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Comparison of Deoxyribose Nucleic Acid Purification Methods of Mangosteen (*Garcinia mangostana* L.) and Its Relatives

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ABSTRACT

Mangosteen (*Garcinia mangostana* L.) and its relatives (*Garcinia hombroniana*, *Garcinia celebica*, *Garcinia forbesii*, *Garcinia malaccensis*, *Garcinia porecta*, *Garcinia subeliptica*, *Chalophylum inophylum*) contain polyphenol compound. The polyphenol compound makes pure deoxyribose nucleic acid is difficult to reveal. The aim of this research was to find the deoxyribose nucleic acid purification method of mangosteen leaves and its relatives. The research was conducted from January to August 2015 at the Center of Horticultural Tropical Studies Laboratory, Bogor Agricultural University. The mangosteen leaves were isolated based on cetyl trimethyl ammonium bromide (CTAB) buffer extraction added 2x chloroform isoamyl alcohol (CIAA 24:1), 3x CIAA (24:1), and sliced gel purification using Fermentas kit extraction. The best treatment was CTAB and added 2x CIAA purification for *Garcinia mangostana* L. and its relatives for purification of deoxyribose nucleic acid. This modified method produced an apparent amplified polymerase chain reaction using PKBT7 inter simple sequence repeat marker. It was applicable to evaluate genetic diversity interspecies.

INTRODUCTION

The deoxyribose nucleic acid (DNA) molecular methods have been known as the tool to determined *interspecies* identification (Zietkiewicz et al., 1994), genetic variability (Mohamad et al., 2017, Ahmad et al., 2010; Sangani, 2017; Daryono et al., 2019), transgenic analysis (Maaty & Oraby, 2019), mapping gene (Amom & Nongdam, 2017), and yield characters' selection (Roberdi et al., 2015). The DNA molecular methods based on polymerase chain reaction (PCR) need high-quality DNA for high quality identification. Optic density (OD) ratio (A_{260}/A_{280}) has been standardized to measure the purity of DNA at 1.8-2.0 (Becker et al., 1996).

Garcinia mangostana L. is well known as a queen of fruit. *Garcinia mangostana* L. belonging to Guttiferae family contains yellow latex in the stem, leaves, flowers, and fruits. Latex is secondary metabolic produced by terpene and phenolic pathway. *Garcinia mangostana* L. and its relatives' leaves contain phenolic compounds (Febrina et al.,

2018; Rohman et al., 2019; Parveen & Khan, 1988) and polysaccharides (Wathoni et al., 2019). The phenolic compound inhibits the process of DNA purification. PCR process is an enzymatic reaction that is sensitive to an inhibitor. The phenol and polysaccharides may damage the enzymatic process by mimicking the nucleic acid structure (Schrader et al., 2012).

DNA isolation is a molecular-based technique to obtain pure DNA from contaminants such as proteins, polysaccharides, phenols compound, and ribonucleic acid (RNA). DNA purification methods is crucial to determine the quality of DNA extraction from mangosteen leaves (*Garcinia mangostana* L.) and its relatives. There are PCR inhibitors during sample extraction (Schrader et al., 2012). Several strategies have been evaluated to remove PCR inhibitors during DNA extraction. A robust method is needed to improve DNA isolation from higher plants (Maaty & Oraby, 2019). Cetyl trimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1987) and the other modification (Jadhav et al., 2015) are known to be used for DNA isolation, but some plants produce different results (Ghaffariyan et al., 2012). The modifications of DNA extraction using higher CTAB concentration (3X), 2-β-mercaptoethanol, sodium chloride (6M), isopropanol for DNA free from polysaccharides (Maaty & Oraby, 2019), and some extraction kit of DNA are used to get clear DNA from contamination (Youssef et al., 2015). The main goal of this study was to evaluate various methods of DNA isolation for high-quality DNA. The process of contaminant removal requires a standard protocol of DNA extraction to detect the genetic diversity.

MATERIAL AND METHODS

Plant materials

The sample leaves of *Garcinia mangostana, Garcinia hombroniana, Garcinia porecta, Garcinia celebica, Garcinia forbesii, Calophyllum inophylum, Garcinia subelliptica*, and *Garcinia malaccensis* were collected from the Center of Tropical Horticultural Studies, Mekarsari Fruit Garden, Bogor Botanical Garden, and the farmer fields (Table 1).

