

Genetic Diversity Evaluation of *Moringa oleifera*, Lam from East Flores Regency Using Marker Random Amplified Polymorphic DNA (RAPD) and Its Relationship to Chemical Composition and In Vitro Gas Production

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ABSTRACT

The research objective was to evaluate the genetic diversity of *Moringa oleifera*, Lam (MO) and its relationship to chemical composition and in vitro gas production (IVGP). Fresh MO leaves were kept frozen in ice gels pack until laboratory analysis. Four methods applied: RAPD marker for measuring DNA concentration and purification; Kjeldhal and HPLC for analysing proximate and amino acid (AA) composition; and IVGP. MO's four distinct morphology found: green, red, reddish green and aromatic green. RAPD result analysis was 68.8-74.7 %, it means those MO had a close genetic similarity. The morphological differences are also related to leaves chemical composition variation. The highest protein and AAs content were found in aromatic green MO. Total IVGP at 96 hours reached 95.9, 99.3, 111, 115 mL per 500 mg DM in aromatic green, green, reddish green, red MO, respectively and statistically among those was highly significant difference ($P < 0.01$). However, DM and OM digestibility did not differ significantly and estimated ME contents were similar suggesting MO leaves had sufficient fermentable nitrogen amount required to ensure rumen microbes normal activities. Conclusively, those MO has a close genetic relationship but the aromatic green MO more beneficial due its higher content of crude protein and AAs.

Keywords: amino acid content; in vitro digestibility; miracle tree; morphological variation

INTRODUCTION

The ubiquitous spread of *Moringa oleifera* (MO) in Indonesia may have been as long as the migration history of Indonesian ancestor from

India and Indochina. This notion stems from the fact that the plant is originated from India but nowadays it has been endemic plant found elsewhere from the low to the highland of tropical regions (Bayé-Niwah & Mapongmetsem, 2014). MO has many local names such as kelor (in Java island); maronggi (Madura island), moltong (Flores island), hau fo (West Timor) and it is generally perceived by people in certain parts of Indonesia as anti black magic trees rather than as source of nutritious vegetables or fodder consumed by either human or animals. Nevertheless, in the last decade many studies on MO's nutritive values and its benefits for livestock feed resources have been accumulated. Soetanto, & Firsoni (2008) reported a significant increase in in vitro organic matter digestibility and rumen microbial biomass production when MO was added to the diet consisted of maize stover, commercial concentrate and urea molasses-block. In other field experiment Soetanto, Chuzaemi, & Marhaeniyanto (2010) revealed that adding MO in the basal diet of Ettawah goats had 83 % more weight gain that can be attributed to MO supplementation. Other authors reported a 14.5 % increase in daily weight gain of fat-tailed growing rams when MO and *Samanea saman* leaves were supplemented to a diet containing 18 % crude protein (Marhaeniyanto, Soetanto, Kusmartono, & Hartutik, 2013). Recently, Dahlanuddin, Yanuarianto, Poppi, McLennan, & Quigley (2014) fed male Bali cattle aged between 6 and 12 months with four tree fodder hay as a sole diet, i.e. Sesbania, Leucaena, MO and Gliricidia and revealed that only the first three fodder hay could increase the body weight from 0.47 to 0.22 kg per head per day while gliricidia hay as the sole diet seemed to satisfy the maintenance requirement only.

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The potential of MO as supplement for sheep has been reported by Mulyati, Kusmartono, Hartutik, & Rusdi (2015) where the daily gain reached 107 g per head per day. There was evidence demonstrated by Rahardja, Fattah, & Toleng (2010) that reproductive performance improved in beefi cattle given MO in the diet. For this reason it is not surprising if MO has been used widely for human and livestock nutrition due to its uniqueness of nutrient composition that satisfy the most requirement of essential nutrients (Price, 2007), such as essential amino acids, minerals and other antioxidant compounds (Nouman *et al.*, 2014). According to Ali (2014) although MO contains phenolic acids (gallic, chlorogenic, ellagic and ferulic acid) and flavonoids (kaempferol, quercetin and rutin) there is no evidence of harmful effect of feeding MO reported in the literature.

