [JB1]: Describe the background. Is this well studied?

Kommentar [LI32R1]: The research background has been described in the introduction section and cannot be displayed in the abstract because the number of words is limited.

Kommentar [JB3R1]: For a research paper, the word limit is 8,000 words, so there are many more words available.

Kommentar [JB4]: Include scientific name on first mention

Kommentar [LI35R4]: The scientific name has been added.

Kommentar [JB6]: How and where were samples collected from?

Kommentar [LI37R6]: The sampling method has been described in the methods section and cannot be shown in detail in the abstract due to the limited number of words.

Kommentar [JB8R6]: Most readers will only read the abstract. As such, the methods need to be included so that readers understand what was done. ...

Kommentar [JB9]: What do you mean here?

Kommentar [LI310R9]: The private band has been changed to a specific band.

Kommentar [JB11]: This doesn't make sense.

Kommentar [JB12R11]: This sentence still doesn't make sense. Please revise

Kommentar [LI313]: It has been revised following the reviewer's ...

Kommentar [JB14]: Some of the key words are already included in the title. ...

Kommentar [LI315R14]: We did not find other terms to replace this word, so ...

Kommentar [JB16R14]: There are many alternative terms. For example, the ...

Kommentar [JB17]: There is no comma required after the et al. as per the ...

Kommentar [LI318R17]: It has been revised following the reviewer's ...

Kommentar [JB19]: Give full term on first mention

Kommentar [LI320R19]: It has been revised following the reviewer's ...

Kommentar [JB21]: Takes less time than what?

Kommentar [LI322R21]: It has been revised following the reviewer's ...

Kommentar [JB23]: Sequences?

Kommentar [LI324R23]: It has been revised following the reviewer's ...

Kommentar [JB25]: Not formatted to Biodiversitas style. Please check guideli ...

Kommentar [JB26]: Please include scientific name on first mention

wild horse milk which is beneficial for health (Prastyowati 2021), while from the socio-cultural aspect, Sumbawa horses are used as a horse racing vehicle called "Main Jaran". This horse racing culture has a high ~~philosophy~~ value for one's social status. In addition, horse racing culture can strengthen brotherhood, preserve culture and serve as people's entertainment (Asidah 2020). Seeing the importance of the existence of the Sumbawa horse, it is necessary to preserve the genetic resources of the Sumbawa horse. Information on the genetic status of the Sumbawa Horse as one of Indonesia's native livestock is very important as a step in developing a conservation strategy in the long term (Sutarno et al. 2015).

Research that reveals the genetic diversity of Sumbawa horses has never been done. So far, research related to Sumbawa horses has focused on milk quality (Saragih et al. 2013). The latest research on the genetic diversity of horses was reported by Lapian (2021). The study is only a review of the results of research that has been carried out by people in several countries and has not revealed the genetic diversity of the Sumbawa Horse. Another study by Wibisono et al. (2017) only revealed the morphological diversity of horses and had not used genetic markers. Therefore, this research is critical because it is one of the pioneers in revealing the genetic diversity of the Sumbawa Horse based on DNA fingerprints. The purpose of this study was to reveal the genetic diversity of Sumbawa horses based on DNA fingerprints using microsatellite DNA markers.

MATERIALS AND METHODS

Sampling and DNA purification

DNA isolation was conducted from horse blood samples. Blood samples were taken from two horse farms with 24 individual horses from two different populations, namely Lalar and Liang, West Sumbawa Regency, Indonesia (Figure 1). Blood was taken from the jugular vein (Sikka and Sethi 2008) located in the jugular groove on each side of the neck from the jaw angle just above the brisket and slightly above the side of the horse's windpipe. Blood samples were collected by a qualified veterinarian from the Faculty of Veterinary Medicine, Universitas Pendidikan Mandalika, Indonesia following the protocol of Zalkovic et al. (2001). Blood samples put into a tube containing EDTA (Ethylenediamine Tetraacetic Acid) solution, and brought to the laboratory for DNA extraction. DNA was extracted from whole blood samples (Griffiths and Chacon-Cortes 2014) using the NucleoSpin Blood QuickPure kit (Macherey-Nagel, Germany) following a predetermined procedure. The extracted DNA was then separated using Agarose Gel Electrophoresis (Lee et al. 2012) with a concentration of 0.5%.