Table 1. Accession *Garcinia mangostana* L. and its relatives

No	Accession	Location
1	*G. hombroniana* (H1)	Bogor Botanical Garden
2	*G. hombroniana* (H2)	Bogor Botanical Garden
3	*G. porecta* (P1)	Bogor Botanical Garden
4	*G. porecta* (P2)	Bogor Botanical Garden
5	*G. celebica* (C1)	Bogor Botanical Garden
6	*G. celebica* (C2)	Bogor Botanical Garden
7	*G. mangostana* (L1)	Leuwiliang Bogor
8	*G. mangostana* (L2)	Leuwiliang Bogor
9	*G. mangostana* (L3)	Leuwiliang Bogor
10	*G. porecta*(AL)	Center of Tropical Horticultural Studies
11	*G. forbesii* (For)	Center of Tropical Horticultural Studies
12	*C. inophylum* (Cal)	Center of Tropical Horticultural Studies
13	*G. subelliptica* (Sub)	Center of Tropical Horticultural Studies
14	*G. malaccensis* (M1)	Mekarsari Fruit Garden

DNA extraction

The DNA was isolated from 0.1 g young leaf tissue by modifying the CTAB method (Doyle & Doyle, 1987) added 1% polyvinyl-pyrrolidone (PVP) and 1% *β*-mercaptoethanol. DNA purification consisted of three methods: addition of 2X chloroform isoamyl-alcohol (CIAA 24:1) (2X CIAA), 3X chloroform isoamyl-alcohol (CIAA 24:1) (3X CIAA) and Fermentas extraction kit. Preheat suspension buffer (pH 8) contained 10%

CTAB, 0.5 M EDTA, 1 M Tris-HCl, 5 M NaCl, 1% PVP, and 1% *β*-mercaptoethanol in a water bath at 65° C. Young leaf tissue (0.1 g) were grounded and added by 1500 µl CTAB solution then transferred into 1.5 mL microcentrifuge tubes. After that, it was incubated in the water bath at 65ºC for 30 minutes. The suspension was centrifuged at 10,000 rpm for 10 minutes at room temperature. The aqueous phase was transferred into new tubes and added double chloroform volume: Isoamyl alcohol (24:1). Then, it was centrifuged at 10,000 rpm for 10 minutes. Some samples were applied the 2X CIAA and 3X CIAA treatment, then fill in chilled isopropanol and kept at -20ºC for 24 hours for precipitating the DNA. Then, it was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was discarded. DNA pellet was added 70% chilled ethanol and centrifuged again at 10,000 rpm for 10 minutes. The DNA pellet was then dried at chamber temperature for 1 hour and added 100 µl of Tris-EDTA buffer (1M Tris-HCl, 0.5M EDTA at pH 8). The Fermentas extraction kit method used slicing DNA on gel electrophoresis. The gel was added 100 µl *binding solution* and incubated at 60ºC for 10 minutes until it turned into a yellow solution. After that, it was added 700 µl *wash buffer* into *column membrane* and 50 µl *elution solution* (Figure 1).

