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Proximate, Mineral, Fatty Acid and Cholesterol Composition of Five Muscles of an Indigenous Swine Breed of North East India

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ABSTRACT

Background: Ghungroo is the first registered indigenous pig breed of India bearing accession number INDIA_PIG_2100_GHUNGROO_09001 reported from Western Assam and North Bengal. The present study determines nutritional content (proximate, mineral, fatty acid and cholesterol) of five muscles that are considered most valued cuts by customers.

Methods: Thirty muscles were dissected from six reared Ghungroo pigs, each from shoulder (*Triceps brachii, Latissimus dorsi*) and from ham region (*Biceps femoris, Gracilis, Tensor fasciae latae*). Two months old piglets were reared from October, 2019 to May, 2020 and slaughtered. They were administered commercial diet *i.e.* starter, grower and finisher feed during different stages of growth.

Result: The highest protein, fat and ash were found in *tensor fasciae latae, gracilis* and *triceps brachii* respectively (P<0.05). In 100 g of meat, potassium, sodium, magnesium and zinc was found highest in *Tensor fasciae latae, Latissimus dorsi* and *Biceps femoris* respectively (P<0.05). The total saturated (SFA's), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids were found highest in *Tensor fasciae latae* and *Latissimus dorsi* respectively (P<0.05), while trans-fatty acid and cholesterol were both found highest in *Gracilis*. The data shall be valuable for muscle specific improvement of meat quality and value addition for global markets.

Key words: Cholesterol, Fatty acid, Ghungroo pig, Indigenous swine breed, Minerals, Porcine muscles, Proximate.

INTRODUCTION

In India, there are around 10.29 million pigs, of which 2 million pigs are found only in Assam, highest among all the states (Livestock census of India, 2019; Singh *et al.,* 2020). Ghungroo is the first registered indigenous pig breed of India with accession number INDIA_PIG_2100_GHUNGROO_09001 (NBAGR, 2008). Due to its high productivity in terms of litter size (9-12 nos.) and good mothering ability, they have gained high rearing popularity in Northeast India. Ghungroo is a medium size pig with large drooping ears and dark-black color with scanty hairs and bristles. Their appearance is that of atypical bull dog face (Zaman *et al.,* 2013).

Pork meat is sold in primal cuts and these primal cuts are formed by various individual muscles which have different characteristics (Kim *et al.,* 2008). It is important to evaluate the chemical composition of different muscles so that its nutritional and human health perspectives are known. A meat is considered to exhibit good nutritional quality when it is rich in protein with a high amount of polyunsaturated fatty acids (Listrat *et al.,* 2016).The amount of fat content in meat is of a great deal of interest due to its organoleptic properties (Essien *et al.,* 1998).

Earlier studies have highlighted the minerals, amino acid and fatty acid contents of *M. longissimus thoracis et lumborum* (LTL) of Ghungroo pig and crossbred pigs (Thomas *et al.,* 2016; Thomas *et al.,* 2018). The present study is aimed to provide detail information on proximate, mineral, fatty acid and cholesterol content of five muscles, namely *Triceps brachii, Latissimus dorsi, Biceps femoris, Tensor fasciae latae* and *Gracilis*.

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MATERIALS AND METHODS

The study was approved by the Institutional Animal Ethics Committee; Bodoland University vide letter no- IAEC/BIOTECH/2019/3. The muscles selected for the study were *Triceps brachii, Latissimus dorsi, Biceps femoris, Tensor fasciae latae* and *Gracilis*. The experiment was conducted with six Ghungroo barrows. They were born in the month of August, 2019. The experiment was undertaken during October 2019 to May 2020 and slaughtered. The reared pig breeds were administered commercial diet-starter, grower and finisher feed during different stages of growth. Prior to slaughter, the pigs were fasted for 12-16 hour with *ad-libitum* supply of water. Pigs were slaughtered in a commercial abattoir for poultry and swine in Tangla market, Assam, with the slaughtering process adapted for pigs. Five muscles *viz. triceps brachii* (291.91±42.0 g) and *Latissimus dorsi* (313.3±9.46 g) were dissected from shoulder; *Biceps femoris*

(305.95±17.79 g), *gracilis* (306.73±19.73 g) and *Tensor fasciae latae* (300.7±10.42 g) were dissected from ham region. The dissected muscles were identified and transferred to Bodoland University laboratory and stored at -20°C. 24-hour post-mortem, the subcutaneous fats from all the muscles were trimmed off by knife and homogenized using a mechanical grinder. The experiments were performed at the Department of Biotechnology, Bodoland University.

The chemical contents *i.e.* moisture, protein, fat and ash content of all the five muscles were determined according to AOAC (2005). The carbohydrate content was calculated by the difference method (FAO, 2003). The nutritive value or calorific value in kcal/100g was calculated with the help of the equation given by James (1995).

The determination of potassium (K), magnesium (Mg), sodium (Na), iron (Fe), zinc (Zn), manganese (Mn) and copper (Cu) were detected by Atomic Absorption Spectrometer (AAS-ICE 3500), Thermo Scientific, UK, at respective wavelengths with Instrument mode - Flame, Gas used - air acetylene. The meat samples were grinded and dried at oven at 105°C. For the digestion, 1 g of the dried sample was taken, into which 5 ml of HNO_3 and 1 ml of $HClO_4$ was added. The samples were left overnight to pre-digest and then placed in an oven at 100°C for 5-8 hrs. The cooled sample was diluted with deionised water to final volume of 50 ml (ASEAN Manual of Food Analysis, 2011).

For fatty acid determination, the dried muscle samples were subjected to lipid extraction with chloroform/methanol (Folch *et al.*, 1957). The lipid extract was esterified with BF_3-methanol (Joseph *et al.*, 1992) for preparation of FAME's (Fatty Acid Methyl Esters). The fatty acid composition of each aliquot was estimated by Gas Chromatography. GC-MS analysis of sample extracts was carried out with Perkin Elmer (USA), Model: Clarus 680 GC and amp; Clarus 600C MS comprising a liquid auto-sampler. The Software used in the system was TurboMass Ver.6.1.2. The peaks were analyzed using data analysis software NIST-2014. The capillary column used is 'Elite- 5MS' having dimensions-length- 60 m, ID- 0.25 mm and film thickness- 0.25 µm and the stationary phase is 5% diphenyl 95% dimethyl polysiloxane. Helium gas (99.99%) was used as carrier gas (*i.e.* mobile phase) at flow rate of 1 ml/minute. An injection volume of 2 µl was employed in split less mode. Injector temperature was 280°C and ion-source temperature 180°C. The oven temperature was programmed at 60°C (for 1 minute), with an increase at the rate 7°C/minutes to 200°C (hold for 3 minutes) then again increased at rate of 10°C/min to 300°C (hold for 5 min).The total run time is ~39 minutes. Solvent delay was kept for 8 minutes. MS Protocol Mass Spectra was taken in Electron Impact positive (EI+) mode at 70 eV. For MS scan, a solvent delay of 8 minute was provided with m/z range 50-600 amu. For the analysis of total cholesterol content, 1 gm of muscle sample was taken and 0.5 ml of 5α-cholestane (internal standard, 1 mg/0.5 ml of cyclohexane) was added. To it 5 ml of saturated methanolic KOH was added in a capped vial. Then the solution was heated for 30 minutes at 80°C. After cooling at room temperature, 5 ml of cyclohexane was added and vortexed for 1 minute (Naeemi *et al.*, 1995). An aliquot of the cyclohexane layer was injected into the Gas Chromatography (Perkin Elmer, Clarus 680, USA).

The mentioned data were statistically analyzed using SPSS, Version 26.0 and demonstrated as mean±SEM (standard error of mean). The differences of readings in respect of muscles are analyzed with the help of ANOVA technique. Tukey's test has been employed to check the pair-wise association for those attributes which have significant effect ($P<0.05$).

Results and Discussion

Proximate composition

The moisture, protein, fat, ash, total carbohydrate and calorie contents of five muscles of Ghungroo pig are shown in Table 1. Muscle *biceps femoris* (79.33%) had the highest moisture and presented an overall mean of 76.86%, while that of protein, highest content was reported in *tensor fasciae latae* with a mean of 21.99% and *gracilis* showing lowest protein value with a mean of 20.10% having a significant value of less than 0.05 ($P<0.05$). The present findings were similar to the results of LTL muscle studied by Thomas (2016) but when compared with the crossbred pigs of Korea in 21 different muscles by Kim (2008), they showed 3.82% less protein content than our present findings. In spite of the differences, the distribution of moisture content was similar across the countries with 90% of the samples having 65-78% moisture.

Intramuscular fat present in meat has a significant effect on its taste and meat quality. Though different regions showed different results, yet there was no huge difference between muscles. Intramuscular fat content varied from a mean value of 3.13% in *triceps brachii* to 4.25% in *gracilis* ($P<0.05$). Higher values of fat content have been reported with a mean of 4.46% for 21 different muscles of crossbred pigs of Korea (Kim *et al.*, 2008) and LTL muscle (Thomas *et al.*, 2016). Ash content did not differ among muscles and were similar to the study of Kim (2008) and Thomas (2016) with overall mean of 0.80%. In any meat, the most abundant chemical composition is water, followed by protein and fat, carbohydrates occur in much smaller amount. *Triceps brachii* (0.44%) has shown lowest amount of total carbohydrate content, whereas *Tensor fasciae latae* (5.43%) has shown highest amount. In 100 g of raw meat, nutritive value or calorific value was found highest in *Triceps brachii* (166.82 kcal/100 g).

Minerals

Differences in the type of tissue, sampling process, production system and seasonal changes can also be the reasons for variations in mineral composition (Tajik *et al.*, 2010). Table 2, represents the results of minerals in five muscles of Ghungroo pig. *Tensor fasciae latae* (154.59 mg/

Table 1: Proximate composition in five muscles of Ghungroo pig.

Parameters	*Triceps brachii*	*Latissimus dorsi*	*Biceps femoris*	*Tensor fasciae latae*	*Gracilis*	Significance (P-value)
Moisture (%)	76.00±3.60	77.00±3.60	79.33±5.03	78.33±3.05	73.66±1.76	0.438
Protein (%)	20.47±0.44	21.58±0.27	20.13±0.22	21.99±0.38	20.10±0.45	*0.012
Fat (%)	3.13±0.08	3.40±0.24	3.99±0.07	3.42±0.23	4.25±0.22	*0.011
Ash (%)	0.84±0.02	0.81±0.02	0.81±0.02	0.84±0.02	0.75±0.00	0.083
Total carbohydrate (%)	0.44±0.01	2.77±0.02	3.84±0.05	5.43±0.16	1.28±0.02	*0.000
Calorie (kcal/100 g)	166.82±2.86	127.74±1.43	133.51±1.73	144.38±2.38	122.27±0.87	*0.000

The values are expressed in mean±SEM. *P<0.05.

Table 2: Mineral composition (mg/100 g) in five muscles of Ghungroo pig.

Parameters	*Triceps brachii*	*Latissimus dorsi*	*Biceps femoris*	*Tensor fasciae latae*	*Gracilis*	Significance (P-value)
Major elements						
K	122.72±1.52	147.71±1.49	142.48±1.37	154.59±1.16	151.40±0.87	*0.000
Na	18.79±0.34	20.86±0.31	20.84±0.28	18.20±0.16	19.44±0.28	*0.000
Mg	10.94±0.66	15.18±0.10	14.09±3.35	14.74±0.38	12.46±0.28	*0.000
Minor elements						
Fe	1.63±0.30	1.53±0.29	1.68±0.26	1.72±0.38	1.54±0.33	0.990
Zn	1.62±0.30	2.56±0.15	3.37±0.23	1.96±0.07	1.65±0.27	*0.001
Mn	0.02±0.00	0.03±0.01	0.03±0.01	0.02±0.01	0.02±0.01	0.972
Cu	0.03±0.00	0.05±0.01	0.03±0.01	0.04±0.01	0.03±0.01	0.907

The values are expressed in mean±SEM. *P<0.05.

100 g) and *Latissimus dorsi* had the highest content of K and Na contents, while *triceps brachii* (122.72 mg/100 g) and *tensor fasciae latae* (18.20 mg/100 g) had the lowest K and Na content, which were significantly different from each other (P<0.05). A study on LTL muscle the amount of K was found to be higher than present study with a mean of 328.28 mg/100 g (Thomas *et al.*, 2016; Thomas *et al.*, 2018). Na and K are important for water and electrolyte metabolism and acid-base equilibrium in the organism (Mienkowska-Stepniewska *et al.*, 2007).

Mg is a basic component for protein metabolism. Present study shows high amount of Mg in *latissimus dorsi* (15.18 mg/100 g) and lowest in *Triceps brachii* (10.94 mg/100 g). While that of LTL muscle, showed low amount of Mg (6.27 mg/100 g) (Thomas *et al.*, 2016; Thomas *et al.*, 2018). Absorption of Fe obtained from meat is approximately 20-30%, while that of plants is only 5% (Nikolic *et al.*, 2015). *Tensor fasciae latae* (1.72 mg/100 g) had highest Fe content, while that of Zn was found highest in *biceps femoris* (3.37 mg/100 g) in the present study. Data of Fe (2.72 mg/100 g) content were similar to that of LTL, but amount of Zn (0.79 mg/100 g) was quite low compared to the present study (Thomas *et al.*, 2016; Thomas *et al.*, 2018). In our study, Mn content ranged from 0.02-0.03 mg/100 g and Cu ranged from 0.03-0.05 mg/100 g, which were quite similar to the results of LTL muscle (Thomas *et al.*, 2016; Thomas *et al.*, 2018).