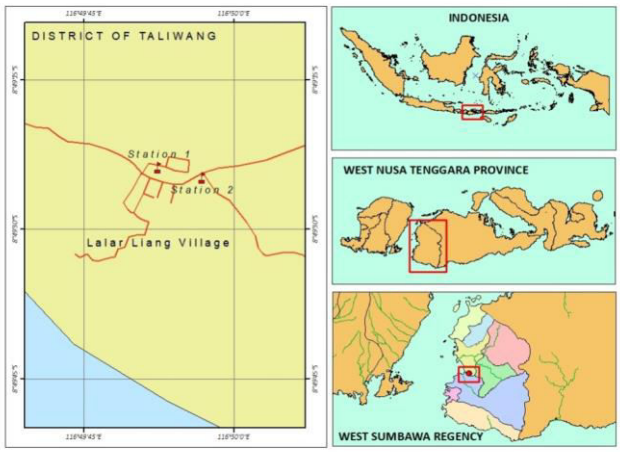


Figure 1. Sumbawa horse sampling location, station 1 = Liang (8°49'23.8"S 116°49'52.4"E), station 2 = Lalar (8°49'24.8"S 116°49'56.8"E)

PCR amplification

A total of 4 microsatellite primers were used in this study. Two microsatellite primers were adopted from buffalo microsatellite primers, namely INRA032 (Navani et al. 2002) and HEL09 (Uffo et al. 2017). Meanwhile, two primers were adopted from horse microsatellite primers recommended by the International Society for Animal Genetics, namely CA425 and AHT4 (ISAG 2016). The primer sequences are shown in Table 1. PCR was carried out with a total volume of 25 µL with a mixture of: 2.5 µL DNA template, 2.5 µL forward primer, 2.5 µL reverse primer, 12.5 µL PCR mix, and 5 µL dH2O (Sukri 2014). The PCR process was carried out in several stages, namely initial denaturation at 95^{0}C for 10 minutes, 30 cycles of 30 seconds at 95^{0}C (Denaturation), 30 seconds at 60^{0}C (Annealing), 1 minute at 72^{0}C (Extension), and finally, final elongation at 72^{0}C for 10 minutes (Moshkelani et al. 2011).

Kommentar [JB27]: Value?

Kommentar [LI328R27]: It has been revised following the reviewer's suggestions.

Kommentar [JB29]: More information is needed here. Please explain:
1. Where were horses kept? Wild or captive?
2. Where on the body were samples taken? E.g. hair, skin, muscle?
3. How were the horses selected? Were any related to one another?
4. Was the study approved by any ethics committees?

Kommentar [LI330R29]: It has been revised following the reviewer's suggestions.

Kommentar [JB31]: Full term on first mention

Kommentar [LI332R31]: It has been revised following the reviewer's suggestions.

Kommentar [JB33]: spacing

Kommentar [JB34]: extension

Kommentar [JB35]: How many cycles were used?

Kommentar [LI336R35]: It has been written at the beginning of the sentence, which is 30 cycles.

Microsatellite analysis

Genotyping of microsatellite DNA polymorphisms was carried out through agarose gel electrophoresis (Asif et al. 2008), then DNA fragments were analyzed using GeneScan and Genotyper® software (Cozzi et al. 2022). Genetic diversity measures include allele frequency, the observed number of alleles, banding patterns and expected heterozygosity, molecular variance, and similarity relationship based on the dendrogram. Allele frequencies, observed number of alleles, and expected heterozygosity were analyzed using POPGENE v.1.32 and GenAlEx 6.5 software (Peakall and Smouse 2012). Heterozygosity analysis was conducted to describe inter-population variability through allele frequency analysis, while analysis of molecular variance (AMOVA) was conducted to determine variations within and between populations using Arlequin v 3.0 software (Excoffier et al. 2005). Cophenetic correlation analysis was performed on the similarity matrix based on DNA fingerprinting data from microsatellite primers using Co-Stat for Windows. Furthermore, the dendrogram was constructed using the clustering method based on the optimum R value cophenetic correlation results using the Multivariate Statistical Package Ver. 3. (Kasiamdari et al. 2019). Finally, the effectiveness of microsatellite primers in revealing the genetic diversity of horses from two populations was measured based on the Shanon Information Index value using the GenAlEx 6.5 software.