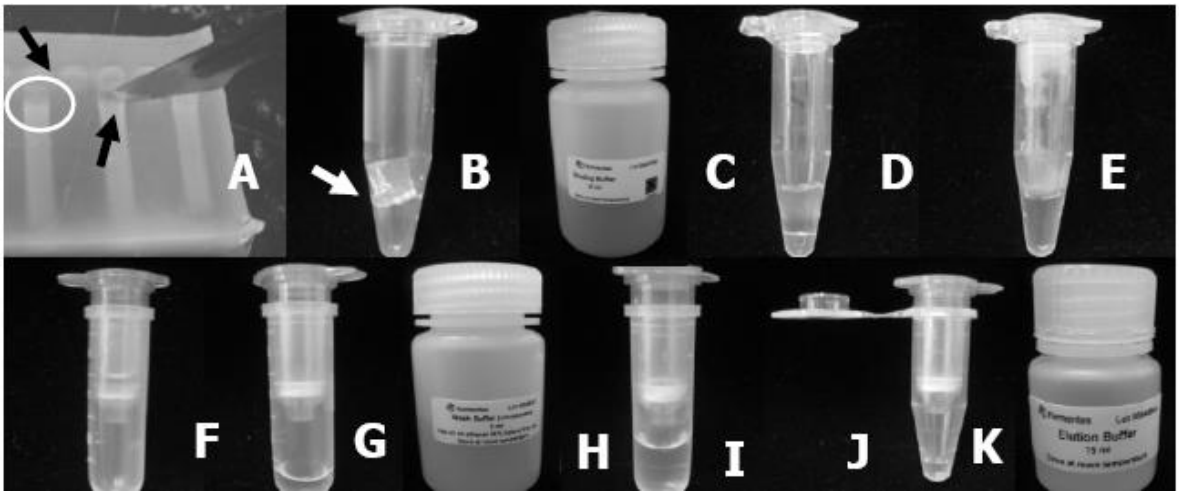

Figure 1 Fermentas extraction kit purification method. Slicing band (A), gel added into new tube (B), addition of binding solution (C), turned into yellow solution (E), fill in column membrane (F), centrifuge (H & I), wash buffer added (G), remove to new tube (J), elution buffer added (K).

Qualitative and Quantitative Analysis of Extracted DNA

The DNA resulted from the previous procedure was detected by using an ultraviolet (UV)-Visible spectrophotometer (Bio-Rad SmartSpec 3000 UV). DNA purity was determined by calculating the absorbance ratio A_{260}/A_{280} nm. While DNA concentration was determined by comparing with 1 µl λ DNA (Promega catalog number D150A). Qualitative analysis was conducted based on PCR results. PCR reactions were carried out in a total volume of 13 µl containing reaction mixture 20 ng of genomic DNA 1 µl, 1 µl primer, 6 µl Go Taq master mix (catalog number M712B) and 5 µl pure water. Amplification was performed in Applied Biosystem 2720 thermal cycler, with 35 cycles after pre PCR for 5 minutes at 94ºC. Each cycle was 1 minute at 9ºC for denaturation, 1 minute at 53ºC for primer annealing, 1 minute at 72ºC for DNA fragment elongation, and post PCR for 5 minutes at 72ºC. Amplified products were electrophoresed on 1.2% agarose gel (Promega catalog number V3121) at 50 volts for one hour in 1X tris acetate EDTA (TAE) buffer (Promega catalog number V4271) and stained with ethidium bromide (Sigma catalog number E8751). DNA bands were visualized under UV light and documented by using a digital camera. In this study, the inter simple sequence repeat (ISSR) of PKBT 7 (GA9A) primers was used.

RESULTS AND DISCUSSION

Quantitative Analysis of Extracted DNA

DNA quantity of mangosteen leaves and its relatives were standardized based on the optic

density ratio (A_{260}/A_{280}). DNA adsorbed ultraviolet on A_{260} and A_{280} nm (Becker et al., 1996). The quality of 2X CIAA, 3X CIAA, and Fermentas kit extraction was poor for most of the samples. A_{260}/A_{280} ratio was less than the optimal limit of 1.8 (Table 2). The poor quality of DNA was caused by protein and phenol contamination (Becker et al., 1996). The strategies to remove PCR inhibitors during sample preparation were carried out using EDTA to deplete magnesium ions that inhibit DNA polymerase activity, mercaptoethanol, and PVP to remove phenols (Schrader et al., 2012; Jadhav et al., 2015).

Fermentas kit method indicated the lowest OD ratio than 2X and 3X CIAA (Table 2). The best treatment was 2X CIAA purification method for Garcinia mangostana L. and its relatives. Multiply purification could decrease DNA concentration (Zhang *et al*., 2013). Purification 2X CIAA showed unpurity DNA for *G. mangostana* (L1 = 1.034, L2 = 1.699, L3 = 1.333), *G. hombroniana* (H2 = 1.667), *G. porecta* (P1 = 0.138, P2 = 1.189), *G. celebica* (C2 = 1.210), *C. inophylum* (Cal = 1521), *G. subelliptica* (Sub = 1.234), *G. porecta* (AL = 0.675) (Table 2). The other cases reported multiply purification increased the pure DNA concentration (Handayani et al., 2016). Purification 3X CIAA showed RNA contamination for *G. hombroniana* (H1=2,838) for 2X CIAA and *G. celebica* (C1=3,019, C2=2,179), *G. mangostana* (L1=2.870, L3=2.878), *G. porecta* (AL=2.481) (Table 2).