Fatty acids

The fatty acid composition (% of total FAME) is tabulated in Table 3. Of all the determined fatty acids SFAs had highest percentage with an average of 44.55% (range, 25.29% to 63.62%). MUFA had an average of 32.38% (14.64% to 59.74%), while PUFA accounted for 15.81% (range, 5.97% to 34.47%) which were significantly different from each other with P- value less than 0.05.

PUFA along with MUFA are considered healthy fats, as they can reduce the risk of heart disease. In our study, omega-3 PUFA fatty acid detected is alpha-linolenic acid (ALA) and for omega-6 PUFA is linoleic acid (LA). ALA was found highest in *tensor fasciae latae* (7.10%), while LA (4.22%) was found highest in *triceps brachii* muscles. Compared to our study, ALA (0.46%) content was low in LTL (0.46%) muscle, but the LA (17.90%) content was high (Thomas *et al.*, 2016). While, LA content on same muscle of Duroc (13.28%), Landrace (12.93%) and Yorkshire (13.63%) was high than our study (Choi *et al.*, 2016). Linoleic acid and alpha-linolenic acid are required in our body in order to synthesize other PUFA (Bentsen, 2017). On the other hand, among MUFAs, oleic acid (range, 3.45% to 20.16%) was found to be most abundant and found highest in *gracilis* (20.16%) muscle. Muscle LTL showed high oleic acid content (32.54%) than the present study (Thomas *et al.*, 2016). Another study, on Duroc (45.33%), Landrace (46.78%) and Yorkshire (46.29%) breeds too showed high oleic acid (Choi *et al.*, 2016). Among SFA's, most abundant fatty acid was palmitic acid (range, 7.47% to11.84%) followed by stearic acid (range, 0.79% to 6.01%) and lauric acid (range, 1.30%to 7.11%), all these were found highest in *tensor fasciae latae* muscle. Compared to our study, stearic

Table 3: Fatty acid and cholesterol composition (% of FAME in 100 g) in five muscles of Ghungroo pig.

Parameters	*Triceps brachii*	*Latissimus dorsi*	*Biceps femoris*	*Tensor fasciae latae*	*Gracilis*	Significance (P- value)
SFA	38.29±1.09	25.29±2.16	42.13±3.11	63.62±1.72	53.44±1.82	*0.000
MUFA	35.22±1.73	59.74±1.97	14.64±0.06	27.55±2.12	24.75±1.66	*0.000
PUFA	34.47±1.96	24.96±8.47	6.88±1.00	6.79±1.12	5.97±0.29	*0.001
Oleic acid (18:1 n-9)	12.34±0.49	17.98±0.28	3.45±0.44	13.36±0.33	20.16±0.16	*0.000
ALA (C18:3 n-3)	5.28±0.54	3.19±0.47	1.28±0.04	7.10±0.44	2.86±0.18	*0.000
LA (C18:2 n-6)	4.22±0.37	2.85±0.35	2.82±0.28	2.68±0.28	2.65±0.08	*0.017
Stearic acid (C18:0)	3.90±0.41	0.79±0.46	2.82±0.18	6.01±0.18	1.86±0.19	*0.000
Lauric acid (C12:0)	3.92±0.44	1.30±0.59	3.33±0.44	7.11±0.30	2.22±0.39	*0.000
Palmitic acid (C16:0)	7.47±2.01	11.29±2.34	7.83±1.70	11.84±2.14	10.69±2.24	0.483
Trans fatty acid	12.69±0.17	18.17±0.38	6.75±0.23	13.28±0.49	19.64±0.33	*0.000
PUFA n-6/n-3	0.72±0.07	0.87±0.05	2.54±0.12	0.47±0.06	1.13±0.04	*0.000
PUFA/SFA	0.62±0.12	1.69±0.09	0.33±0.15	0.06±0.02	0.13±0.08	*0.000
Cholesterol	75.92±5.16	63.91±4.70	63.35±4.35	70.06±6.04	70.07±9.32	0.608

The values are expressed in mean±SEM, SFA= Saturated fatty acid, MUFA= Monounsaturated fatty acid, PUFA= Polyunsaturated fatty acid, ALA = Alpha-linolenic acid, LA= Linoleic acid. *P<0.05.

acid (10.75%) and palmitic acid (22.46%) contents were high on LTLmuscle (Thomas *et al.,* 2016). Similarly, palmitic acid (range, 22.91-23.34%) and stearic acid (range, 12.74-13.78%) content was high on Duroc, landrace and Yorkshire breeds (Choi *et al.,* 2016). Other than these fatty acids, the short chain fatty acid (SCFA) such as propionic acid, acetic acid and butyric acid were too detected in some muscles in small amounts (0.45 to 1.36%). SCFA play an important role in maintaining a healthy body and help in proliferation of microbes (Hussein *et al.,* 2021).

Trans-fatty acid (TFA) was found highest in *gracilis* (19.64%) muscle with an overall mean of 14.10%. Study on LTL (3.48%) muscle showed lower content of TFA than our result (Thomas *et al.,* 2016). The ratio between n-6 and n-3 PUFA is considered vital because of its influence on human health. The n-6 fatty acid *i.e.* LA and the n-3 fatty acids, ALA, EPA and DHA collectively protect against coronary heart disease (Wijendran *et al.,* 2004). In our study, high amount of n-6/ n-3 was found in *biceps femoris* (2.54%) muscle, while other studies showed high PUFA n-6/n-3 content with a mean of 15.98% on LTL muscle (Thomas *et al.,* 2016).

Cholesterol content in five muscles of Ghungroo pig is also shown in Table 3. Highest cholesterol content was found in *Triceps brachii* (75.92%) muscle and lowest in *biceps femoris* (63.35%) muscle. Cholesterol content of 55.9%, 53.1% and 59.7% for *Longissimus dorsi, Semi-membranosus* and *Semi-tendinosus* muscles respectively, were found in crossbred pigs of USA (Bohac *et al.,* 1998). These results were considerably lower than our result. Cholesterol is required to our body to synthesize hormones, vitamin-D and digestive fluids.

CONCLUSION

This study contributes to the proximate composition, mineral content, fatty acid and cholesterol content of Ghungroo pig obtained from five porcine muscles. Our results showed high amount of potassium, sodium and magnesium content which can provide adequate amount of recommended daily allowances. SFA's are known to cause various health diseases, mainly related to heart but these SFA fatty acids can be substituted with PUFA n-6 and PUFA n-3. Moreover; it is found that muscle *tensor faciae latae* has the highest content of SFA's. The study establishes information on nutrient composition on Ghungroo pig meat, which can be traded and will be invaluable for nutritionist and dieticians towards improvement of meat quality and value addition for human consumption.

Conflict of interest: None.

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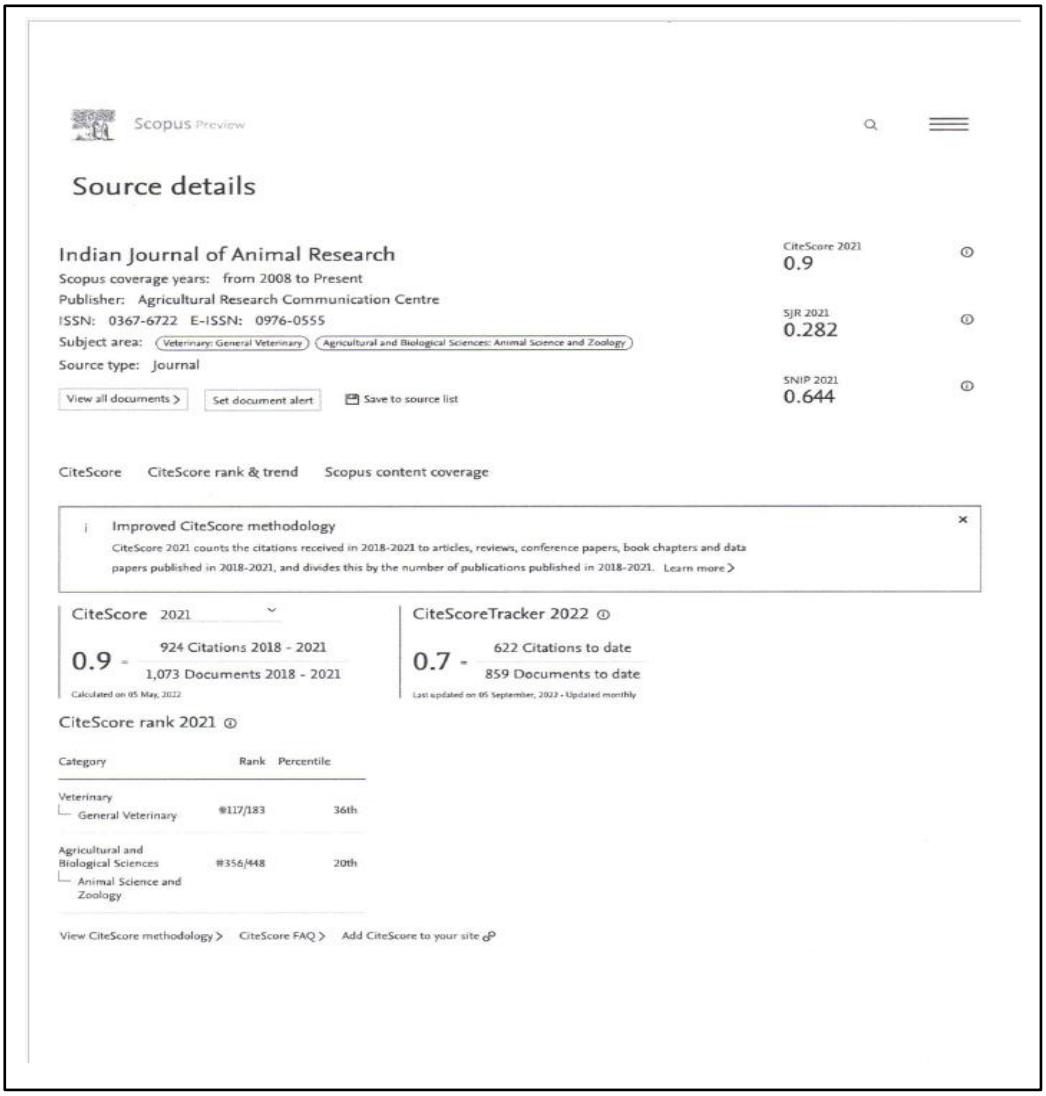

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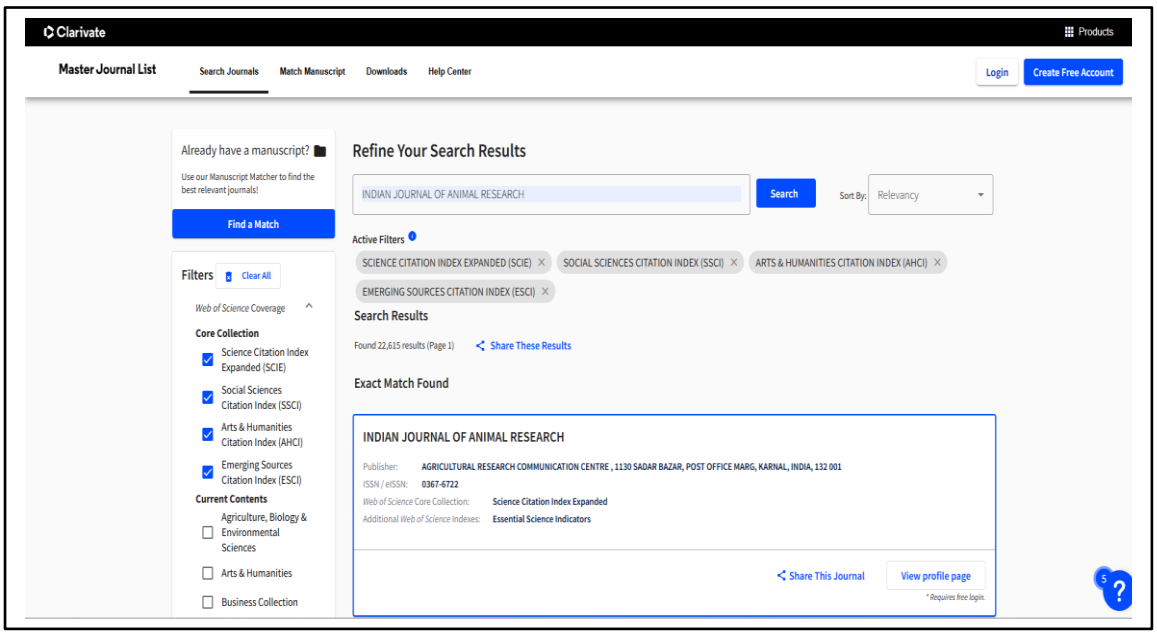

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Nutritional Characterization of Edible Viscera of an Autochthonous Swine Breed of Assam, India

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Abstract | Scientific information about the nutritional quality on meat by-products is very scarce when compared with muscle meats (e.g., physicochemical composition, meat quality, sensory and their utilization etc.). The meat by-products are widely accepted as edible foods by the consumers. Therefore, the current study aims to examine the nutritional composition of edible viscera namely, liver, kidney, spleen, heart, large intestine and small intestine of Doom pig of Assam, India. The samples were collected from semi-extensively reared pig farms of Kokrajhar district, Assam. The approximate composition range of these edible viscera was found to be: moisture 50.67-68.47%, fat 0.47-9.85%, ash 0.16-0.92%, protein 6.85-22.36%, carbohydrate 10.18-24.65% and calorie 124.31-198.16 k/cal/100g. Among the samples the maximum protein, carbohydrate and calorie were found in liver. Most numbers of the essential amino acids was found in spleen with lysine contributing the largest amount. Fat content was maximum in large intestine, similarly the saturated fatty acid content too was found highest in large intestine. Furthermore, the saturated fatty acid, mono unsaturated fatty acid and poly unsaturated fatty acid content range from 35.97-51.42%, 2.03-3.44% and 2.05-18.79% respectively. The study reports that the edible viscera are excellent sources of nutrients. The data may be utilized by the nutritionist and dieticians which may help in value addition and the promotion of the consumption of edible viscera, as well as their utilization in meat processing industry.