Kommentar [JB37]: Shannon is not explained here. Please explained this.

Kommentar [LI338R37]: It has been revised following the reviewer's suggestions.

Table 1. Characteristics of the microsatellite markers used

No	Name	Sequence	Number of bases
1	CA425	F: AGCTGCCTCGTTAATTCA	18
		R: CTCATGTCCGCTTGTCTC	18
2	AHT4	F: AACCGCCTGAGCAAGGAAGT	20
		R: CCCAGAGAGTTTACCCT	17
3	INRA032	F: AAACTGTATTCTCTAATAGCTAC	23
		R: GCAAGACATATCTCCATTCCTTT	23
4	HEL09	F: GGAAGCAATGAAATCTATAGCC	22
		R: TGTTCTGTGAGTTTGTAAGC	20

RESULTS AND DISCUSSION

A total of 49 alleles were detected in 24 individuals of the two tested populations (Lalar and Liang). Each microsatellite marker produced various alleles in the two tested populations. On average, the Lalar population had more alleles than the Liang population. This result was an early indication of the genetic variation between these populations since a different number of the allele was an essential parameter of genetic variation based on microsatellite marker (Ustyantseva et al. 2019)

Kommentar [JB39]: marker revealed various alleles in the two

Kommentar [LI340R39]: It has been revised following the reviewer's suggestions.

Table 2. Allele frequencies and estimated diversity each primer

Marker	Population	Na	Ne	I	Ho	He
HELL09	Lalar	1.750	1.342	0.366	0.233	0.254
	Liang	2.000	1.491	0.484	0.313	0.341
	Mean	1.875	1.417	0.425	0.273	0.297
INRA032	Lalar	2.000	1.900	0.665	0.472	0.515
	Liang	1.000	1.400	0.318	0.222	0.242
	Mean	1.500	1.650	0.492	0.347	0.379
CA415	Lalar	2.000	1.552	0.493	0.325	0.355
	Liang	1.600	1.476	0.424	0.283	0.309
	Mean	1.800	1.514	0.459	0.304	0.332
AHT4	Lalar	2.000	1.432	0.452	0.286	0.312
	Liang	2.000	1.557	0.540	0.356	0.388
	Mean	2.000	1.495	0.496	0.321	0.350

Na : Number of alleles per locus
Ne : Number of effective alleles per locus
I : Shannon's information index
Ho : Observed heterozygosity
He : Expected heterozygosity

Kommentar [JB41]: Surely this should be an integer (e.g. 1, 2 or 3). How can you get 1.75 alleles per locus?

Kommentar [LI342R41]: This is the average value of the number of alleles found at each locus, resulting in a decimal number.

Kommentar [JB43]: How was this generated? Make sure this is explained fully

Kommentar [LI344R43]: It has been described in the methods section.

Several parameters were computed to measure genetic variability between the Lalar and Liang populations (Table 2). The number of alleles per locus (Na) in the Lalar population and Liang population was always higher than the number of

effective alleles per locus (Ne) on all markers (except for the INRA032 marker). A genetic population study on horse breeds based on horse microsatellite markers commonly resulted in higher Na than Ne (Fornal et al. 2020). Higher Ne than Na on the INRA032 marker may be due to this marker was not a specific microsatellite marker for horse breed.

The number of different alleles per locus (Na) was a common genetic variation in horse breed based on microsatellite markers. Western Arabian Horse Na varied from 3–5 (Khanshour et al. 2013), Iranian horse has Na from 3–4 (Moshkelani et al. 2011), Polish Konik Horse has Na varied from 5–6 (Fornal et al. 2020), Akhal–Teke horse has Na varied from 3–4 (Ustyantseva et al. 2019). Sumbawa–Indonesian horse in this study has a lower number of Na than horse breeds from other regions of the world since these horse breeds only have a maximum of two numbers of alleles per locus. The lalar population has an average Na value higher than the Na value of the Liang population.