The main mineral contents of Na, Fe, Mn, and Zn are about 192.95 ± 4.4 ; 107.48 ± 8.2 ; 81.65 ± 2.3 ; and 60.06 ± 0.3 ppm, respectively (Ogbe & Affiku, 2011). It therefore is likely that MO can be used as the source of indispensable minerals for animal and human beings (Dasola, Tunbosun, Adeyemi, & Abidemi, 2014). MO also called a life-tree since it can be used as a source of food to overcome malnutrition (Sixl-Daniell, Sixl, W., Sixl, G., & Fuchs, 2011; Azeez *et al.*, 2013), as nutritional supplement for weaning infant and nursing mother (Kesharwani, Prasad, Roy, & Sahu, 2014) and having anti fungal properties (Patel, P., Patel, N., Patel, D., Desai, & Meshram, 2014). Nowadays, the plant has a highly value and cultivated largely in the tropics and sub-tropical areas. The plant cultivation is done in order to be used for food, herbs or medicine, and industrial objectives (Moyo, Masika, Hugo, & Muchenje, 2011; Anwar, Latif, Ashraf, & Gilani, 2007). MO seed can be used as coagulant for water purification (Dalen, Pam, Izang, & Ekele, 2009; Padmapriya, Thamaraiselvi, Nivethini, & Thirunalasundari, 2014), and the leaf can be used to increase blood haemoglobin (Hb) concentration, when it is used for feeding (Otitoju, Nwamarah, Otitoju, Okorie, Stevens, & Baiyeri, 2014). The richness of MO leaves in essential amino acids, vitamin A and minerals such as Na, Fe, Mn and Zn (Ogbe & Affiku, 2011) may be used as a strategy to eliminate nutrient deficiencies for human as well as for animals in certain regions such as East Nusa Tenggara where a long dry season causes poor growth of ruminant animals. Unfortunately, most people in East Nusa Tenggara do not aware of the

nutritive values of MO other than recognize it as a natural vegetable source for their daily consumption. In Flores island and other parts of East Nusa Tenggara, MO can be easily found anywhere and it usually grows surrounding house yard and dry land without irrigation. Nonetheless, the information on MO genetic diversity from this area is scarce and people generally descriminate them mainly based on the morphological differences of leaf petioles color and fragrance exposure that may reflect their genetic variability and adaptation to environmental conditions. Nevertheless, with the advancement of technology, genetic variation of plants can be determined more accurately using DNA-based analysis such as random amplified polymorphic DNA (RAPD) and, hence, it allows the application for plant breeding and selection as has been reported by Rufai *et al.* (2013) in MO for human food and animal fodder.

The objectives of this study were to ascertain the genetic diversity of MO in East Flores regency using marker RAPD and its relationship to the chemical composition and in vitro gas production.

MATERIALS AND METHODS

MO Leaves Collection

Samples of fresh MO leaves were collected from March to June 2014 on the basis of four distinctive colour differences growing at the village of Lewolere, district of Larantuka, East Flores Regency, East Nusa Tenggara Province at $08^{\circ}20'50.9$ SL and $122^{\circ}57'53.5$ EL, at 50 m above sea level (Fig. 1).

The fresh leaves were placed in the plastic bags containing wet cotton layers to prevent water loss from the leaf cells, then put in the box cotaining iced gels and transported immediately to the central life science laboratory of University of Brawijaya for further DNA analysis. While other fresh leaves were sun dried under the shed and ground through a 1 mm sieve diameter for protein and amino acids analysis.

Isolation and DNA Purification

DNA isolation was carried out according to the modified method of Doyle, J. J. & Doyle, J. L. (1990) on the fresh leaves samples as follow: weighing 0.2 g fresh kelor leaves and wrapped with aluminium foil and stored at -20°C . While frozen it was grinded in the sterilized mortar and added liquid nitrogen and 500 μl CTAB buffer solution, decanted into 1.5

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ml sterilized plastic tube, gently mixed and covered with parafin and incubated in the water bath at 65 °C for 30 minutes. They were then centrifuged at 15493 g for 10 minute at 25 °C. The supernatant was transferred into 1.5 ml sterilized tubes, added with aliquot volume of chloroform, and re-centrifuged at 15493 g for 5 minute. The same procedure was repeated as in step 2 but chloroform was replaced by ammonium acetate and vortexed prior adding 2.5 times the volume with absolute ethanol, mixed until a white treath like form appereared. They were then stored overnight in the -20 °C freezer, centrifuges at 15493 g for 15 minutes at 4 °C until pelleted DNA was formed. 500 µl 70 % ethanol was added to the pelleted DNA and centrifuges at 15493 g for 10 minutes at 4 °C. The pelleted DNA was then air dried , added with 50 µl TE buffer (pH=7.6) and stored at -20 °C for further analysis.