Keywords | Edible viscera, Autochthonous Doom pigs, India, Proximate, Amino acids, Fatty acids.

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INTRODUCTION

The Doom, commonly known as 'Desi' by rearers, is an autochthonous pig breed found in western part of Assam. It has good meat texture, taste and palatability and its meat is sold at higher market prices than other cross-breed pigs. The pig has larger body size, high prolificacy and survival rate even in the low input system that is highly polymorphic in nature (Zaman et al., 2014). Doom is the first registered indigenous pig breed of Assam with accession no. INDIA_PIG_0200_DOOM_09006 (NBAGR, 2008). The Doom pig's viscera are extensively consumed by the local population as edible foods. Consumption of edible viscera is advocated by nutritionists because of its high content of essential amino acids (EAA), vitamins and minerals (Kicinska et al., 2019).

Till recently meat scientists has focused on muscle meats (Thomas et al., 2016a; Thomas et al., 2016b; Daimari et al., 2022) whereas now focus has been shifted to the edible viscera which is also consumed as low cost human food. Due to lack of scientific studies limited data has been generated on its nutritional content. Edible viscera are rich in nutrient content such as minerals (P, Fe, Cu, Mg, I, Ca, K, Na, Se, Zn, Mn) required by human body (Cordian et al., 2002). They are also known for high quantities of es-

sential fatty acids such as arachidonic acid, the omega-3 fatty acids, EPA and DHA (Cordian et al., 2002; Park et al., 1993; Nicklas et al., 2014). Edible viscera also contain folate, choline and B12 which are difficult to obtain elsewhere (Oloruntuba et al., 2019).

Therefore, the present study determines the proximate, amino acid and fatty acid content in six edible organs namely; heart, kidney, liver, small intestine, large intestine and spleen of Doom pig of Assam, India.

MATERIALS AND METHODS

Sample collection

The study was approved by the Institutional Animal Ethics Committee; Bodoland University vide letter no- IAEC/BIOTECH/2019/3. Samples of Doom pig's edible viscera were obtained from six semi-extensive indigenous pig farms in Kokrajhar district of Assam, India, with live weights of around 48-51 kg. These indigenous pigs were given locally available feed like local vegetation, remnants of crop and kitchen waste. Ten months old pigs (n=12) were slaughtered in a commercial abattoir, later six edible viscera samples were collected from each of the 12 pigs. The heart (53.46±1.35 g), liver (887.31±23.30 g), kidney (189±2.42 g), spleen (78.36±1.38 g), small intestine (432.26±21.52 g) and large intestine (1432±64.30 g) were conventionally chilled for 24 hours in a chiller at 4 ° C. 24-hours post-mortem, the viscera's were homogenized using a mechanical grinder. The experiments were performed at the Department of Biotechnology, Bodoland University.

Proximate Analysis

The proximate composition (moisture, ash, fat, protein, carbohydrate and calorie) of the edible viscera were determined according to the methods of analysis of the AOAC (2005). The carbohydrate content was calculated by the difference method, following conversion formula (FAO 2003):

Available Carbohydrate (%) = 100- [Moisture (%) + Ash (%) + Crude Protein (%) + Fat (%)]

The nutritive value or calorific value in kcal/100 g was calculated with the help of the following equation (James 1995):

Calorific value (kcal/100 g) = 4 × Protein (%) + 9 × Fat (%) + 4 × Carbohydrate (%)

Amino Acid Analysis

The amino acid content of the samples was determined on 0.001 g of oven dried and defatted samples. The samples were dissolved in 2mL of milliQ-water and incubated at 45°C in thermomixer for 30 minutes. 8mL of methanol was added to precipitate the proteins and incubated overnight at -20° C. The solutions were centrifuged at 4000 rpm for 30 minutes and the supernatant was transferred to another tube. The supernatant was evaporated under nitrogen gas at 60 ° C to complete dryness. Derivatization was done by adding 350 µL of Borate buffer, 20 µL of AccQ-Tag ulta reagent to the sample and incubated for 10 minutes at 55 ° C. After incubation 2 µL is loaded on the instrument, which is quantified using a Sigma standard. Buffer for Mobile Phase A: AccQ- Tag Ultra eluent A1 and for Mobile Phase B: AccQ-Tag Ultra eluent B. Amino acids were determined using a WATERS Acquity (make) UPLC system.

Fatty Acid Analysis

For fatty acid determination, the dried samples were subjected to lipid extraction with chloroform/methanol (Folch et al., 1957). The lipid extract was esterified with BF_3- methanol (Joseph et al., 1992) for preparation of FAME's (Fatty Acid Methyl Esters). The fatty acid composition of each aliquot was estimated by Gas Chromatography. GC-MS analysis of sample extracts was carried out with Perkin Elmer (USA), Model: Clarus 680 GC & amp; Clarus600C MS comprising a liquid auto-sampler. The Software used in the system was TurboMass Ver.6.1.2. The peaks were analyzed using data analysis software NIST-2014. The capillary column used is 'Elite- 5MS' having dimensions- length- 60 m, ID- 0.25 mm and film thickness- 0.25 µm and the stationary phase is 5% diphenyl 95% dimethylpolysiloxane. Helium gas (99.99%) was used as carrier gas (i.e. mobile phase) at flow rate of 1 ml/minute. An injection volume of 2 µl was employed in split less mode. Injector temperature was 280°C and ion-source temperature 180°C. The oven temperature was programmed at 60°C (for 1 minute), with an increase at the rate 7°C/minutes to 200°C (hold for 3 minutes) then again increased at rate of 10 ° C/min to 300 ° C (hold for 5 min). The total run time is ~ 39 minutes. Solvent delay was kept for 8 minutes. MS Protocol Mass Spectra was taken in Electron Impact positive (EI+) mode at 70 eV. For MS scan, a solvent delay of 8 minute was provided with m/z range 50-600 amu.

The mentioned data were statistically analyzed using SPSS, Version 26.0 and demonstrated as mean ± SEM (standard error of mean).

RESULTS AND DISCUSSION

Proximate Composition

The mean values of proximate composition of Doom pig are tabulated in Table 1. Spleen and small intestine had the highest moisture content (68.47% and 64.72%, respectively), while kidney, heart and liver had significantly lower

Table 1: Proximate composition of edible viscera Doom pig.

	Kidney	Small Intestine	Spleen	Liver	Heart	Large Intestine
Moisture (%)	61.93±1.43	64.72±2.18	68.47±0.99	50.67±1.22	55.25±1.57	64.11±1.26
Fat (%)	2.28±0.28	0.58±0.03	0.47±0.02	1.12±0.17	1.16±0.56	9.85±0.51
Ash (%)	0.78±0.18	0.51±0.02	0.92±1.04	1.19±0.09	0.75±0.13	0.16±0.00
Protein (%)	20.93±0.18	10.12±0.40	19.83±0.11	22.36±0.52	18.23±0.48	6.85±0.48
Carbohydrate (%)	14.15±1.016	24.05±1.83	10.18±1.05	24.65±1.03	24.59±1.98	19.02±1.13
Calorie (kcal/100g)	160.94±7.26	141.97±8.63	124.31±3.57	198.16±5.40	181.80±3.58	192.17±6.85

moisture contents (61.93%, 55.25% and 50.67% respectively). Also the crossbred pigs of Thailand had high moisture content (79.96%) than the present study (Chanted et al., 2021). Another study by Seong et al., (2014), determined the proximate content in pork-by products of crossbred pigs (Landrace×Yorkshire×Duroc) of South Korea, when they found high content of moisture with an overall mean value of 76.38%.

Among the edible viscera determined, large intestine had the highest fat content (9.85%), followed by kidney (2.28%), heart (1.16%), liver (1.12%), small intestine (0.58%) and spleen (0.47%). The present study had similar data with that of fat content of crossbred pigs of Thailand (Chanted et al., 2021). Another study reported high amount of fat content in large intestine (19.54%), followed by heart (4.55%) and lowest in spleen (0.97%) (Seong et al., 2014), which was similar with our study, even though contents were high. High content of fat (5.86-25.97%) was also found in the muscles of different beef breeds (Ba et al., 2013; Moon et al., 2006). Fat not only helps in building up energy but also helps in vitamin absorbance.

Liver had the highest content of ash (1.19%) followed by spleen (0.92%) and large intestine had the least ash content with a mean of 0.16%. Similar data were also found in the pork by-products of crossbred Korean pigs (Seong et al., 2014). The ash content determines one of the most important properties of food i.e. the nutritional value, quality and physiochemical factors (Keran et al., 2009).

Liver and kidney had the highest protein content with a mean of 22.36% and 20.93% respectively, followed by spleen (19.83%) and heart (18.23%) while lowest was found in small intestine (10.12%) and large intestine (6.85%). Previous studies by Seong et al. (2014), too reported high protein content in liver (22.05%) and lowest in large intestine (8.45%). Similar values of protein content were also reported in the liver of similar species (Kim et al., 2008).

The carbohydrate content determined by difference was found to be highest in liver (24.65%), heart (24.59%) and small intestine (24.05%) respectively, while lowest was found in spleen with a mean of 10.18%.

The nutritional calorie/energy value of edible viscera showed that liver had the highest energy content (198.16 kcal/100g), followed by large intestine (192.17 kcal/100g). While heart (181.80 kcal/100g) and kidney (160.94 kcal/100g) had significantly lower content whereas small intestine (141.97 kcal/100g) and spleen (124.31 kcal/100g) had the lowest energy content. On the other hand, studies by Seong et al. (2014), reported highest calorie content in large intestine, followed by pancreas and liver respectively.

Amino Acid Content

The amino acid content in edible viscera of Doom pig is depicted in Table 2. Our data show a large variation among the determined edible viscera of both essential and non-essential amino acids. Among the edible viscera, spleen had the highest levels of most Essential Amino Acids (EAA) such as lysine, phenylalanine, isoleucine and leucine, whereas heart and large intestine had the lowest levels of most EAA. Spleen had the highest content of lysine (10.33 %), followed by kidney (5.29%), liver (5.02%) and heart (5.02%). Methionine (0.94%) too was found highest in spleen. Studies by Seong et al. (2014), too showed similar results where lysine was found highest in liver, while that of methionine was found highest in pancreas and liver, followed by spleen. Small intestine and heart had the highest content of valine. On the other hand isoleucine was found highest in kidney (8.57%), followed by large intestine (8.54%) and lowest was found in heart (0.03%). Leucine (2.34%) and phenylalanine (8.61%) were both found highest in spleen and that of tryptophan (0.85%) was found highest in liver. Valine was found highest in pancreas and liver as reported by Seong et al. (2014). It is found that most of the EAA are found highest in pancreas followed by liver and heart, which was quite similar with our findings (Seong et al., 2014). While that of amino acid content in muscle *longissimus thoracis et lumborum* of Ghungroo pigs of Assam reported quite similar findings with the edible viscera of present study (Thomas et el., 2016b).

Human body cannot produce EAAs and must be provided from outside. Human body cannot function normally without these amino acids. Earlier studies have reported

Table 2: Amino acid composition in edible viscera of Doom pig.

Moles %	Kidney	Small Intestine	Spleen	Liver	Heart	Large Intestine
Essential Amino acids						
Lysine	5.29±0.04	1.24±0.01	10.33±0.01	5.02±0.01	5.02±0.01	1.45±0.01
Methionine	0.15±0.02	0.03±0.01	0.94±0.01	0.23±0.01	0.01±0.00	0.02±0.01
Valine	0.14±0.00	5.34±0.02	0.04±0.01	0.15±0.02	4.11±0.00	0.87±0.00
Isoleucine	8.57±0.33	5.89±0.33	5.11±0.01	0.25±0.01	0.03±0.01	8.54±0.30
Leucine	2.15±0.02	0.15±0.02	2.34±0.01	1.34±0.01	0.15±0.02	0.51±0.01
Phenylalanine	1.14±0.02	3.92±0.01	8.61±0.14	5.84±0.01	0.03±0.01	0.44±0.02
Tryptophan	0.07±0.01	0.12±0.03	0.46±0.02	0.85±0.02	0.04±0.01	0.26±0.02
Non-Essential Amino Acids						
Serine	0.11±0.012	5.22±0.02	2.02±0.02	8.87±0.01	3.68±0.21	1.87±0.04
Glutamine	1.22±0.01	7.61±0.31	0.83±0.04	7.23±0.28	1.75±0.00	0.05±0.02
Arginine	1.60±0.02	1.76±0.31	4.20±0.05	12.67±0.05	2.26±0.02	3.32±0.02
Glycine	4.92±0.02	5.34±0.32	2.98±0.31	1.79±0.12	9.32±0.02	9.30±0.01
Aspartic acid	1.46±0.01	5.22±0.02	0.82±0.03	13.05±0.02	2.15±0.02	1.77±0.00
Glutamic acid	8.92±0.02	10.82±0.09	2.61±0.04	17.12±0.32	0.16±0.00	1.77±0.04
Alanine	0.03±0.00	0.02±0.00	4.46±0.01	4.54±0.07	0.02±0.01	0.01±0.00
Proline	4.12±0.01	0.05±0.01	3.33±0.02	13.81±0.01	0.03±0.00	0.36±0.12
Cysteine	1.34±0.02	1.79±0.05	1.45±0.01	2.13±0.02	1.34±0.00	0.01±0.00
Tyrosine	0.23±0.01	0.04±0.01	1.25±0.01	1.57±0.01	0.04±0.01	0.02±0.01

Table 3: Fatty acid composition (expressed in FAME %) in edible viscera of Doom pig.