The number of observed heterozygosity (Ho) in the Sumbawa-Indonesia horse population was lower than the number of expected heterozygosity (He). The low value of Ho (below 0.5) indicates that this horse population has suffered a loss of heterozygosity due to increased inbreeding. However, this result also indicates the unique genetic characteristic of the Sumbawa-Indonesian breed compared to the genetic characteristic of horse breeds from Asia, Africa, and Europe. Most horse populations from Asia, Europe, and Africa have Ho values higher than 0.5 (Benahamadi et al. 2020; Dorji et al. 2018; Gómez et al. 2017). Sumbawa-Indonesian horse population has heterozygosity characteristics similar to the American (Brazilian) horse breed with a Ho value less than 0.5 (Reis et al. 2008). Moreover, the Brazilian American horse mostly has a Ho value lower than the He value, just like Indonesia –Sumbawa horse (Table 3)(Silva et al. 2012).

Table 3. Comparison of the Sumbawa horses' Ho and He values and other horses in the world

Population	Ho	He	Reference
Lalar Sumbawa Horse	0.31	0.36	This research
Liang Sumbawa Horse	0.29	0.32	This research
Brazilian-American Horse	0.32	0.57	(Reis *et al.*, 2008)
Algerian-African Horse	0.67	0.71	(Benahamadi *et al.*, 2020)
Bhutan-Asian Horse	0.79	0.78	(Dorji *et al.*, 2018)
Marismeno-Europe Horse	0.77	0.78	(Gómez *et al.*, 2017)
Akhal Teke Horse	0.72	0.70	(Ustyantseva *et al.*, 2019)
Iranian-Arab Horse	0.75	0.73	(Moshkelani, Rabiee and Javaheri-Koupaei, 2011)

Molecular variation based on microsatellites (Single Sequence Repeats) has become a typical analysis for population genetic study (Vieira et al. 2016). Microsatellite marker specified for horse breed has been developed to reveal genetic variation in different horse breed (Mahrous et al. 2011). AHT4 in this study was a specified microsatellite marker for a horse that could reveal genetic diversity among the Sumbawa-Indonesia horse population compared to other markers. AHT4 marker could produce the highest Shannon's information index among other markers. This marker also produces a consistent number of alleles per locus on the Lalar and Liang populations. This result implicated that AHT4 was a useful microsatellite marker for the genetic population study of the Indonesian horse breed.

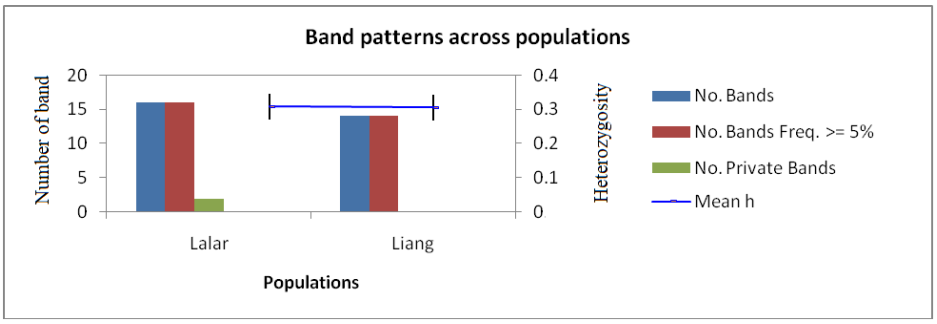


Figure 2. Band pattern analysis using all primers

The banding pattern across the population graphic at Figure 2 has shown that the Lalar population has a higher number of bands and band frequency than that of the Liang population. The ~~lalar~~ Lalar population also has several private bands representing unique allele loci to a single population. Banding patterns and heterozygosity graphics were standard analyses for horse genetic population diversity studies (Mahrous et al. 2011; Seo et al. 2016). This result indicates that the genetic diversity of the Lalar population was higher than the genetic diversity of the Liang population.

Kommentar [JB45]: Could you use a table or figure to compare your results against these from the data?

Kommentar [LI346R45]: It has been revised following the reviewer's suggestions.

Kommentar [JB47]: These citations still need to be revised

Kommentar [JB48]: This figure is difficult to read. Please ensure you provide:
X and Y axis labels.
An axis for the y – what are the numbers here?
Error bars where appropriate.

Kommentar [LI349R48]: The graphic has been replaced according to the reviewer's suggestion.

Kommentar [JB50]: Make sure place names are consistently capitalised.

Kommentar [LI351R50]: It has been revised following the reviewer's suggestions.