RAPD PCR Amplification

PCR amplification was performed using Go Taq Green PCR Mix kits (Promega) as follow:

mixing 1 µl Primer 10 pmol µl⁻¹ with 3 µl double distilled water, 5 µl PCR mixture and 1 µl DNA. An initial denaturation temperature of 95 °C for 3 minutes and denaturation for 1 minute, annealing at 37 °C for 1 minute, extensions at 72 °C for 2 minutes and last extension at 72 °C for 5 minutes and back to 95 °C for 45 cycles.

DNA fragment pattern produced were translated in binary data. The data, then, were processed and analyzed using Multi Variate Statistical Package (MVSP) with method of UPGMA (Unweight Pair Grouping Method with Arithmetic Averaging) (Sokal & Michener, 1958).

Chemical Analysis

Samples of MO leaves collected from the area under study were sun dried under the shed canopy and ground to pass the 2 mm size sieve prior to proximate analysis (AOAC, 1990) particularly crude protein content of MO leaves determined by Kjeldhal method.

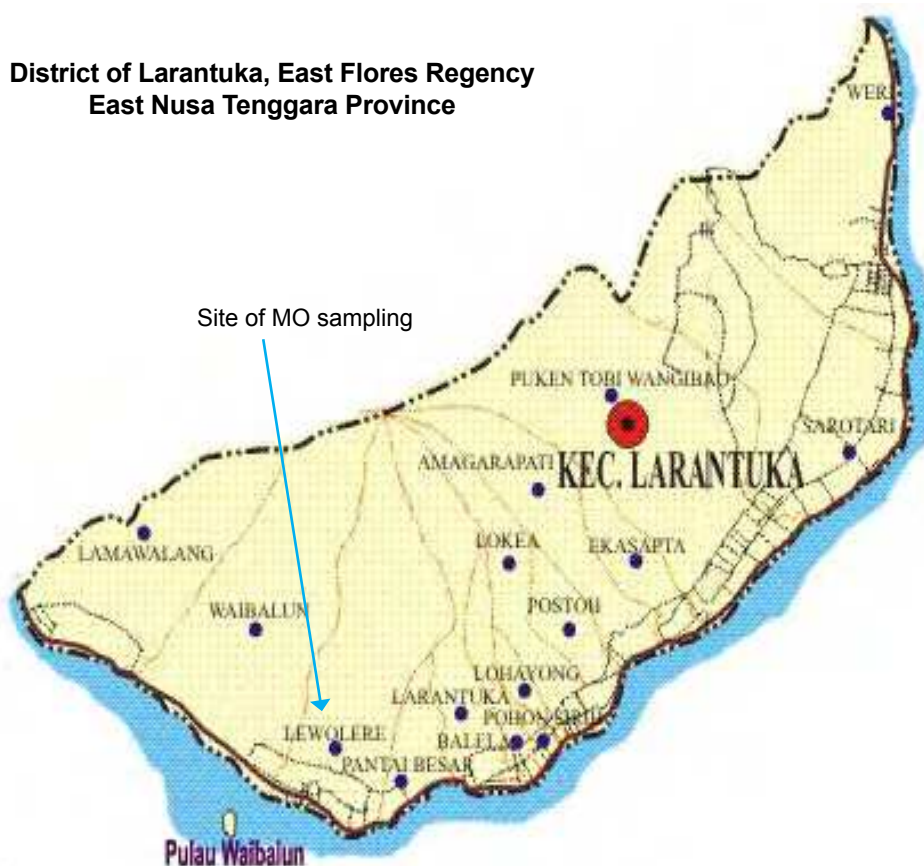


Fig. 1. The site of MO collection

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Analysis of Amino Acid

Amino acid content of each sample of MO leaves was determined by HPLC method (Lookhart & Jones, 1985) on the basis of:

$$AA (\%) = \frac{\mu\text{mol AA} \times \text{Mr AA} \times 100}{\mu\text{g sample}}$$