Table 2 Quantitative analysis of extracted DNA mangosteen leaves and its relatives DNA purified by addition of 2X CIAA, 3X CIAA and Fermentas kit extraction based on optic density ratio (A_{260}/A_{280})

No	Accession	2X CIAA (λ nm)			3X CIAA (λ nm)			Fermentas kit extraction (λ nm)		
		260	280	OD ratio	260	280	OD ratio	260	280	OD ratio
1	*G. hombroniana* (H1)	0.086	0.030	2.838	0.194	0.183	1.059	0.093	0.099	0.938
2	*G. hombroniana* (H2)	0.089	0.054	1.667	0.212	0.192	1.102	0.139	0.139	0.997
3	*G. porecta* (P1)	0.167	0.138	1.212	0.185	0.174	1.063	0.140	0.144	0.974
4	*G. porecta* (P2)	0.210	0.177	1.189	0.172	0.161	1.065	0.136	0.146	0.929
5	*G. celebica* (C1)	0.072	0.041	1.758	0.042	0.014	3.019	0.115	0130	0.889
6	*G. celebica* (C2)	0.182	0.150	1.210	0.080	0.037	2.179	0.114	0.136	0.838
7	*G. mangostana* (L1)	0.122	0.118	1.034	0.052	0.080	2.870	0.002	0.016	0.116
8	*G. mangostana* (L2)	0.026	0.016	1.699	0.073	0.046	1.589	0.097	0.104	0.931
9	*G. mangostana* (L3)	0.146	0.129	1.333	0.067	0.023	2.878	0.004	0.006	0.747
10	*G. porecta* (AL)	0.012	0.018	0.675	0.060	0.024	2.481	0.148	0.137	1.086
11	*G. forbesii* (For)	0.143	0.081	1.763	0.178	0.176	1.013	0.139	0.142	0.978
12	*C. inophylum* (Cal)	0.205	0.166	1.521	0.029	0.020	1.453	0.031	0.027	1.133
13	*G. subelliptica* (Sub)	0.066	0.043	1.234	0.172	0.160	1.070	0.121	0.124	1.024
14	*G. malaccensis* (M1)	0.041	0.023	1.795	0.054	0.048	1.133	0.007	0.008	0.868

Note: OD = optic density; 2X CIAA = 2X chloroform isoamyl-alcohol (CIAA 24:1) ; 3X CIAA = 3X chloroform isoamyl-alcohol (CIAA 24:1)

Qualitative Analysis of Extracted DNA

The qualitative extracted DNA analysis was separated on 0.8% gel agarose. The absence of smears indicated the high purity of extracted DNA. The DNA compared to control (λ) DNA Promega catalog number D150A (Figure 2). The 2x CIAA purification showed smear DNA what are protein and phenol contamination. CTAB buffer containing (0.3%) of 2-β-mercaptoethanol, which successfully removed polyphenols, produced a clear translucent DNA pellet (Sahu *et al.,* 2012), but 1% *β*-mercaptoethanol followed by 2X CIAA for mangosteen and relatives leaves showed the poor quality of DNA.

Purification using 3X CIAA showed smear that could be protein and phenol contamination compared to control (λ) DNA Promega catalog number D150A (Fig. 3). The addition of 1% *β*-

mercaptoethanol for removing phenol combined with the highest concentration level of 3X CIAA also produced poor quality DNA. Phenolic contamination was indicated by a sticky and brownish pellet. Phenolic compounds were difficult to handle (Sahu et al., 2012). Polyphenol is a major component in woody plants. Polyphenols bind DNA and remove it, PVP can be used during grinding the leaf tissue (Jadhav et al., 2015). Chloroform removes protein by trapping the gas when chloroform contacts with the air, causing foaming. Chloroform with isoamyl alcohol stabilizes separation between organic and aqueous layer interphase (Jadhav et al., 2015).