Parameters	Kidney	Small Intestine	Spleen	Liver	Heart	Large Intestine
SFA	39.03±0.01	46.88±3.20	43.55±0.02	35.97±0.31	39.98±0.34	51.42±0.29
MUFA	2.69±0.15	2.72±0.19	3.44±0.11	2.03±0.02	2.55±0.27	2.06±0.64
PUFA	2.91±0.48	6.26±0.28	18.79±0.15	12.72±0.89	5.28±0.43	2.05±0.35
Palmitic acid (C16:0)	18.68±0.59	24±23±0.24	20.58±0.33	12.26±0.58	19.20±0.29	26.40±0.92
Stearic acid (C18:0)	12.38±0.13	18.74±0.11	16.00±0.05	18.62±0.01	14.81±0.14	22.57±0.18
Lauric Acid (C12:0)	2.61±0.39	3.29±0.56	3.00±0.05	1.90±0.13	1.60±0.34	2.08±0.49
Myristic acid (C14:0)	2.60±0.30	3.57±0.58	1.86±0.49	1.53±0.34	2.43±0.56	2.75±0.56
Behenic acid (C22:0)	2.11±0.24	2.65±0.30	2.74±0.56	1.70±0.26	1.65±0.31	2.37±0.30
Arachidic acid (C20:0)	0.77±0.33	0.75±0.02	0.34±0.01	0.74±0.02	1.30±0.01	0.74±0.02
Alpha Linolenic acid-ALA (C18:3-cis, n-3)	0.25±0.07	0.23±0.09	0.36±0.06	0.42±0.15	0.38±0.18	0.14±0.05
Linolenic acid-LA (C18:2-cis)	0.14±0.02	0.03±0.01	0.16±0.02	0.31±0.02	0.04±0.01	0.05±0.00
Arachidonic acid-ARA (C20:4 C, n-6)	2.26±0.02	5.76±0.02	18.24±0.02	13.44±0.06	4.82±0.02	1.65±0.02
Oleic acid (C18:1n-9)	0.65±0.02	1.08±0.01	1.24±0.01	0.86±0.02	0.26±0.02	0.74±0.01
Palmitoleic acid (C16:1n-7)	1.76±0.32	1.58±0.32	0.96±0.07	1.06±0.07	1.56±0.34	1.07±0.09
Myristoleic acid (C14:1n-7)	1.07±0.34	0.92±0.68	1.76±0.32	0.49±0.31	1.07±0.31	0.64±0.30

that the levels of EAA in edible viscera are not reduced after cooking or due heating treatment because of the low reducing sugar content of edible viscera do not cause secondary degradation reactions (Aristoy and Toldra, 2011). From our present study, it is observed that the edible viscera especially spleen, liver and heart are good sources of EAA's.

The non-essential amino acids detected in edible viscera of Doom pig are serine, glutamine, arginine, glycine, aspartic acid, glutamic acid, alanine, proline, cystine and tyrosine. Among the non-essential amino acids (NEAA), glutam-

ic acid (17.12%), proline (13.81%), aspartic acid (13.05%) and arginine (12.67%) represented the highest amount of non-essential amino acids.

Fatty Acid Content

The fatty acid composition (expressed in FAME %) are depicted in Table 3. The highest percentage of saturated fatty acid (SFA) was in the small intestine (52.54%) and large intestine (51.42%). Lower and similar percentages of SFA's were found in spleen (43.55%), heart (39.98%), kidney (39.03%) and lowest value was obtained in liver (35.97%).

Spleen, small intestine and kidney showed significantly higher mono-unsaturated fatty acid (MUFA) percentages (3.44%, 2.72% and 2.69%, respectively), whereas lower and significantly similar percentages were found in large intestine and liver (2.06% and 2.03%, respectively). Higher percentages of poly-unsaturated fatty acid (PUFA) was obtained in spleen and liver (18.79% and 12.72%, respectively), whereas lower and similar percentages are obtained in small intestine (6.26%) and heart (5.28%). Lowest was reported in kidney and large intestine (2.91% and 2.05%).

When compared with the SFA's, MUFA's and PUFA's content in muscle tissues of Ghungroo pig breed, they reported higher content (44.55%, 32.38% and 15.81% respectively) (Daimari et al., 2022). Earlier studies reported high SFA content in large intestine and spleen of crossbred pigs of Korea which was similar with our findings, similarly that of MUFA was found highest in large intestine and that of PUFA was found highest in liver and spleen (Seong et al,. 2014). Another study determined the fatty acid content in liver, kidney, heart and tongue of veal calves and suckler beef, where SFA's was reported highest in liver and that of MUFA was obtained highest in tongues and heart of beef and calves, while that of PUFA was reported highest in heart and kidney of beef (Florek et al., 2012).

SFA's are regarded as bad fatty acid as they are known to trigger various diseases mainly related to coronary heart disease. In regard to this, the Food and Agriculture Organization (FAO) and World Health Organization (WHO) has recommended dietary intakes of total fat and fatty acids for adult humans i.e. SFA content should be less than 10%, 15-20% MUFA and 6-11% PUFA (Burlingame et al., 2009). Therefore, nutritionist recommends reducing the intake of SFAs and thereby increasing the intake of PUFA's is encouraged.

The SFA's detected in our study are palmitic acid (C16:0), stearic acid (C18:0), lauric acid (C12:0), myristic acid (C14:0) and behenic acid (C22:0). Palmitic acid was detected the highest among the SFA's followed by stearic acid. The MUFA's detected in our study are oleic acid (C18:1n-9), palmitoleic acid (C16:1n-7) and myristoleic acid (C14:1n-7). And the omega-3 PUFA detected are alpha Linolenic acid-ALA (C18:3-cis, n-3) and omega-6 PUFA detected are linolenic acid-LA (C18:2-cis) and arachidonic acid-ARA (C20:4 C, n-6).

Limitations Of The Study

The data obtained in the study could not be compared with earlier studies on the nutritional content of edible offal of the same pig breed or any other autochthonous pig breeds of India as only limited data has been generated from similar works.

CONCLUSION

The present study determined the nutritional characteristics of pork by-products i.e. edible viscera on the basis of their proximate composition, amino acid and fatty acid content. The data obtained from our study defines that edible viscera especially spleen, liver and heart are good sources of nutrients such as proteins, EAA and PUFA (omega-3 and omega-6) as an essential fatty acid. Furthermore, when compared with the fatty acid content of the muscles, the edible viscera had lower fatty acid content. Thus, it may be concluded that the pork by-products i.e. edible viscera are suitable for human consumption. Also these by-products may be suitable for processing them into other final products in order to increase economic benefits. This is the first study to characterize the nutritional compositions of edible viscera which would provide not only the useful information for consumers but also the important databases for further investigations. Further; the data shall be of great importance in promotion of consumption of edible pork by-products as well as their utilization in meat processing. Attempts may be made to increase the commercial values of edible offal by using them in various meat products.

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CONFLICT OF INTEREST

The authors report there are no competing interests to declare.

NOVELTY STATEMENT

The study highlights that the edible viscera of Doom swine breed are excellent sources of nutrients. The viscera by-products may be suitable for processing into other fi-

nal products in order to increase economic benefits to the rearers.2

AUTHOR'S CONTRIBUTION

Rijumoni Daimari: Conceptualization of the study, manuscript writing, analysis and interpretation of data, formulation of proposed strategies. Silistina Narzari: Assisted in laboratory work and editing of the manuscript. Jatin Sarmah: Designed the study, manuscript finalization and revisions necessary.

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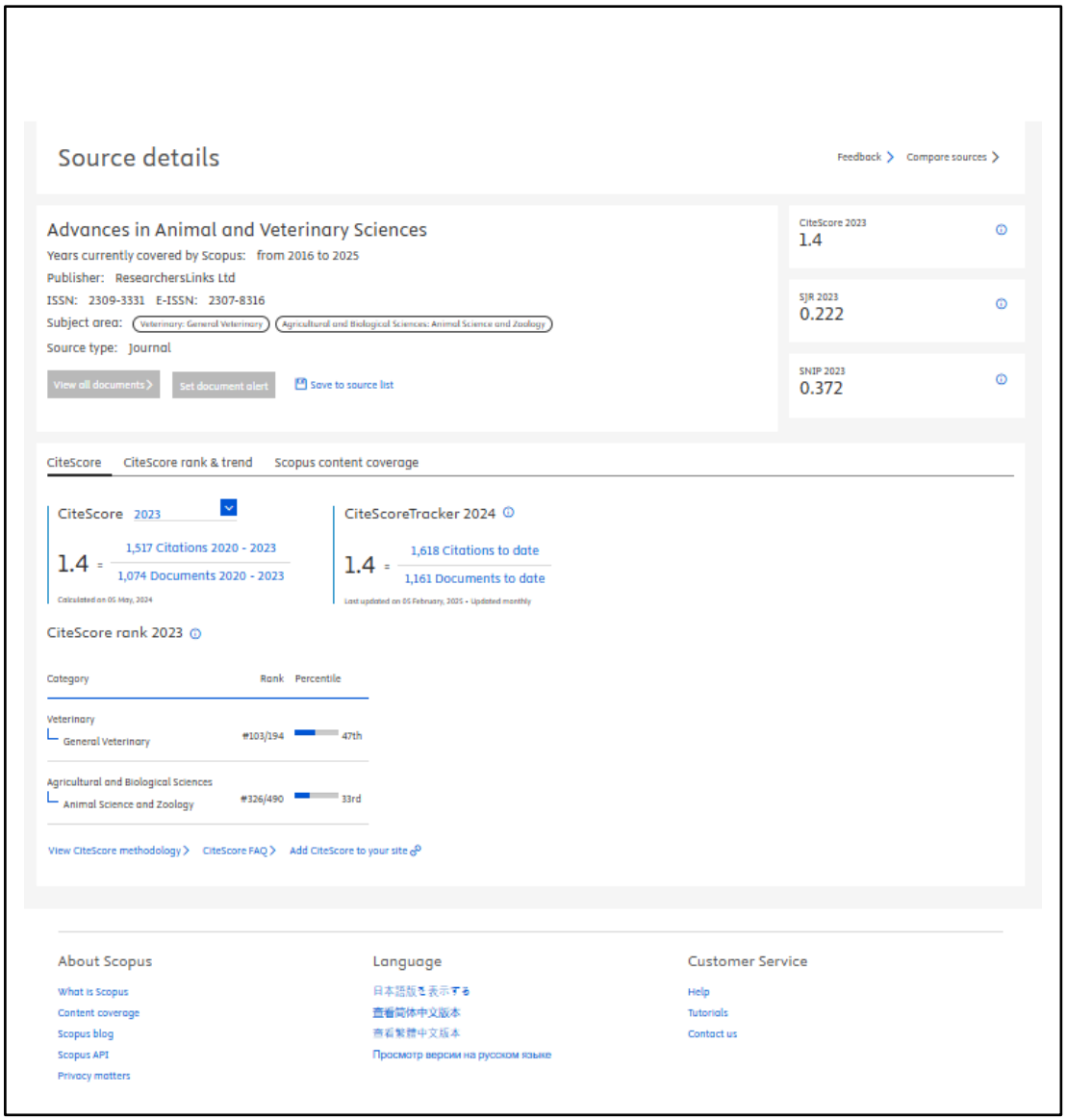
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Composition of Chemical Elements in Edible Offal and Muscle of Semi- extensively Reared Indigenous Doom Pig Breed of Northeast India and its Correlation with Feed and Environment

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Abstract

With the increase in anthropogenic activities and other long-range transport of pollutants, there is a high probability of accumulation of heavy metals in foods consumed by pigs which in turn may amass in edible offals. Therefore, the present study was undertaken to evaluate sixteen chemical (heavy and non-heavy) elements in edible offal and *Longissimus dorsi* muscle of indigenous Doom pig breed of India. Additionally, the values for highly toxic elements were compared with maximum residue limits (MRLs) stated by the regulatory authorities. The elements namely- Sodium (Na), Magnesium (Mg), Potassium (K), Calcium (Ca), Manganese (Mn), Iron (Fe), Zinc (Zn), Selenium (Se), Copper (Cu), Cobalt (Co), Chromium (Cr), Nickel (Ni), Arsenic (As), Cadmium (Cd), Mercury (Hg) and Lead (Pb) were determined in the tissues as well as in feed, drinking water and soil by Inductively Coupled Plasma-Optical Emission Spectrometry. Among the elements determined, the non-heavy metals (K, Na, Mg and Ca) were found to be the highest both in tissues (edible offal and muscle) and feed, drinking water and soil followed by Fe, Zn and Mn which are essential heavy metals. Ni was found to exceed the European Food Safety Agency allowed limits. Spearman correlation test shows significantly ($p<0.0001$) positive relationships between the element of tissues and feed, drinking water and soil. The work underscores the elemental analysis on hitherto understudied consumable edible offal for value addition to the food industry.

KEYWORDS
Doom pig, Edible offal, ICP-OES, Muscle, Non-heavy, Heavy elements.