Table 4. Analisis of molecular variance result of lalar population and liang population

Source	df	SS	MS	Est. variance	Variance percentage
Among Population	1	6.125	6.125	0.287	10%
Within Population	22	58.917	2.678	2.678	90%
Total	23	65.042		2.965	100%

Analysis of Molecular Variance (AMOVA) based on F-statistics calculation is one of the most frequent methods to determine a population's genetic structure (Meirmans 2012). AMOVA result has shown that variation within the population was higher than variation among the population (Table 4). This genetic structure indicated the high level of gene flow between the Lalar population and Liang population. The same result was found in the AMOVA result of the Bosnian mountain horse population which variation within the population was much higher than variation among the population. It is suggested that the high gene flow in several horse populations is due to a high level of inbreeding between populations (Rukavina et al. 2021).

Table 5. Comparison different methods for constructing similarity matrices and dendrogram

Similarity matrices	Algorithm	Co-phenetic coefficient	S.E. of r
Simple Matching	UPGMA	0.827	0.034
Simple Matching	Single Linkage	0.697	0.043
Simple Matching	Complete Linkage	0.827	0.034
Jaccard	UPGMA	0.940	0.021
Jaccard	Single Linkage	0.925	0.023
Jaccard	Complete Linkage	0.900	0.026
Nei and Li (Dice)	UPGMA	0.889	0.028
Nei and Li (Dice)	Single Linkage	0.929	0.022
Nei and Li (Dice)	Complete Linkage	0.893	0.027

UPGMA = un-weighted pair-group method using arithmetic average

Various banding patterns of all microsatellite primers were tabulated and sorted based on their molecular weight. These bands were treated as DNA fingerprinting characters for similarity value calculation. Similarity value was essential for clustering analysis for dendrogram construction (Wangiyana et al. 2022). This systematic step has been used recently for genetic diversity studies based on microsatellite DNA fingerprinting (Driskill et al. 2022; Fiore et al. 2022). This research also applied microsatellite DNA fingerprinting to study horse population genetic diversity. It could update different approaching methods for genetic diversity study on horse populations based on microsatellite markers that commonly only focus on allele frequency, heterozygosity, and polymorphism information (Mahrous et al. 2011).

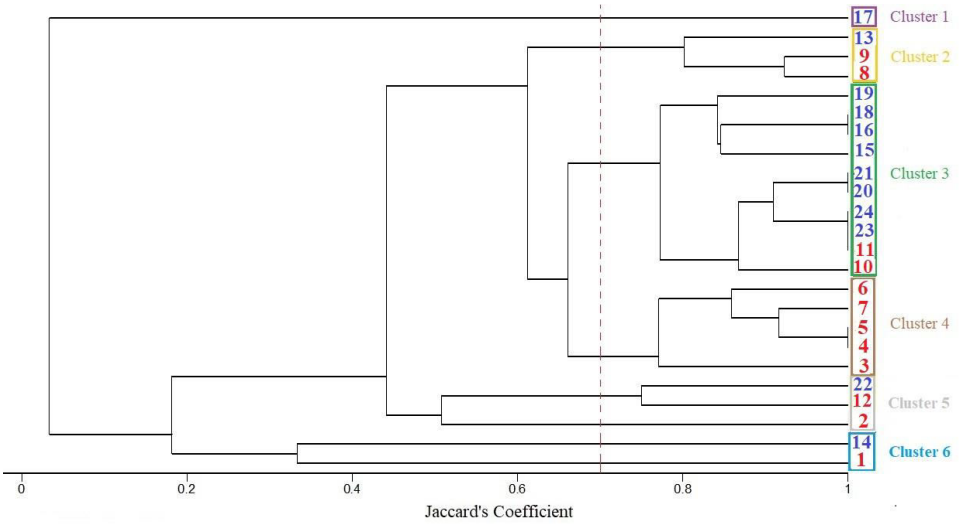
Similarity matrices and dendrograms based on microsatellite DNA fingerprinting were constructed using different methods (Table 5). Co-phenetic correlation analysis was used to examine the correlation between the similarity value represented on the dendrograms (sorted similarity matrix) and the actual similarity value calculated for each dendrogram (unsorted similarity matrix). Co-phenetic correlation coefficient (r) could determine the distortion between the unsorted similarity matrix and the sorted similarity matrix (Carvalho et al. 2019). UPGMA algorithm and Jaccard's similarity coefficients have the highest r-value (0.940) among the other methods. This result is similar to the co-phenetic correlation analysis based on microsatellite DNA fingerprinting that used different similarity matrices (Jaccard, Dice, and simple matching) and algorithms (UGPMA, complete linkage, single linkage) on flue-cured tobacco genotypes (Gholizadeh et al. 2012). However, this result could be considered a novel finding on microsatellite DNA fingerprinting on horse samples since no co-phenetic correlation analysis has been reported in horse population genetic study.