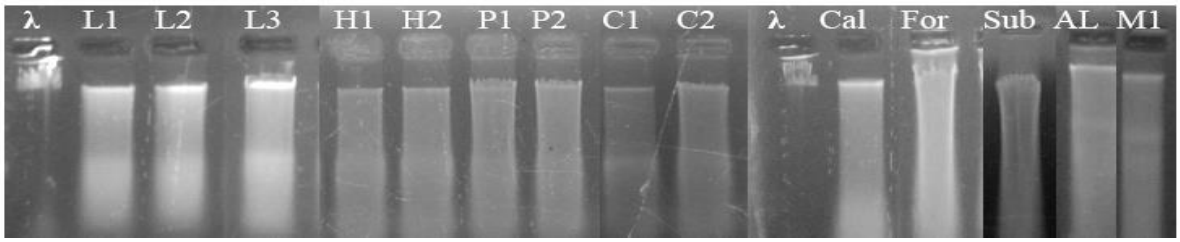

Figure 2 Genomic DNA isolated from *Garcinia mangostana* and relatives leaves resolved under 0.8% agarose gel. First line was control (λ) DNA (Promega catalog number D150A). The DNA were isolated by using 2X CIAA for *G. mangostana* (L1, L2, L3), *G. hombroniana* (H1, H2), *G. porecta* (P1, P2), *G. celebica* (C1, C2), *C. inophylum* (Cal), *G. forbesii* (For), *G. subelliptica* (Sub), *G. porecta* (AL), *G. malaccensis* (M1).

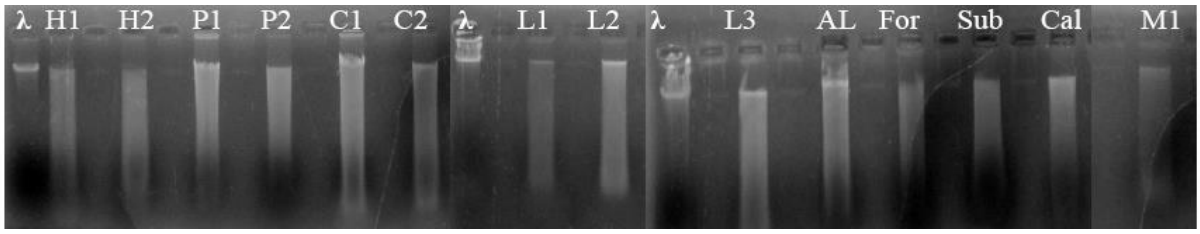

Figure 3 Genomic DNA isolated from *Garcinia mangostana* and relatives leaves resolved under 0.8% agarose gel. The first line was control (λ) DNA (Promega catalog number D150A). The DNA was isolated by using 3X CIAA for *G. hombroniana* (H1, H2), *G. porecta* (P1, P2), *G. celebica* (C1, C2), *G. mangostana* (L1, L2, L3), *G. porecta* (AL), *G. forbesii* (For), *G. subelliptica* (Sub), *C. inophylum* (Cal), *G. malaccensis* (M1).

Table 3 DNA concentration of *Garcinia mangostana* L. and its relatives on CTAB, CIAA and Fermentas extraction kit methods

No	Accession	DNA Concentration (ug/ml)		
		2X CIAA	3X CIAA	Fermentas kit extraction
1	*G. hombroniana* (H1)	857.29	1935.54	927.54
2	*G. hombroniana* (H2)	893.44	2120.14	1389.37
3	*G. porecta* (P1)	1669.55	1851.22	1401.31
4	*G. porecta* (P2)	2100.29	1719.24	1357.12
5	*G. celebica* (C1)	718.58	416.37	1153.93
6	*G. celebica* (C2)	1818.90	797.83	1141.04
7	*G. mangostana* (L1)	1218.31	516.23	19.04
8	*G. mangostana* (L2)	263.92	730.65	970.25
9	*G. mangostana* (L3)	1461.28	674.48	43.40
10	*G. porecta* (AL)	124.87	600.39	1483.35
11	*G. forbesii* (For)	1433.50	1780.83	1388.69
12	*C. inophylum* (Cal)	2054.21	289.85	305.18
13	*G. subelliptica* (Sub)	659.42	1717.84	1235.37
14	*G. malaccensis* (M1)	411.00	540.31	73.48

Note: 2X CIAA = 2X chloroform isoamyl-alcohol (CIAA 24:1) ; 3X CIAA = 3X chloroform isoamyl-alcohol (CIAA 24:1)