INTRODUCTION

In Northeast India, pig holders practice low input pig rearing system (Das *et al.*, 2005), where feed is mainly consists of local vegetations, remnants of crop and kitchen waste. This type of rearing and feeding system causes uncertainty about availability of nutrients, especially minerals. Doom pig breed is distributed in western region of Assam. This breed is known to have good meat quality, taste and palatability and its meat and meat by-products have high demand among the consumers. They are sold at higher prices than other pig breeds.

Offal of pig is considered as edible and is recommended by nutritionist because they contain essential amino acids, vitamins, and minerals (Kicinska *et al.*, 2019). Even though offal is recommended for human diet, there is a high probability of accumulation of heavy metals in animal organs which has been a great concern of discussion in recent years. Toxic elements in muscles are lower than those found in offal products (Lopez-Alsonso *et al.*, 2007). Studies suggest that even in low amount, the toxic elements (Pb, Cd, Hg and Al) are known to cause many disorders to humans (Dlugaszek, 2019). They are also known to accumulate in the food chain (Nikolic *et al.*, 2017).

Dlugaszek (2019) pointed out that, chemical elements cannot be synthesized in animal body and can only be obtained through feed and drinking water. Animal feed is regarded as one of the most important factors that influence the accumulation of elements in the tissues (Kicinska *et al.*, 2019). Previous studies on the mineral status of feed and in the blood serum of exotic (Hampshire and Large White Yorkshire) breeds and indigenous pigs of Mizoram, India, reported high mineral content of Ca, P and Na in indigenous than exotic pigs (Kumaresan *et al.*, 2009).

The present study determines the concentration of sixteen elements in edible offal (heart, kidney, liver, small intestine, large intestine, and spleen) and muscle (*Longissimus dorsi*) of Doom pig breed (accession no. INDIA_PIG_0200_DOOM_09006 of Northeast India, NBAGR, 2008), and its correlation with the feed and environmental samples (drinking water and soil i.e. 0 cm-depth).

MATERIALS AND METHODS

Sample collection

The study was approved by the Institutional Animal Ethics Committee, Bodoland University, Assam, India (Vide No. IAEC/BIOTECH/2019/3). Samples were collected from five semi-extensive indigenous pig farms of Kokrajhar district, Assam, India. The pigs are reared under semi-extensive (local feed i.e. non

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specified diet, free-roaming within the paddocks) system. Local feed mainly included local vegetations, remnants of crop and kitchen waste. Ten months old pigs (n=12) were slaughtered in a commercial abattoir, with live weights of around 48-51 kg. Six edible offal and one muscle samples from each of the 12 pigs were collected namely: heart (41.66±1.45 g), liver (900.33±63.80 g), kidney (201±1.52 g), spleen (98.66±0.88 g), small intestine (562.66±31.52 g), large intestine (1696±54.30 g) and *Longissimus dorsi* muscle from loin region (169.23±9.4 g). The samples were conventionally chilled for 24 h in a chiller at 4°C and were homogenized using a mechanical grinder. Drinking water and soil were collected from the farm where the pigs were reared. All the samples were stored at -20°C in polyethylene bags until further analysis.

Digestion of samples and analysis

The samples were digested using wet digestion procedures as reported by ASEAN Manual of Food Analysis (2011). 1.5 g of the oven dried sample was digested in 8 mL of concentrated HNO_3 and 2 mL of $HCLO_4$ overnight to predigest the sample. Next day, the samples were kept in oven for 5 hrs at 100°C. They were cooled down and checked for clear/transparent samples. After analyzing the samples, they were diluted up to 100 mL with deionized water. The solution is filtered with Whatman filter paper No. 541 and analyzed.

The analysis of the sixteen elements (Na, Mg, K, Ca, Mn, Co, Se, Pb, As, Cr, Cu, Fe, Ni, Zn, Hg and Cd) was performed by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry), Model No. 7600, Thermo Fisher Scientific.

Statistical Analysis

The mentioned data were statistically analyzed using SPSS, Version 26.0. Partial Least squares discriminant analysis (PLS-DA) was applied to differentiate between the investigated samples according to element data. Spearman correlation test was applied to determine the correlation between investigated tissues and feed and environmental samples (soil and drinking water). P-values <0.05 were considered statistically significant.

RESULTS

Non-heavy metals in edible offal

The non-heavy metals analyzed in the study (Na, Mg, K, and Ca) is presented in Table 1. Na (122.843 mg/kg) was found to be the highest in spleen followed by kidney (97.196 mg/kg) while lowest was found in *Longissimus dorsi* (13.640 mg/kg). While that of K was found the highest in *Longissimus dorsi* (210.880 mg/kg), followed by liver (101.458 mg/kg) and the lowest in small intestine (32.150 mg/kg).

Similar to K, Mg was found the highest in *Longissimus dorsi* (14.063 mg/kg), followed by liver (5.230 mg/kg) and the lowest was reported in large intestine (2.424 mg/kg). Ca content was found to be the highest in liver (7.813 mg/kg), followed by small intestine (6.021 mg/kg) whereas that of kidney and large intestine were quite similar (4.140 and 4.980 mg/kg respectively) and the lowest was detected in heart (2.243 mg/kg). It is seen that both K and Mg were the highest in *Longissimus dorsi*.

Heavy metals in edible offal

Among the heavy metals, the non-toxic elements that were analyzed (Mn, Zn, Fe, Se, Cu and Co) are depicted in Table 1. Mn was found the highest in small intestine (15.576 mg/kg) and the lowest was in *Longissimus dorsi* (0.197 mg/kg). Fe and Zn were found the highest in liver (11.706 mg/kg and 1.879 mg/kg respectively) while the lowest was in *Longissimus dorsi* (0.769 mg/kg) and that of Zn was seen in heart (0.326 mg/kg). Se, Cu and Co was the lowest among the analyzed non-toxic elements ranging from 0.608 to 0.005 mg/kg.

The other heavy metals that are analyzed are regarded as potentially toxic elements (Cr, Ni, As, Cd, Hg and Pb) depicted in Table 2. Considering the effects of these toxic elements on human health, it is necessary to monitor the levels of these elements

Table 1. Concentration of non- heavy and heavy metals in edible offal and muscle of Doom pig (mg/kg).

Parameters	Non-heavy metals				Heavy metals (Non-toxic)					
	Na	Mg	K	Ca	Mn	Zn	Fe	Se	Cu	Co
Liver	77.578	5.23	101.458	7.813	0.217	1.879	11.706	0.013	0.157	0.006
Kidney	97.196	3.031	78.564	4.14	0.726	1.701	7.354	0.056	0.608	0.008
Heart	31.205	2.766	52.76	2.243	0.593	0.326	2.551	0.01	0.09	0.084
Small Intestine	29.882	3.716	32.15	6.021	15.576	1.727	2.635	0.012	0.047	0.018
Large Intestine	32.233	2.424	34.209	4.98	2.882	0.722	0.89	0.011	0.009	0.054
Spleen	122.843	3.275	60.203	5.49	0.421	0.737	9.594	0.017	0.036	0.005
Longissimus dorsi	13.64	14.063	210.88	4.722	0.197	1.626	0.769	0.005	0.007	0.004

Table 2. Concentration of potentially toxic heavy metals in edible offal and muscle of Doom pig (mg/kg).

Parameters	Potentially toxic heavy metals					
	Cr	Ni	As	Cd	Hg	Pb
Liver	0.032	0.063	0.012	0.012	0.006	0.14
Kidney	0.006	0.022	0.005	0	0.004	0.107
Heart	0.01	0.027	0	0	0.004	0.114
Small Intestine	0.035	0.048	0	0	0.004	0.029
Large Intestine	0.031	0.055	0	0.015	0.003	0.035
Spleen	0.037	0.039	0.005	0.019	0.004	0.015
Longissimus dorsi	0.007	0.008	0.004	0.004	n.d.	0.06

and analyze with their maximum residue limits (MRL's). The mean values of Cr ranged from 0.037 mg/kg in spleen to 0.006 mg/kg in heart, while that of *Longissimus dorsi* showed 0.004 mg/kg. The obtained results were below the allowed range provided by the WHO of 0.1 mg/kg. Ni concentrations ranged from 0.008 to 0.063 mg/kg. The daily allowed intake for Ni is 0.0028 mg/kg and 0.005 mg/kg body weight for adults according to European Food Safety Agency (2015) and the data from this study in all the samples was found to exceed the allowed range.

The maximum limit proposed for As by the China's National standards (GB2762-2012) is 0.5 mg/kg. The arsenic content determined in the investigated samples (0.012 – 0.004 mg/kg) did not exceed the given limited allowance. The highest Cd levels was found in spleen (0.019 mg/kg), followed by liver (0.012 mg/kg), while that of *Longissimus dorsi* was found to be lowest (0.004 mg/kg).

Hg was found highest in liver (0.006 mg/kg), followed by heart (0.004 mg/kg) and kidney (0.004 mg/kg). According to USDA Foreign Agricultural Service (2006), the maximum limit allowance of Hg is 0.05 mg/kg and it can be concluded that the studied samples did not exceed the limit. The Pb content in all the edible offal ranged from 0.140 to 0.015 mg/kg, while that of *Longissimus dorsi* muscle was found to be 0.060 mg/kg. According to FAO and WHO, the maximum daily allowance intake of Pb is 0.214 mg/kg for an average adult (Gu *et al.*, 2015) and did not exceed the limit allowance.

Partial Least squares discriminant analysis (PLS-DA) was applied to the investigated samples according to element data. The PLS-DA loading plot shown in Fig 1, shows that kidney, liver and spleen were easily discriminated from rest of the edible offal.

Non-heavy and heavy metals in environmental samples

The concentration of non-heavy and heavy elements in environmental samples (drinking water, 0 cm-depth soil) and feed are provided in Table 3. K (23.439 mg/kg) was reported to be the highest in feed, followed by Ca (21.508 mg/kg). In soil Mg content was found the highest with a mean of 15.215 mg/kg. Among essential heavy metals, Fe (60.650 mg/kg) was found to be the highest in soil.

Fig. 2, showing all the investigated elements including both non-heavy and toxic heavy metals (also depicted in Table 4) of the feed, drinking water and 0 cm-depth soil were clearly separated from each other using PLS-DA plots. The Hg was in the middle as it was not found in any of the determined parameters.

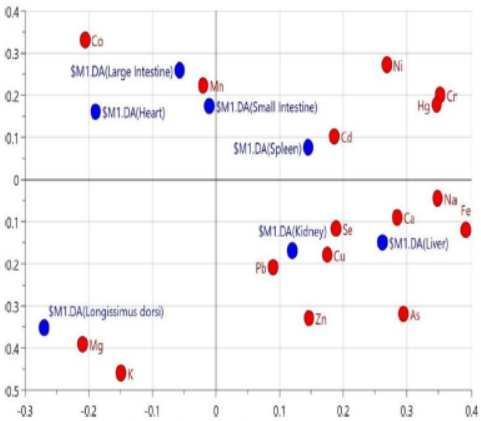

Fig. 1. PLS-DA score (R^2X =1, R^2Y =1 and Q^2 = 1) and loading plots of the sixteen chemical elements of the edible offal and muscle of indigenous Doom pig. Red dots represent the investigated elements and blue dots represent liver, kidney, spleen, large intestine, small intestine, heart and *Longissimus dorsi*.

Correlation between edible offal and muscle with feed and environmental samples

Correlation between the edible offal and environmental samples (drinking water, 0 cm-depth soil) and feed are given in Table 5. The relationship between the sixteen element profiles of tissues (edible offal and muscle) and feed and environmental samples are analyzed based on Spearman's correlation coefficient (r). The correlation seen between the edible offal and environmental and feed was positively significant ($p<0.0001$).

The liver, kidney, heart, small intestine, large intestine, spleen

Table 3. Concentration of non-heavy and heavy metals in drinking water, feed and 0 cm-depth soil (mg/kg).

Parameters	Non-heavy metals				Heavy metals (non-toxic)					
	Na	Mg	K	Ca	Mn	Zn	Fe	Se	Cu	Co
Drinking water	10.306	0.835	8.895	4.18	2.38	0.012	3.33	0.009	0.032	0.006
Feed	11.177	8.601	23.439	21.508	1.907	0.559	2.001	0	0.014	0.009
0 cm-depth soil	4.773	15.215	18.467	4.717	9.672	0.858	60.65	0.002	0.152	0.812

Table 4. Concentration of potentially toxic heavy metals in drinking water, feed and 0 cm-depth soil (mg/kg).

Parameters	Potentially toxic heavy metals					
	Cr	Ni	As	Cd	Hg	Pb
Drinking water	0.044	0.047	0.002	0.001	n.d.	0.048
Feed	0.005	0.015	0.01	0.006	n.d.	0.056
0 cm-depth soil	0.029	0.129	0.022	0.002	n.d.	0.018

Table 5. Correlation matrix of elemental composition between edible offal and muscle with feed, drinking water and soil.

Variables	Liver	Kidney	Heart	Small Intestine	Large Intestine	Spleen	*Longissimus dorsi*
Feed	0.9109 (<0.0001)	0.8698 (<0.0001)	0.9072 (<0.0001)	0.8851 (<0.0001)	0.8830 (<0.0001)	0.8477 (<0.0001)	0.9197 (<0.0001)
Drinking water	0.9110 (<0.0001)	0.9029 (<0.0001)	0.8904 (<0.0001)	0.9198 (<0.0001)	0.8765 (<0.0001)	0.8735 (<0.0001)	0.8866 (<0.0001)
0 cm-dept soil	0.8262 (<0.0001)	0.8359 (<0.0001)	0.8800 (<0.0001)	0.8697 (<0.0001)	0.8344 (<0.0001)	0.8374 (<0.0001)	0.8010 (<0.0003)

and *Longissimus dorsi* has the highest correlation with the drinking water. Small intestine had the strongest correlations with drinking water. Relatively higher r values were also found between edible offal and feed, ranging from 0.844 to 0.919.