Remarks:

AA = Amino Acid

Mr = relative masses

In Vitro Gas Production

Sample preparation and measurement of total gas production protocol were essentially according to the method of Makkar, Blümmel, & Becker (1995). Rumen liquor was obtained from a Frisien Holstein cross bred cow which is equipped with a rumen cannulae and fed with fresh elephant grass and concentrate mixture in proportion of 60:40 to meet the above maintenance requirement. The animal was kept at the field laboratory of University of Brawijaya and the using of this animal for experiment has obtained a permit from the ethical commission of University of Brawijaya. A mixture of rumen fluid and digesta was taken by aspirating it directly from the cannulae and placed it in a pre-warmed thermo flask and carried to the nutrition laboratory where in vitro gas production measurement was performed. A mixture of rumen fluid and digesta was screened using four layer cheese cloth under continuous flow of CO₂ gas. Approximately 340 ml screened rumen fluid was added to mineral buffer to get 1500 ml of solution. 50 ml of rumen fluid buffer mixture was added to each syringe containing approximately 500 mg of MO samples, blank and standard, respectively and they were incubated at 39 °C in the waterbath to measure gas production at time intervals 0,2,4,6,8,12,16,24,36,48,72, and 96 hours.

The data of each parameter was estimated using exponential equation model according to Ørskov & McDonald (1979), with a Neway computer packet program (Boga, Yurtseven, Kilic, Aydemir, & Polat, 2014) according to exponential equation:

$$Y = a + b(1 - e^{-ct})$$

Where:

- Y = amount of gas produced at t time
- a = amount of gas produced by soluble fraction (ml per 500 mg DM)
- b = amount of gas produced by insoluble fraction (ml per 500 mg of DM)
- c = constant of gas production from insoluble fraction (ml per hour)

a + b = total production of gas from fermented fraction (ml per 500 mg DM)

t = time of incubation (hour)

e = 2.72 (natural logarithm)

Digestibilities of DM and OM were calculated as differences between samples and residues obtained after 96 hours incubation. All data obtained from IVGP was subjected to statistical analysis using a statistical package program of SPSS version 18 (Levesque, 2007).

RESULTS AND DISCUSSION

Morphological Differences and Genetic Diversity

There were four distinctive morphological differences of MO trees growing in the area of study based on the colours of petiole and leaf odor (Fig. 2). This is also supported by evidence that in nature each type showed genetic diversity as demonstrated by the results of RAPD analysis. In general the purity of DNA analysis may be interfered by the presence of polysaccharides and polyphenolic compounds (Lutz, Wang, Zdepski, & Michael, 2011). This interference may be reduced by addition of CTAB as an inhibitor compound. This study showed that the purity of DNA found from the samples were high as indicated by the ratio of UV absorbance wave length at Å260/Å280 which denotes as purity fell between 1.88 and 2.07, suggesting that neither polysaccharides nor polyphenolic interfered the purity of DNA (Table 1).

The result of PCR analysis using RAPD marker demonstrated that from 20 OPA primers only 18 resulted reproducible bands. A total of 79 DNA fragments had successfully been amplified from the genome of four MO samples resulting in 44 polymorphic band or equal to 55.9 % polymorphic. Number of amplified fragments per primer varied between 250 bp and 2500 bp as shown in Fig. 3. This indicates that among four MO samples there is different sequence of DNA from the DNA fragment being amplified. Table 2 depicts the sequence of each primer, the number of band and polymorphism percentage.