Polymerase Chain Reaction Analysis

The polymerase chain reaction (PCR) process can be inhibited by contaminants (Rezadoost et al., 2016). The PCR is an enzymatic reaction and sensitive to inhibitor at annealing of the primers to the DNA template (Schrader et al., 2012). Phenol and polysaccharides degrade DNA polymerase and disturb the enzymatic process by mimicking the nucleic acid structure (Schrader et al., 2012). PCR needs a high concentration for inhibitory effect (Schrader et al., 2012). DNA concentration varied for 14 accessions (Table 3). *G. porecta* (P2=2100.9 ug/ml) produced the highest concentration of DNA by using 2X CIAA. On the other hand, *G. hombroniana* (H2=2120.14 ug/ml) produced the highest concentration by using 3X CIAA (Table 3). The concentration was needed for calculating DNA volume on PCR.

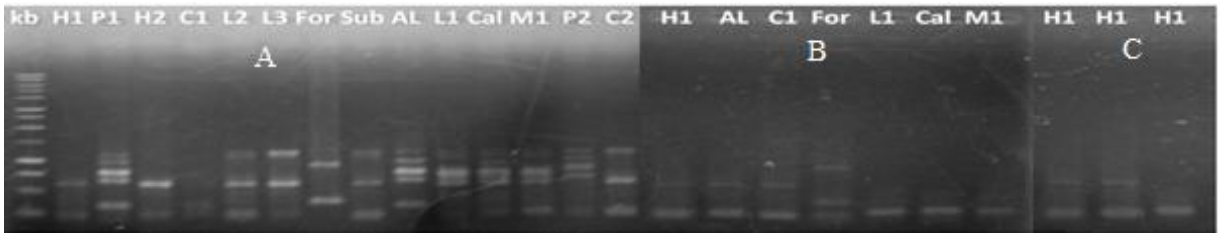

Figure 4 Separation of Polymerase chain reaction products under 1.2% agarose gel using inter simple sequence repeat primer (PKBT7) for *G. mangostana* (L1, L2), *G. hombroniana* (H1, H2), *G. porecta* (P1, P2), *G. celebica* (C1, C2), *C. inophylum* (Cal), *G. forbesii* (For), *G. subelliptica* (Sub), *G. porecta* (AL), *G. malaccensis* (M1). The first lane was a 1 kb ladder. Amplified 14 accessions of bands by adding 2X chloroform isoamyl-alcohol (CIAA 24:1) (A), 7 accessions purified by using 3X chloroform isoamyl-alcohol (CIAA 24:1) (B) and 1 accession (*G. hombroniana*) for 2X, 3X, Fermentas kit extractions methods (C).

Determination of the successful PCR amplification of the DNA extracted was carried out using an ISSR primer (Zietkiewicz et al., 1994) of PKBT7 (GA9A). Purification using 2X CIAA showed clearly differentiated band patterns and produced polymorphic bands (Figure 4A). PCR inhibitors could be organic or inorganic substances (Schrader et al., 2012). *G. hombroniana* (H1), *G. porecta* (AL), *G. celebica* (C1), *G. forbessi* (For), *C. inophylum* (Cal), and *G. malaccensis* (M1) showed unclear bands used 3x CIAA purification (Figure 4B) (Figure 4B). Fermentas kit extraction showed only one band at 250 bp on *G. hombroniana* (H1). However, *G. Hombroniana* (H1) produced two bands at 250 and 500 bp used 3x CIAA method (Figure 4C). The best method was 2x CIAA to separated polymorphic bands of *Garcinia mangostana* L and its relaives. The method of 2X CIAA was cheaper and faster method (Naurin & Qaiser, 2017) than kit extraction that was more expensive (Santos et al., 2018).

CONCLUSION AND RECOMMENDATIONS

The CTAB method added by 2X CIAA was the best treatment for *Garcinia mangostana* L. and its relatives for DNA purification. It produced a clear amplified PCR using the PKBT7 ISSR marker. Multiply purification by the addition of 3X CIAA and Fermentas kit extraction decreased the DNA concentration. Purification by addition of 2X CIAA was cheaper and faster method than the other. Thus, it could be applied for evaluating genetic diversity interspecies.

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