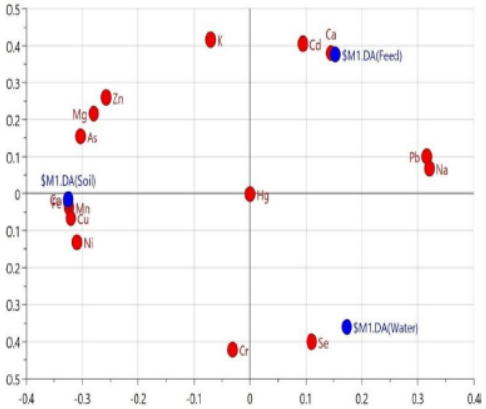

Fig 2. PLS-DA score (R^2X =1, R^2Y =1 and Q^2 = 1) and loading plots of the feed, drinking water and 0 cm depth soil. Red dots represent the investigated elements and blue dots represent the feed, drinking water and 0 cm-depth soil.

DISCUSSION

Mineral content in pork is affected by various factors like, breed of pig, feed type, management practices and environment. Kidney is an organ that helps to determine toxicity level, (Lentini *et al.*, 2017). The non-heavy metals (K, Na, Mn, Mg and Ca) analyzed in this study was the highest in the kidney of Doom pig, followed by iron content which is an essential heavy metal. Among all the minerals, K and Na concentration were found to be the highest in *Longissimus dorsi*. Earlier study on muscles of Ghungroo pig too had high content of K and Na (Daimari *et al.*, 2022) than other minerals. The present study revealed high content of Na and Fe in spleen. It may be due to the ability of spleen to removes old red blood cells, store blood, reusing element iron, and participating in body's immune system (Zhou *et al.*, 2019).

Ca content was found to be the highest in liver, Nikolic (2017) too found high concentrations of Ca in liver. Mn (15.576 mg/kg) was found to be the maximum in small intestine which is more than *Longissimus dorsi* (0.197 mg/kg) and liver (0.217 mg/kg). Nikolic (2017) too showed similar results in liver, kidney, and muscle (*longissimus thoracis et lumborum*).

Fe and Zn are essential heavy metals. They are known to involve in many body functions (Dlugaszek *et al.*, 2019). In the present study, both Fe and Zn were found to be the highest in liver, while that of *Longissimus dorsi* was found to be lowest. Earlier studies on cross breed pigs from Serbia revealed high content of Fe and Zn in liver and low in muscle (Nikolic *et al.*, 2017). The mean of Se, Cu and Co detected in edible offal and muscle of semi-extensively reared Doom pig were very low in the present study.

Determination of potentially toxic elements (Cr, Ni, As, Cd, Hg and Pb) in edible offal and muscle of Doom pig is important as it affects the human health. Cr is regarded as an essential trace element (Tuzen, 2009). Cr is considered important part of diet as it is involved in insulin function and lipid metabolism (Bratakos *et al.*, 2002), also its excess consumption is known to cause gastrointestinal bleeding and necrosis of the kidney (Ihedioha *et al.*, 2014). Cr was found to be the highest in spleen and the lowest in kidney. Earlier study by Mi (2020) in liver, kidney and large intestine of Tibetan pigs found high Cr than MRL concentration (more than 0.1 mg/kg provided by WHO), while the obtained findings were below the allowed range.

Ni is considered an essential element for humans (Yipel *et al.*, 2017). Ni content was detected in all tissues and was found to be above the allowed range in this study. Again, in the study conducted by Nikolic (2017) Ni was not detected in any of the tissues (muscle, liver, and kidney).

As (arsenic) is known as a carcinogen causing lung, liver, skin, and bladder cancers (Kapaj *et al.*, 2006). Similar to results from this study, other studies too presented low concentration of As (Mi *et al.*, 2020). Also, it was seen that arsenic content did not exceed the allowed limit. Accumulation of Cd can hamper kidney function, cause skeletal damage, and reproductive deficiencies (Commission of the European Communities, 2001). Cd is reported to be carcinogenic and teratogenic (Simoniello *et al.*, 2011). Cd was detected in all offals and *Longissimus dorsi* but did not exceed the maximum tolerance level. The MRL provided by European Commission (2006) for Cd in liver and kidney for human consumption are 0.5 and 1 mg/kg respectively and the samples in the present study did not exceed the limit.

Hg was detected in all offals, but not in *Longissimus dorsi*. However, in none of the samples Hg exceeded the allowed limit. Hg is toxic to the developing fetus and has a high carcinogenic property (Ikem and Egilla, 2008).

The cognitive development and intellectual performances are affected by lead intake and known to increase blood pressure and cause cardiovascular diseases in adults (Commission of the European Communities, 2001). Mi (2020) reported that in liver and kidney of Tibetan pigs, Pb exceeded the maximum allowance limit. In the present study, lead was detected in all tissues but that was within the limit.

Feed is the main source of essential and non-essential elements. Non-essential elements like, Cd, Pb and Cr remain in small concentration in environment (Sager, 2007). Soil too, is known to have heavy metals (Kabata-Pendias, 2001). In semi-intensive production system, soil ingestion has a significant role in soil exposure as pigs are known to show rooting behavior even though there is enough food (Beattie and O'Connell, 2002).

Feed was found to contain the high number of elements, followed by soil and drinking water. Among heavy metals, Fe was found to be the highest in soil which was in accordance with the study of intensive and extensive pigs from Serbia where iron content of soil was the highest compared to concentrated feed and forage (Nikolic *et al.*, 2017). Among the non-heavy metals, K was found to be the highest in this study. Concentrations of Se, Cu and Co were quite low in feed, soil and drinking water. Studies done by Nikolic (2017), concentrations of Se and Co were similar with the obtained results except for soil content, but Cu content was higher than finding from the current study.

Arsenic, lead, mercury and cadmium are regarded as the most toxic elements to be found in feed. The maximum tolerance level for arsenic, cadmium, mercury, and lead in animal feeds are 30 mg/kg, 10 and 0.5 mg/kg, 0.2 and 2 mg/kg and 10 and 30 mg/kg respectively (NRC, 2005; AAFCO, 2019). As, Cd, Hg and Pb content in feed were 0.01 mg/kg, 0.006 mg/kg, 0.00 mg/kg and 0.056 mg/kg. Therefore, the feed in this study was considered harmless with respect to the level of tolerance for consumption.

Positive correlation is seen between the tissues (i.e. edible offal and muscle) and environmental samples and feed. Conclusion can be drawn that accumulation of metals in meat and meat by-products are directly associated with environmental status of the living area which is in conformity with Kumaresan (2009). It is found that the tissues (liver, kidney, heart, small intestine, large intestine, spleen, and *Longissimus dorsi*) have the highest correlation with the drinking water. Out of all organs, small intestine had the strongest correlations with drinking water. This might be due to the biological function of the small intestine which can absorb ~90% of nutrients and water from the food (Liao *et al.*, 2018).

CONCLUSION

All edible offal and muscle (*Longissimus dorsi*) have levels of Ni, which exceed the maximum tolerance level. The toxic elements in feed, drinking water and soil did not exceed and are

within the safe ranges. Moreover, feed, soil, drinking water and pig's rooting behavior have a strong impact on the elemental composition of edible offal and muscle of Doom swine breed. The present study highlights the elemental status of the Doom pig's offal. The findings will be helpful for possible modification for feeding strategies by the rearers, so that good quality and safe products can be served to consumers.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related.

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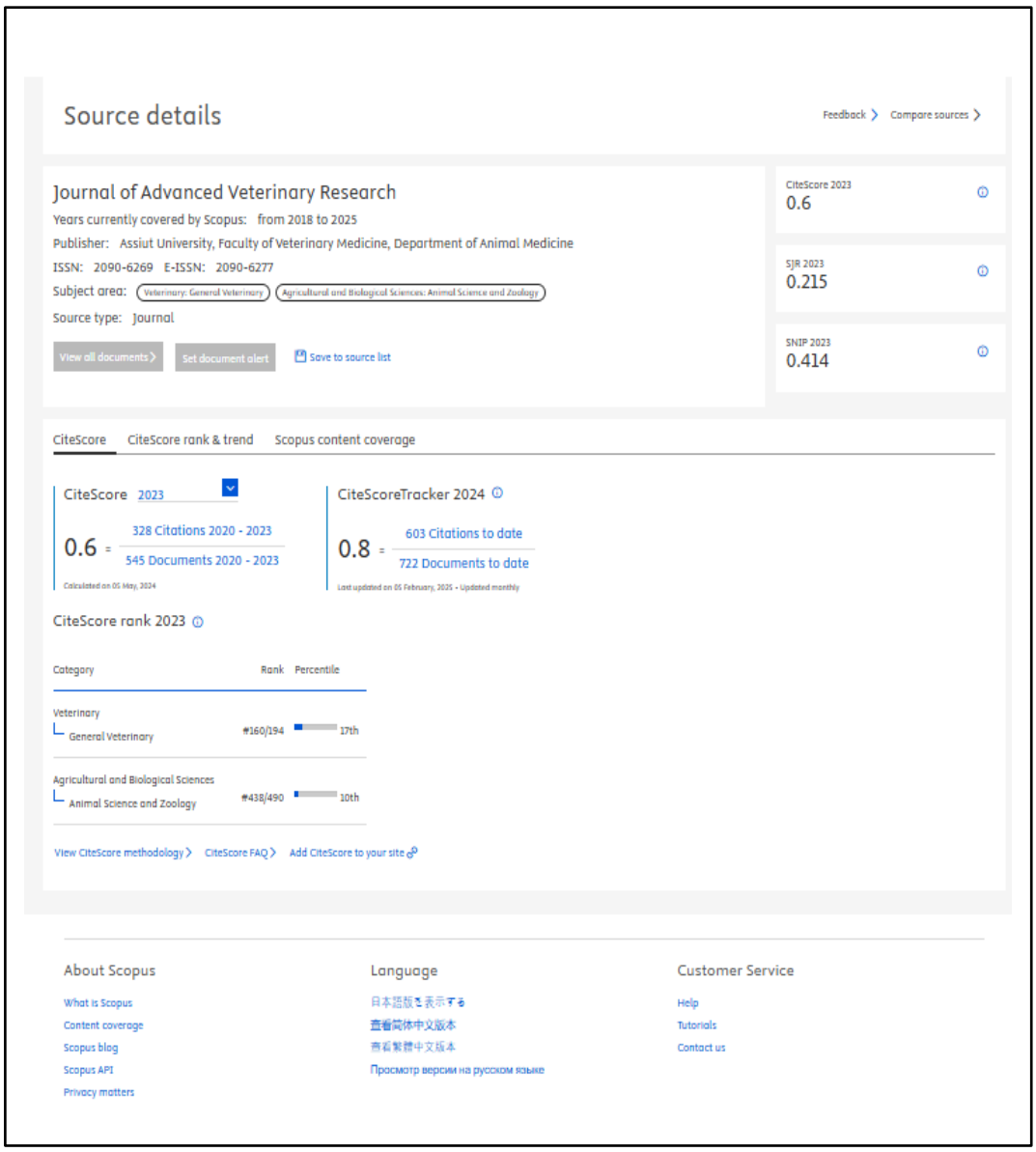
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Genetic diversity and phylogenetic relationship of domestic pig breeds of northeast India based on Cytochrome b gene analysis

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Abstract

Northeast India contributes about 38.5% of the country's pork production. However, extensive crossbreeding between indigenous and exotic breeds in this region has caused decline in native pig populations. To conserve and restore the native pig breeds, characterising them at molecular level is a must. Therefore, current study determines genetic diversity and phylogenetic analysis of domestic pigs (Doom and Ghungroo pigs) of northeast India, using cytochrome b gene. Blood samples from 24 pigs were collected, followed by DNA extraction and gene was amplified using universal primers and sequenced. Sequence variation sites, genetic distance and phylogenetic analysis were performed at MEGA 11. A total of 18 polymorphic sites was found where 6 sites were common to Doom and Indian wild pig (15940, 16344, 16350, 16355, 16356 and 16357).

Doom pig showed least genetic distance with wild boars while Ghungroo pig showed the farthest distance. Phylogenetic analysis depicted that Doom and Ghungroo, clustering in one clade with Indian wild pig, indicates of having common maternal ancestor. This study highlights the evolutionary history and genetic diversity of domesticated pigs of northeast, India. Identifying the pig-specific mitochondrial DNA sequences will help in distinguishing domestic pigs from wild boars as well as in forensic and vegeto-legal cases.

Keywords: Indigenous pig breeds, cytochrome b gene, genetic variation, phylogenetic analysis, MEGA 11.

Introduction

Domestication of pigs was found to have occurred about 9000 – 10000 years before present (YBP) at various places across Asia and Europe. The present-day pigs are the development of local breeds that have occurred throughout the centuries, characterized by distinct phenotypes and productive capabilities[2]. Mitochondrial DNA (mt-DNA) is a powerful tool to study the genetical structure and to carry out matrilineal origin of domesticated pigs[1]. Compared to nuclear DNA, mitochondrial DNA has higher rate of mutation and does not undergo recombination, thereby making it ideal to study divergence between the animal species[4,20]. Cytochrome b gene is one of the regions of mitochondrial DNA, commonly used as a marker for identification of species and to establish phylogenetic relationships[5].