The percentage of polymorphic of four MO accession in this study was only 55.9 % which is considered lower than those reported by other authors such as Ojuederie, Igwe, Okuofu, & Faloye (2013) who reported polymorphism of 10 MO from Western Nigeria was 81.5 % while Popoola (2014) found the corresponding figure for 13 MO accession in Nigeria was 77.9 %. Nevertheless, this finding

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is higher if compared by the report of Rufai *et al.* (2013) that found only 32.7 % from five accessions of MO in Malaysia. Furthermore, following a MVSP analysis of DNA fragment pattern using UPGMA (Sokal & Michener, 1958) the four MO accessions were found having close genetic similarity ranged between 68.8 % and 74.7 % (Fig. 4) suggesting that in the area under study the existing MO trees

might come from similar genus but due to genetic and environment interaction they differ phenotypic in petiole colors and odor shown in Fig. 2 table 2. These differences may be reflected in chemical composition such as CP and AAs profile that are beneficial for selection of MO to produce a source of essential nutrition either for human or animals.



A. Green MO



B. Reddish green MO



C. Red MO



D. Aromatic green MO

Fig. 2. The differences of MO trees growing in the area of study based on the colours of petiole and leaf odor

Table 1. DNA concentration of MO from East Flores, Indonesia

No	Sample Name	Absorbance (A0)		Purity	Concentration (ng µl ⁻¹)
		260	280		
1	A1	18.1	9.30	1.95	907
2	A2	9.10	4.69	1.94	454
3	B1	21.5	10.4	2.07	1,072
4	B2	8.66	4.37	1.98	433
5	C1	8.56	4.34	1.97	428
6	C2	7.42	3.72	1.99	371
7	D1	13	6.55	1.99	651
8	D2	10.8	5.73	1.88	538

Remarks: Samples of A1 and A2 = green MO; B1 and B2 = reddish green MO; C1 and C2 = red MO; D1 and D2 = aromatic green MO

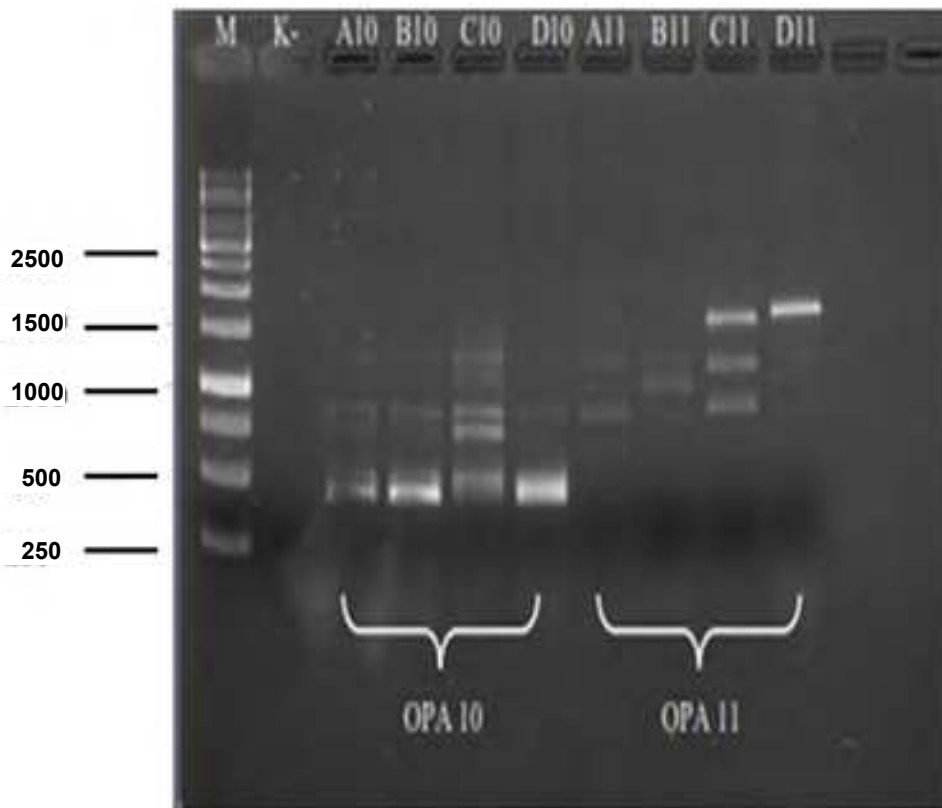


Fig. 3. Example of RAPD analysis of MO leaves using OPA10-OPA11 primer

Table 2. Sequence of 18 OPA primers with the number of scorable, amplified and polymorphic bands