Kommentar [JB52]: This is the first time AMOVA is discussed. Please introduce this in your methods section, data analysis subheading.

Kommentar [LI353R52]: It has been described in the method section according to the reviewer's recommendations.

Kommentar [JB54]: Please provide the P values

Kommentar [LI355R54]: P-value was not used in this analysis. This analysis is only used to determine the optimum model to be used in constructing the dendrogram.



1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 = Lalar Population
13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 = Liang Population

Figure 3. Dendrogram based on UPGMA algorithm and Jaccard coefficient

Dendrograms were constructed using the UPGMA algorithm and Jaccard Similarity coefficient based on co-phenetic correlation analysis (Figure 3). Cluster member on this dendrogram could be determined by the cut of value on the Jaccard coefficient, recommended at 0.7 (Wangiyana et al. 2021). Based on the cut of value, there were 6 clusters on this dendrogram with various members. The uniqueness of this dendrogram is that most cluster has both Lalar population individuals and Liang population individual. Cluster 5 is the only cluster with Lalar Population individuals without mixing with Liang population individuals. This result has emphasized the high gene flow between Lalar Population and Liang Population that was previously described in the AMOVA result.

Similarity relationship on the horse by dendrogram construction based on microsatellite marker could be tested its validity compared with other markers. RAPD marker could be chosen as a comparison since this marker was commonly used in the similarity relationship study of the horse with dendrogram construction (Abdulrazaq et al. 2019). Dendrogram based on microsatellite marker has lower similarity value than dendrogram based on RAPD marker (Hassan et al. 2019). This result indicated that the microsatellite marker could differentiate Organism Taxonomical Unit (OTU) on particular similarity values that could not differentiate by the RAPD marker.

Genetic diversity analysis of Sumbawa horses showed that there was a specific band that became a unique allele in the Lalar population. In addition, the higher number and frequency of bands in the Lalar population makes it a population with higher genetic diversity than the Liang population. In general, Sumbawa horses are unique compared to other horses, as indicated by the low value of Na per locus and a higher Ho value than He with a value of less than 0.5. The molecular analysis of variance results indicated the presence of gene flow in the two observed populations. This is evidenced by the value of variation within the population higher than between populations. This result was also strengthened by the similarity analysis, which showed a cluster consisting of individual horses from the two observed populations. The Shanon Information Index analysis results revealed that the AHT4 primer had the best ability to reveal the genetic diversity of Sumbawa horses compared to other primers.

ACKNOWLEDGEMENTS

We want to thank the Lembaga Pengelola Dana Pendidikan (LPDP) and Kemendikbudristek for funding this research through the Riset Keilmuan Scheme in 2021.

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Kommentar [JB56]: The numbers don't make sense. Why start at 15?

Kommentar [LI357R56]: The graphic has been replaced according to the reviewer's suggestion.

Kommentar [JB58]: Should be Shannon

Kommentar [JB59]: Provide a conclusion here

Kommentar [LI360R59]: A conclusion has been added at the end of the discussion

Kommentar [JB61]: Currently, the references are not formatted to the style of Biodiversitas. Please review the author guidelines and reformat all references. Specifically, pay attention to:

- Author punctuation should be formatted as per the guidelines
- The year should not be in brackets
- The journal name should not be in italics. It should be abbreviated
- The Volume, issue and pages should be provided
- The DOI should be provided for all journals
- Scientific names should be in italics.
-

Kommentar [LI362R61]: The reference has been revised according to the reviewer's suggestion.

Kommentar [JB63R61]: Many scientific names (e.g. Camelia) have not yet been italicised.
Many DOIS are still missing for journals.

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