Doom (accession no. INDIA_PIG_0200_DOOM_09006) and Ghungroo (accession no. INDIA_PIG_2100_GHUNGROO_09001) are indigenous pig breeds from northeast India[7, 8]. They are protected and recognized by ICAR-NBAGR with the aim to utilize their genetic resources and creating a database on indigenous livestock species of India[11]. Indigenous pig breeds are more promising than commercial ones due to their high prolificacy, hardiness, disease resistance, adaptability and low maintenance[3]. However, due to high rate of crossbreeding for better pork productivity, these local breeds are declining at an alarming rate. Therefore, conserving and restoring local germplasm is essential for protecting the genetic makeup of indigenous pig breeds.

On the other hand, it also helps in preserving the cultural and economic benefits that these breeds provide to local farmers. Previous studies used 'cytochrome b' as a molecular marker to identify the genetic differences between Indian wild pigs and domestic pigs[10]. Another study determined the molecular characterization of cytochrome b gene by microsatellites in indigenous pig of India[15]. Despite the importance of cytochrome b gene in species identification and determining phylogenetic relationships, there are very few studies on domesticated pig breeds in India that utilize this marker.

Evaluating the phylogenetic relationships of domestic pigs (Doom and Ghungroo) will provide insights of their genetic diversity and the origin based on maternal lineages. The present study determines the genetic diversity and phylogenetic relationship of domestic pigs based on partial sequence of cytochrome b gene that will provide a base for future studies and conservation of these novel genotypes.

Material and Methods

Collection of samples: The study was conducted during October 2019 to July 2020 at Udalguri district of northeast, India. A total of 72 blood samples were collected from 24 (12 Doom and 12 Ghungroo) pigs by certified veterinarian of Animal Husbandry and Veterinary Department of Government of Assam, India. About 2 mL of blood was withdrawn from the auricular vein of the ear and collected in clean EDTA vials[13]. Each sample were labelled accordingly and stored at -20 ◦C till extraction of DNA.

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Extraction of DNA: DNA was extracted from the samples using DNeasy Blood and Tissue kit (QIAGEN, Germany) in a final elution volume of 50-100 µL and the DNA samples were stored at -20°C.

PCR amplification and DNA sequencing: Cytochrome b sequences were amplified using universal primers: Forward primer: 5'TACCATGAGGACAAATATCATTCTG3' and Reverse primer: 5'CCTCCTAGTTTGTTAGGGATTG ATCG3'[20]. PCR amplification was performed in a MiniAmp Plus thermal cycler (Applied BioSystems). PCR amplification was conducted in a final volume of 25 µL with 100 ng of extracted DNA, using 2.5 µL of 10X PCR buffer, 0.5 µL of 10 mM dNTPs, 5 pmol/µL of each forward and reverse primer and 3 units/µL of Taq DNA polymerase (Applied Biosystems). The PCR was performed using the following conditions: initial denaturation at 95° C for 5 minutes, 35 cycles of denaturation at 94° C for 45 s, annealing at 52°C for 50 s, extension at 72°C for 1 minute with a final extension of 72°C for 7 minutes.

The PCR products were loaded on to 2% agarose gel containing ethidium bromide. The amplified DNA was run on a horizontal electrophoresis at 100 V for 40-45 minutes. The gel was visualized at gel documentation system (Life Technologies). The PCR products were sequenced using Automated DNA Sequencer (Model 3730XL, from Applied BioSystems, USA).

Ethical approval: The Ethical approval was obtained from the Institutional Animal Ethics Committee (IAEC), Bodoland University vide letter no.-IAEC/BIOTECH/2019/3. All procedures were conducted according to the guidelines mentioned by the IAEC.

Submission of nucleotide sequences to NCBI: The partial fragment sequences of cytochrome b sequences of Doom and Ghungroo pig breeds obtained in FASTA format were submitted to NCBI (National Centre for Biotechnology Information) for generating accession number.

Analysis of nucleotide sequences: BLASTN search was performed on the Doom and Ghungroo pig cytochrome b gene sequences to generate similarity scores (i.e. 90 to 100 %) with other suidae sequences. The cytochrome b gene sequences of the present study along with sequences retrieved from gene bank were run for multiple sequence alignment and pairwise sequence alignment using Clustal W[17]. Among all the models tested using MEGA 11, the general time reversible (GTR+G) using discrete gamma distribution model has the lowest Bayesian Information Criterion (BIC) score. Therefore, it is regarded as the most suitable model for nucleotide substitution to determine the variation sites.

Kimura 2-parameter model was used to generate the genetic distance between the samples of the current study and all the suidae sequences retrieved from gene bank[12,16]. The aligned sequences were used to establish phylogenetic relationship by neighbour-joining tree method bootstrapping at 1000 replications using Kimura-2 parameter using MEGA 11[16].

Results

Accession numbers of cytochrome gene of Doom and Ghungroo pig breeds: The 437 bp of cytochrome b gene sequences of Doom and Ghungroo pig breeds were submitted to NCBI for generation of accession number. The accession numbers of Doom and Ghungroo pig as published in NCBI are tabulated in table 1.

BLAST similarity score of cytochrome b gene of Doom and Ghungroo pig: The partial fragment of cytochrome b sequences of Doom and Ghungroo pig generated a homology of 99 % with 25 domestic pig breeds around the world and 5 wild boars retrieved from gene-bank. After generating the BLAST similarity scores, indigenous pig breeds and wild boars were selected according to their closest similarity score to generate variation position/sites by Clustal W at MEGA 11, outlined in table 2. The indigenous pigs and wild boars selected based on closest similarity included 11 indigenous pig breeds from India, China, Japan and European countries and 5 wild boars of Asian-European origin. The same indigenous pigs and wild boars were also used for generating genetic-distances and phylogenetic tree to ensure a comprehensive understanding of their genetic relationships with the samples of the current study.

Variation position/sites of cytochrome b gene of Doom and Ghungroo pig: For generating variation position or sites of sequences of cytochrome b gene of Doom and Ghungroo pigs, the nucleotide sequences of the current samples including sequences retrieved from NCBI were run for multiple sequence alignment and pairwise sequence alignment using Clustal W at MEGA 11. The variable positions of 437 bp of cytochrome b gene of Doom and Ghungroo pig are shown in table 2. The complete genome mitochondrial sequence of *Sus scrofa domesticus* (acc. no. ON715893) was taken as a reference to generate the number of nucleotide positions. In the table, "." (dots) represents identical nucleotides.

The NCBI gene bank accession number of DNA sequence is given in brackets ( ) consisting of alpha-numeric letters. A highly variable region was found between 15924 and 16357 sites. There was a total of 18 polymorphic sites that were identified (Table 2) with no observed insertions and deletions in cytochrome b gene.

The sample of the current study i.e. Doom pig (DB-S1; accession no. PP951122) showed identical nucleotides with Indian wild pig (IWP; accession no. PP951121) at 6 positions. These positions have substitutions of A in place of C at 15940, C in place of T at three positions 16344, 16350 and 16355, T in place of A at 16356 and A in place of G at 16357. Doom pig sample of the present study also showed identical nucleotide with Ryukyu wild boar (RWB;

accession no. AB015073) at one position (16350) where T is substituted by C. The Doom pig (MZ846190) retrieved from NCBI showed similar nucleotides with the Asian (Indian wild pig, Lanyu wild boar, Ryukyu wild boar, Yunan wild boar) and European wild boars at 4 positions 16110, 16185, 16218 and 16332.

At position 16110, C is substituted by T, at position 16185, G is substituted by A, at position 16218, T is substituted by C and at 16332, C is substituted by T. The sample of the present study Ghungroo pig (GB-S2) only showed identical nucleotides at two positions with Indian wild pig and Ryukyu wild boar. At position 16344, T is substituted C and at position 16350, again T is substituted by C. The sequences of Ghungroo pig (OM634652) retrieved from NCBI did not show any similar nucleotides with sequences of indigenous and wild pigs.

Genetic distance analysis of Cytochrome b of Doom and Ghungroo pig: The genetic distance between the samples of the current study and sequences of wild boar and indigenous pig breeds was generated based on Kimura 2-parameter model[12,16] presented in table 3. In the table, lower triangular matrix values depict the mean genetic distances and upper triangular matrix depicts the standard errors. The Doom pig (0.0140±0.0059) of the current study was found to have closest distance with Indian wild pig with a mean of 0.0238±0.0079, Lanyu wild boar (mean, 0.0301±0.0014) and European wild boar (mean, 0.0119±0.0009). It also showed close distance with indigenous pig breeds of European origin i.e. Pietran (mean, 0.0123±0.0009) and Mangalica (mean, 0.0123±0.0009). The Doom pig (mean, 0.0104±0.0008) sequence retrieved from NCBI too showed close genetic distance with the same wild boars and indigenous pigs.

The indigenous pig breeds: Indian breeds (Ghungroo, Tenyi Vo, Niang Megha, Zovawk), Chinese breeds (Ya Chen, Ma Shen) and Japanese breeds (Ohmini miniature pig, Satsuma), all showed the close genetic distance among the groups. Ghungroo breed sample of the present study was found to have the nearest genetic distance with Satsuma (S) indigenous pig breed of Japan with a mean of 0.0044 ± 0.0020 (Table 3) and with Ohmini miniature pig (OMP) of Japan (0.0026 ± 0.0015). The Ghungroo pig sample retrieved from NCBI of mean 0.0002±0.0008 had closest genetic distance with that of indigenous pig breeds of India that included Tenyi Vo (0.0014±0.0003), Niang Megha (0.0014±0.0003) and Zovawk (0.0016±0.0003).

Phylogenetic tree of Cytochrome b of Doom and Ghungroo pig: Neighbour-Joining (NJ) tree method was applied for phylogenetic construction, bootstrapping at 1000 replications using Kimura-2 parameter using MEGA 11 (Fig. 1). The *Babyrousa babyrussa* (Bb) was taken as an outgroup for the construction of phylogenetic tree. The NJ phylogenetic tree revealed that the samples of the current study including the Indian wild pig (IWP) are confined to one cluster, placed next to Asian indigenous pig breeds.

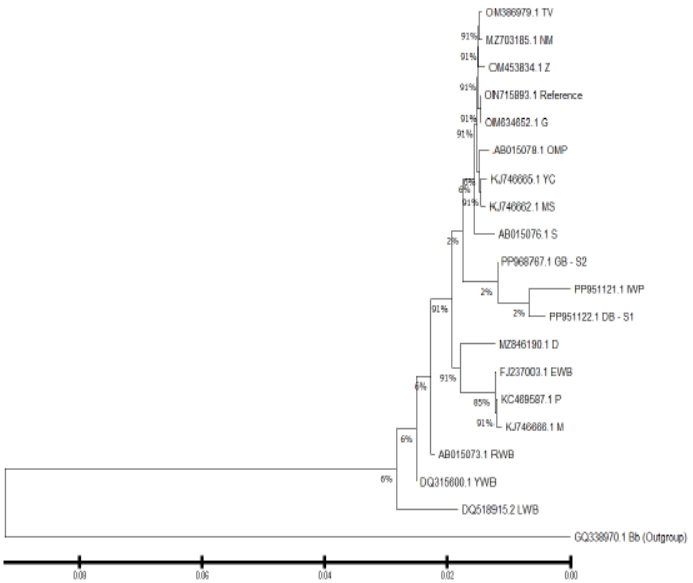

Figure 1: Phylogenetic tree of partial fragment of Cytochrome b gene. DB-S1= Doom, GB–S2= Ghungroo, IWP = Indian wild Pig, LWB = Lanyu Wild Boar, RWB= Ryukyu Wild Boar, YWB= Yunnan Wild Boar, EWB= European Wild Boar, G= Ghungroo, D= Doom, TV= Tenyi Vo, NM= Niang Megha, Z= Zovawk, YC= Ya Cha, MS= Ma Shen, OMP= Ohmini Miniature Pig, S= Satsuma, P= Pietran, M= Mangalica, Bb (Outgroup)= *Babyrousa babyrussa*.

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Table 1
Accession number of Doom and Ghungroo pig breeds

S.N.	Pigs	NCBI Accession numbers
1.	Doom pig	PP951122
2.	Ghungroo pig	PP968767

Table 2
Variable positions of 437 bp of cytochrome b gene of Doom and Ghungroo pig.

	Nucleotide positions																	
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	9	9	9	0	0	1	1	1	2	2	2	3	3	3	3	3	3	3
	2	3	4	0	3	1	8	9	1	2	7	2	3	4	5	5	5	5
	4	6	0	0	5	0	5	9	8	2	7	3	2	4	0	5	6	7
Animals																		
Reference (ON715893)	G	T	C	G	T	C	G	T	T	C	T	C	C	T	T	T	A	G
DB – S1 (PP951122)	.	.	A	.	.	.	.	.	.	.	.	.	.	C	C	C	T	A
GB – S2 (PP968767)	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	.	.
Wild boars																		
IWP (PP951121)	.	.	A	.	.	T	A		.	.	.	T	T	C	C	C	T	A
LWB (DQ518915)	.	.	.	.	.	T	A	.	.	.	.	.	T	.	.	.	.	.
RWB (AB015073)	.	.	.	.	.	T	A	.	C	.	.	.	T	.	C	.	.	.
YWB (DQ315600)	.	C	.	.	.	T	A	.	.	.	.	.	T	.	.	.	.	.
EWB (FJ237003)	.	.	.	.	C	.	A	.	.	.	.	.	T	.	.	.	.	.
Indigenous pig breeds																		
G (OM634652)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D (MZ846190)	.	.	.	.	.	T	A		C	A	.	.	T	.	.	.	.	.
TV (OM386979)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NM (MZ703185)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Z (OM453834)	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
YC (KJ746665)	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
MS (KJ746662)	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
OMP (AB015078)	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
S (AB015076)	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P (KC469587)	.	.	.	.	C	.	A	.	.	.	.	.	T	.	.	.	.	.
M (KJ746666)	.	.	.	.	C	.	A	.	.	.	C	.	T	.	.	.	.	.