Primer Name	Sequence (5'-3')	Total Number of band	Number of polymorphic band	Percentage of polymorphism
OPA 1	CAG GCC CTT C	5	2	40.0
OPA 2	TGC CGA GCT G	4	3	75.0
OPA 3	AGT CAG CCA C	4	0	0
OPA 4	AAT CGG GCT G	3	1	33.3
OPA 7	GAA ACG GGT G	2	2	100
OPA 8	GTG ACG TAG G	3	2	66.7
OPA 9	GGG TAA CGC C	4	2	50.0
OPA 10	GTG ATC GCA G	6	4	66.7
OPA 11	CAA TCG CCG T	6	6	100
OPA 12	TCG GCG ATA G	7	5	71.4
OPA 13	CAG CAC CCA C	5	1	20.0
OPA 14	TCT GTC CTG G	2	1	50.0
OPA 15	TTC CGA ACC C	3	2	66.7
OPA 16	AGC CAG CGAA	6	2	33.3
OPA 17	GAC CGC TTG T	4	3	75.0
OPA 18	AGG TGA CCG T	2	0	0
OPA 19	CAA ACG TCG G	8	7	87.5
OPA 20	GTT GCG ATC C	5	1	20.0
Total		79	44	55.9

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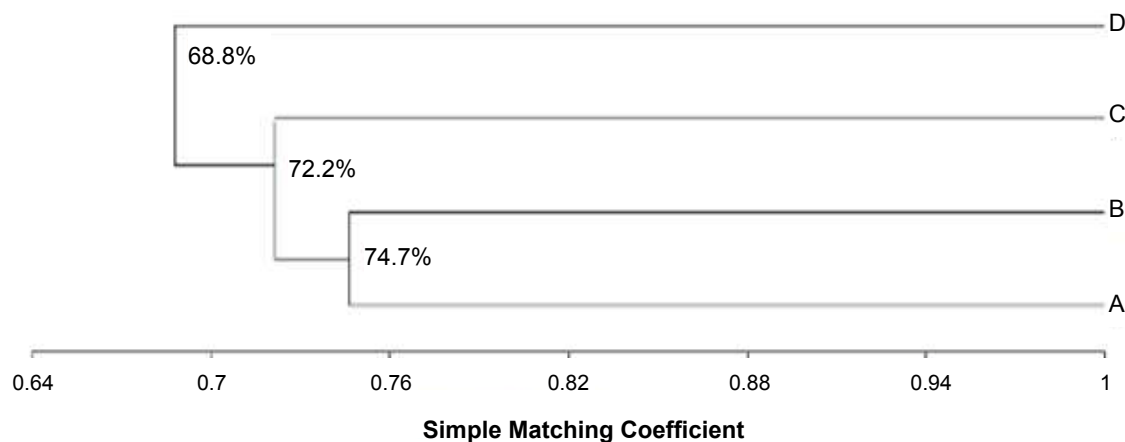


Fig. 4. Phylogenetic relationship among the studied MO accessions using UPGMA dendrogram based on RAPD Primary result OPA 1-20

Table 3. Proximate analysis of four accessions of MO leaves from East Flores

Items	Morphology			
	Green	Reddish Green	Red	Aromatic Green
Dry Matter (%)	88.8	87.2	88.3	86.2
Organic Matter (% DM)	89.1	90.1	91.3	88.3
Crude Protein (% DM)	33.9	27.5	28.4	36.5
Crude Fiber (% DM)	6.84	8.73	7.14	7.74
Crude Lipid (% DM)	7.42	9.99	8.99	6.97
NFE (% DM)	40.8	43.8	46.8	37.1
Ash (% DM)	10.9	9.92	8.70	11.7

Table 4. Amino acid profile of MO leaves varying in morphology (%)