Note: DB-S1= Doom, GB–S2= Ghungroo, IWP = Indian wild Pig, LWB = Lanyu Wild Boar, RWB= Ryukyu Wild Boar, YWB= Yunnan Wild Boar, EWB= European Wild Boar, G = Ghungroo, D= Doom, TV= Tenyi Vo, NM= Niang Megha, Z= Zovawk, YC= Ya Cha, MS= Ma Shen, OMP= Ohmini Miniature Pig, S= Satsuma, P= Pietran, M= Mangalica.

Within this cluster, Doom pig sample of the current study was in close relationship with Indian wild pig. The Doom breed (retrieved from gene bank) forms a clade along with European indigenous pigs. Within the Asian indigenous clade, Satsuma pig (S) of Japan with low bootstrap value showed separate cluster. At the level of the European clade, the Ryukyu wild boar (RWB) with 91% bootstrap probability and Yunan and Lanyu wild boar, both with low bootstrap value are designated as an isolated clade.

The northeast Indian pigs, included Tenyi Vo and Niang Megha with 91% bootstrap probability (Fig. 1). Within the northeast Indian pigs, Zovawk indigenous pig breed is inclined in a separate cluster, with bootstrap value of 91%. The minor clade included Ghungroo and complete genome of *Sus scrofa domesticus* (reference) that generated bootstrap probability of 91%.

Discussion

The results of the current study provide an outline of the partial cytochrome b gene of domestic pigs (Doom and Ghungroo pig) comparing them with the other pig sequences obtained from gene bank NCBI. Cytochrome b gene which is a mitochondrial DNA (mt-DNA), is utilized as a molecular marker for exploring the evolutionary lineage of various animal species[6, 21]. The cytochrome b gene when compared with other phylogenetic markers was found to show greater compatibility than standard mammalian phylogeny marker, showing nucleotide variation at greater level in short sequences[10,18]. Earlier studies have confirmed that the cytochrome b gene can be applied to distinguish between the two subspecies i.e. wild pig and domestic pig[10].

Assessing the variable positions showed that Indian wild pig had identical nucleotides with Asian and European wild

boars at three sites and these nucleotide substitutions can be used for differentiating wild boars and indigenous pigs of Asian and European origin as suggested by previous reports[10]. The Doom pig sample of the current study is showing six similar nucleotides with the wild boars and that of Ghungroo pig showing only two similar nucleotides with the wild boars. Also, the Ghungroo pig gene sequences retrieved from NCBI did not show any nucleotides similarity.

Table 3
Matrix output of genetic distance of the indigenous pig breeds and wild boars.

	Reference	DB-S1	GB-S2	IWP	LWB	RWB	YWB	EWB	G	D	IV	NM	Z	YC	MS	OMP	S	P	M	Bb (Outgroup)
Reference		0.0059	0.0032	0.0079	0.0014	0.0031	0.0031	0.0009	0.0001	0.0008	0.0003	0.0003	0.0003	0.0003	0.0003	0.0015	0.0020	0.0009	0.0009	0.0168
DB-S1	0.0140		0.0093	0.0119	0.0075	0.0076	0.0080	0.0076	0.0059	0.0083	0.0059	0.0059	0.0063	0.0064	0.0064	0.0064	0.0063	0.0076	0.0080	0.0301
GB-S2	0.0045	0.0023		0.0055	0.0052	0.0054	0.0059	0.0053	0.0032	0.0062	0.0032	0.0032	0.0039	0.0041	0.0039	0.0040	0.0039	0.0053	0.0057	0.0277
IWP	0.0238	0.0024	0.0049		0.0062	0.0063	0.0067	0.0072	0.0079	0.0071	0.0079	0.0079	0.0075	0.0083	0.0083	0.0082	0.0082	0.0072	0.0075	0.0303
LWB	0.0301	0.0213	0.0114	0.0164		0.0023	0.0024	0.0016	0.0014	0.0015	0.0014	0.0014	0.0014	0.0014	0.0014	0.0033	0.0033	0.0015	0.0015	0.0168
RWB	0.0098	0.0213	0.0114	0.0164	0.0062		0.0022	0.0033	0.0031	0.0030	0.0031	0.0031	0.0030	0.0030	0.0030	0.0031	0.0031	0.0033	0.0034	0.0167
YWB	0.0098	0.0238	0.0138	0.0189	0.0062	0.0053		0.0033	0.0031	0.0034	0.0031	0.0031	0.0030	0.0031	0.0031	0.0032	0.0032	0.0033	0.0034	0.0165
EWB	0.0119	0.0213	0.0114	0.0213	0.0365	0.0117	0.0117		0.0009	0.0008	0.0009	0.0009	0.0009	0.0010	0.0010	0.0037	0.0038	0.0002	0.0003	0.0168
G	0.0002	0.0140	0.0045	0.0238	0.0302	0.0098	0.0098	0.0121		0.0009	0.0003	0.0003	0.0003	0.0004	0.0004	0.0015	0.0020	0.0009	0.0009	0.0168

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	Bb (Outgroup)	M	P	S	OMP	MS	YC	Z	NM	TV	D
DB-S1	0.1696	0.0123	0.0123	0.0044	0.0026	0.0020	0.0021	0.0016	0.0014	0.0014	0.0104
GB–S2	0.1936	0.0238	0.0213	0.0164	0.0164	0.0164	0.0164	0.0164	0.0140	0.0140	0.0262
IWP	0.1735	0.0138	0.0114	0.0068	0.0068	0.0068	0.0068	0.0068	0.0045	0.0045	0.0161
LWB	0.1936	0.0238	0.0213	0.0263	0.0263	0.0263	0.0263	0.0213	0.0238	0.0238	0.0213
RWB	0.1663	0.0358	0.0358	0.0117	0.0117	0.0309	0.0309	0.0307	0.0310	0.0308	0.0343
YWB	0.1666	0.0126	0.0117	0.0108	0.0108	0.0098	0.0098	0.0089	0.0098	0.0098	0.0098
EWB	0.1651	0.0126	0.0117	0.0108	0.0108	0.0098	0.0098	0.0089	0.0098	0.0098	0.0117
G	0.1696	0.0012	0.0007	0.0154	0.0135	0.0128	0.0131	0.0118	0.0121	0.0121	0.0112
D	0.1696	0.0125	0.0125	0.0044	0.0026	0.0021	0.0023	0.0017	0.0016	0.0016	0.0106
TV	0.1633	0.0122	0.0121	0.0172	0.0172	0.0112	0.0115	0.0109	0.0113	0.0111	
NM	0.1696	0.0128	0.0126	0.0044	0.0026	0.0023	0.0026	0.0016	0.0011		0.0009
Z	0.1696	0.0128	0.0127	0.0044	0.0026	0.0025	0.0028	0.0018		0.0003	0.0009
YC	0.1681	0.0125	0.0124	0.0053	0.0035	0.0025	0.0027		0.0003	0.0003	0.0009
MS	0.1726	0.0131	0.0131	0.0044	0.0026	0.0019		0.0004	0.0004	0.0004	0.0009
OMP	0.1726	0.0130	0.0128	0.0044	0.0026		0.0003	0.0004	0.0004	0.0004	0.0009
S	0.1711	0.0144	0.0135	0.0053		0.0015	0.0016	0.0018	0.0015	0.0015	0.0040
P	0.1726	0.0163	0.0154		0.0022	0.0020	0.0020	0.0022	0.0020	0.0020	0.0040
M	0.1696	0.0010		0.0038	0.0037	0.0009	0.0009	0.0009	0.0009	0.0009	0.0008
Bb (Outgroup)	0.1711		0.0002	0.0040	0.0038	0.0009	0.0010	0.0009	0.0009	0.0009	0.0008
		0.0169	0.0168	0.0173	0.0170	0.0172	0.0173	0.0167	0.0168	0.0168	0.0163

Note: DB-S1= Doom, GB–S2= Ghungroo, IWP = Indian wild Pig, LWB = Lanyu Wild Boar, RWB= Ryukyu Wild Boar, YWB= Yunnan Wild Boar, EWB= European Wild Boar, G= Ghungroo, D= Doom, TV= Tenyi Vo, NM= Niang Megha, Z= Zovawk, YC= Ya Cha, MS= Ma Shen, OMP= Ohmini Miniature Pig, S= Satsuma, P= Pietran, M= Mangalica, Bb (Outgroup)= *Babyrousa babyrussa*.

Furthermore, Ghungroo pig generating the farthest distance from Doom pig and other wild boars. This shows the low genetic diversity of Ghungroo pig indicating the existence of inbreeding within the population. Recent study showing low haplotype genetic diversity of Ghungroo pig population supports the current findings in regard to Ghungroo pig[14].

The Doom pig having the nearest genetic distance from the wild boars' states that it is the most recent domesticated pig breed as also revealed by Das et al[9] in their study analysing the mitochondrial genome of Indian wild pigs.

The phylogenetic tree constructed depicted that the samples of the present study are grouped under one clade originating from Asian indigenous pig breeds. The results of the phylogenetic tree corroborate with the genetic distance i.e. the close distance between Indian wild pig and Doom breed pig suggests that they are closely related to each other and Doom pig is the most primitive among the indigenous pig breeds of India. Recent study too revealed through D-loop sequences of Indian pigs that Doom pig has the least genetic distance with Indian wild pig[9]. The inclusion of Doom pig of northeast India in the European clade could suggest of common maternal haplotypes due to practice of crossbreeding carried out in India for better production[14,19].

Conclusion

It can be concluded that the study investigated the partial fragment sequence of cytochrome b gene of mitochondrial DNA of two domestic pigs (Doom and Ghungroo), comparing them with the other sequences of wild boars and indigenous pigs generated from NCBI gene bank. The sequence variation sites revealed that Doom pig had more identical nucleotides with Indian wild pig than Ghungroo pig. Therefore, these sites common only to Doom and Indian wild pig can be used to distinguish between indigenous pigs and wild boars.

Furthermore, the least genetic distance between Doom pig and Indian wild pig also shows their close resemblance maternally. Even though the analysed cytochrome b sequences are short, they are unambiguous and will provide a foundation in future studies in determining the forensic cases as well as vegeto-legal cases including conservation of these novel genotypes.

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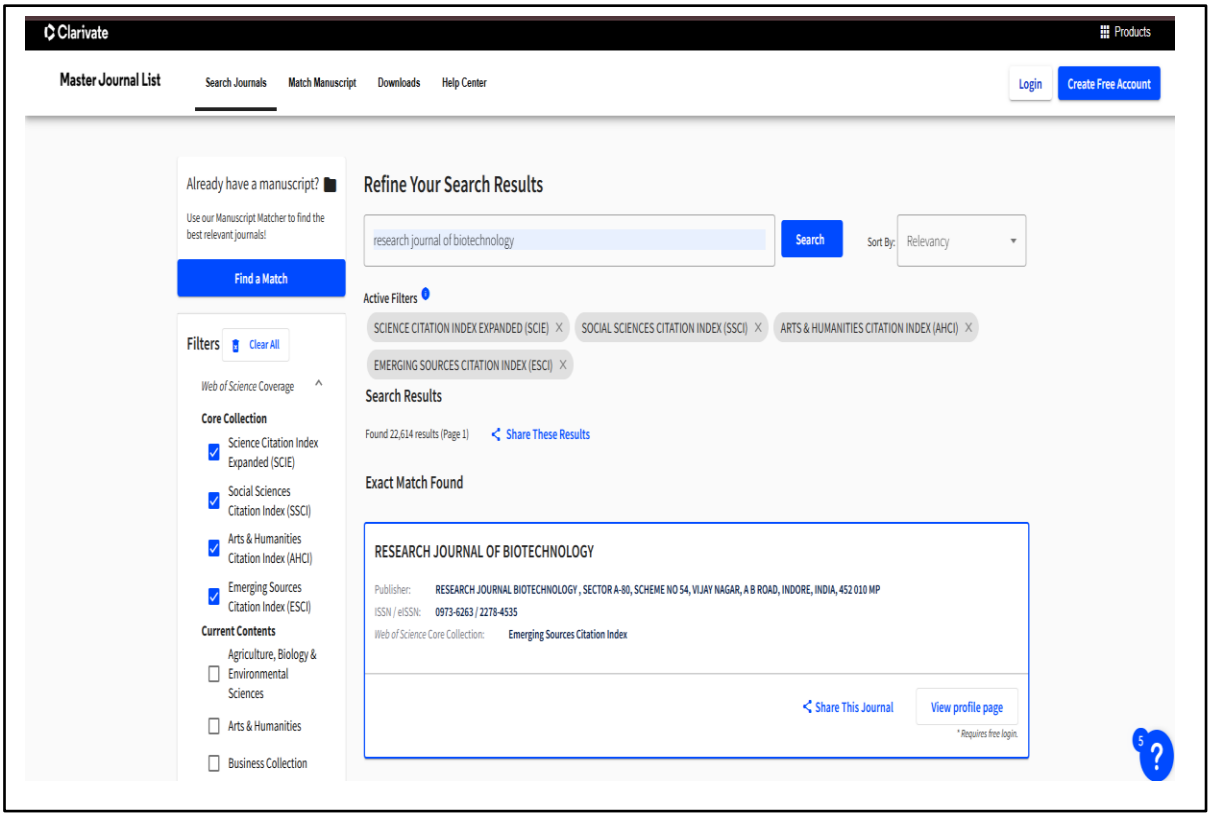
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