Parameter	Morphology			
	Green	Reddish Green	Red	Aromatic Green
Amino Acid Essential				
Arginine	1.59	1.09	1.11	1.94
Histidine	0.57	0.41	0.41	0.73
Leucine	2.20	1.68	1.70	2.70
Isoleucine	1.34	1.00	1.01	1.61
Lysine	1.38	1.04	1.05	1.47
Methionine	0.30	0.26	0.27	0.34
Threonine	1.11	0.82	0.83	1.43
Phenylalanine	1.77	1.32	1.34	2.17
Valine	1.61	1.19	1.21	1.91
Non Essential				
Aspartic Acid	3.03	2.00	2.03	3.89
Glutamic Acid	3.67	2.82	2.86	4.25
Serine	1.14	0.84	0.86	1.38
Glycine	1.23	0.89	0.91	1.39
Alanine	1.62	1.25	1.27	1.91
Tyrosine	1.64	1.15	1.17	1.75
Cystine	0.33	0.21	0.16	0.69
Proline	0.99	0.84	0.90	1.28

Chemical Composition and Amino Acids Profile

Table 3 describes the result of proximate analysis of four MO accession leaves in which the aromatic green type was found containing the highest CP followed in descending order by green, red and reddish green types, respectively. In contrast, the crude fiber (CF) content was relatively similar among them. From the survey of literature, the CP content of MO leaves varies between 18.4 and 39.1 % (Ogbe & Affiku, 2011; Agbogidi & Ilondu, 2012; Madukwe, Ugwuoke, & Ezeugwu, 2013; Offor, Ehiri, & Njoku, 2014; Moyo, Masika, Hugo, & Muchenje, 2011; Mbailao, Mianpereum, & Albert, 2014; Melesse, 2011; Sodamade, Bolaji, & Adeboye, 2013). While for CF content the corresponding figures are 5.43 to 11.2 (Mbailao, Mianpereum, & Albert, 2014; Melesse, 2011; Ogbe & Affiku, 2011; El-Sohaimy, Hamad, Mohamed, Amar, & Al-Hindi, 2015; Sodamade, Bolaji, & Adeboye, 2013).

The result of AA analysis presented in Table 4 does not include essential AA tryptophan due to limitation of our laboratory to obtain chemical standard for tryptophan and some non-essential AAs. Therefore, only 9 and 8 essential and non-essential AAs, respectively, are presented in Table 4.

In general, AA profile of aromatic green type showed superior compared to the other three MO leaf type, even when compared with the results of Moyo, Masika, Hugo, & Muchenje (2011) and Khalel *et al.* (2014). While for other types of MO leaves contain either essential or non-essential AAs within the value range reported by many investigators (Zaku, Emmanuel, Tukur, & Kabir, 2015; Mbailao, Mianpereum, & Albert, 2014; Nouman *et al.*, 2014).

In Vitro Gas Production

There is generally agreed that protein content in feed affects rumen microbial activity in favor of gas production. Melesse (2011) reported a positive correlation between CP content and IVGP of two species of MO leaves. In contrast this current study was not in accordance with the report of Melesse (2011) as the aromatic green MO type that contained higher CP produced gas at a lesser volume than other MO leaf accessions (Table 3 and Table 5). In addition, there was a lag phase (Table 6 and Fig. 5) found in all MO types suggesting that there is an anti-nutritional factor e.g. tannin in the leaf affecting the commencement of microbial fermentation in the rumen. Total IVGP at 96 hours reached 95.9, 99.3, 111 and 115 ml per 500 mg DM in aromatic green, green, reddish green and red MO leaves, respectively and statistically among those was highly significant difference ($P < 0.01$). Nevertheless, IVGP of this present finding fell within the gas production ranges reported by previous studies (Asaolu, Odeyinka, Binuomote, Odedire, & Babayemi, 2014; Karim, Amin, Moniruzzaman, Sarker, & Kabir, 2015; Melesse, 2011).

As shown in Table 6 both DM and OM digestibility were surprisingly not significant difference among them but higher than reported by Nezarati, Nazer, Ramin, & Abolfazi (2014) and hence the estimated ME content was similar and addition of protein in the diet usually has catalytic effect on rumen microbial activity if there is nutritional deficiency for microbial growth leading to improvement in feed digestion (Leng, 1993). However, this condition may not always operate especially when the ratio of protein and carbohydrate is low.

Table 5. In vitro gas production of MO leaves varying in morphology (ml per 500 mg DM)

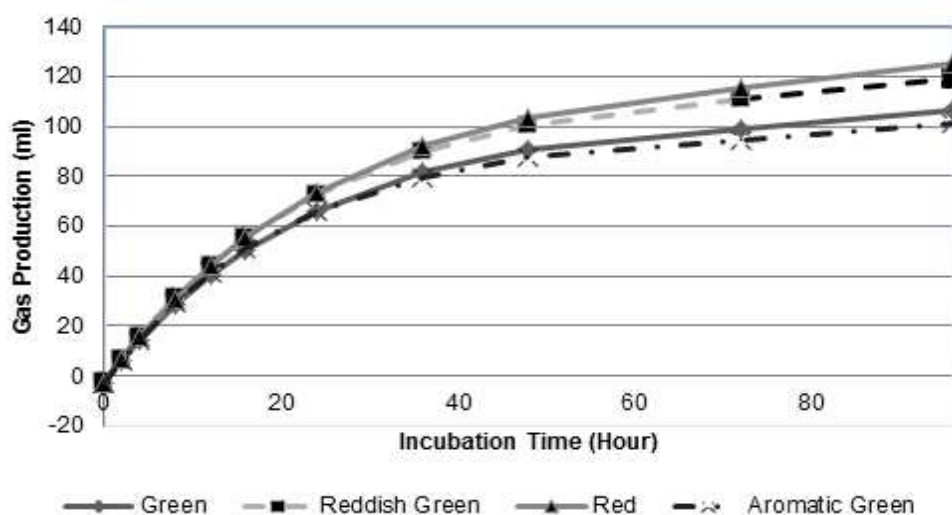
Duration of Incubation (hour)	Morphology				SEM	Prob.
	Green	Reddish Green	Red	Aromatic Green		
2	4.22	5.07	4.92	4.55	0.191	0.619
4	10.9a	12.9b	13.0b	11.4a	0.545	0.021
8	27.3a	31.5b	31bc	28.3ac	1.02	0.034
12	47.7	52	51.3	47.9	1.12	0.080
16	61.3a	66b	66.8b	61.4a	1.47	0.026
24	76.4a	80.7b	84.6b	75.2a	2.16	0.011
36	85.7a	94.2b	98.5c	83.4a	3.56	0.000020
48	91.6a	102b	106c	87.9a	4.33	0.000003
72	96.8a	109b	112b	93.7a	4.46	0.000013
96	99.3a	111b	115b	95.9a	4.68	0.000045

Remarks: Values bearing different letter in the same row differ significantly at $P < 0.01$. SEM = standard error of mean; Prob. = probability

Table 6. Coefficient digestion of dry matter and organic matter of MO leaves

Item	MO type				SEM	Prob.
	Green	Reddish Green	Red	Aromatic Green		
a (ml)	-3.01	-2.3	-2.76	-2.73	0.147	0.689
b (ml)	106 ^a	119 ^b	125 ^c	101 ^a	5.62	0.000207
c (ml per hour)	0.044	0.041	0.039	0.047	0.002	0.065
a + b (ml)	109 ^a	121 ^b	128 ^c	104 ^a	5.56	0.000033
DMD (%)	63.3	66.8	67.1	64.0	1.13	0.595
OMD (%)	89.4	91.3	91.4	89.7	0.729	0.740
ME (MJ/kg DM)*	8.31	8.15	8.45	8.40	0.014	0.244

Remarks: Values bearing different superscript in the same row differ significantly at $P < 0.01$; * = ME estimated from equation $ME = 2.2 + (0.136 \times Gv) + (0.057 \times CP)$ (Makkar, 2002), where: ME = metabolizable energy; CP = crude protein in percent; and Gv = the net gas production in ml from 200 mg dry sample after 24 h of incubation, SEM = standard error of mean; Prob. = probability

**Fig. 5.** In vitro gas production of different MO leaves collected from East Flores (ml per 500 mg DM)

CONCLUSION

From this present study it can be concluded that MO found in East Flores Regency has a close genetic relation each other despite distinct morphological, crude protein and amino acids differences. Nevertheless, the difference in CP content did not affect in vitro DMD and OMD suggesting that MO leaves as a single ingredient has sufficient amount of fermentable nitrogen ensuring a normal activity of rumen microbes to digest food. Further studies under in vivo conditions on the effect of MO leaves supplementation are warranted.

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