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Next generation sequencing reveals plants consumed by the vulnerable ebony langur (*Trachypithecus auratus*) in a fragmented mountain forest

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Abstract. *Karyanto P, Bagasta AR, Nayasilana IN, Nor SMD, Atmoko SSU, Susilowati A, Sunarto 2022. Next generation sequencing reveals plants consumed by the vulnerable ebony langur (*Trachypithecus auratus*) in a fragmented mountain forest. Biodiversitas 23: 4759-4769.* Many mountain forests on Java Island have suffered from forest degradation, fragmentation, and alien species invasion that cause a significant change in vegetation structure. This changing floristic structure may affect the foraging substrate of the foliage eater ebony langur, *Trachypithecus auratus*. Hence, ascertaining the plants eaten by the langur may contribute significantly to informing important ecological data about its foraging adaptation and conservation. We analyzed six fecal samples of the langur from three forest sites in Mount Merbabu National Park, Indonesia. This research used the plant mini barcode to sequence the ribulose-biphosphate carboxylase gene (*rbcl*) in the mitochondrial DNA of the plants eaten by the langur using the Next Generation Sequencing. We compare the NGS results to floristic reference data from a vegetation survey preceding the fecal analysis. The NGS found 238 OTUs that belong to 32 taxa. Most of the langur's diet belongs to the lower crop community. The study's results suggest that the ebony langur's dietary composition shows an adaptation to the new floristic composition. However, since the habitat is continuously degraded, the stakeholders must perform appropriate home-building-based habitat management practices to conserve this vulnerable species.

Keywords: Diet, genetic metabarcoding, next generation sequencing, plants, *Trachypithecus auratus*

INTRODUCTION

Java is Indonesia's most degraded island with a long history of rapid forest conversion for many purposes, for example, agricultural activities, industries, and settlements (Lukas 2014). All provinces on this island have suffered from forest degradation and fragmentation that cause many negative externalities towards biodiversity and the environment (Lavigne and Gunnell 2006). In addition to having a rapid rate of habitat degradation, alien plant invasion has become a common phenomenon attacking some remnant protected areas in Java (Tsujino et al. 2016; Padmanaba et al. 2017). Invasive species such as *Chromolaena odorata*, *Persicaria chinensis*, and *Acacia decurrens* are plant species that massively invade the mountain forest, causing a new vegetation structure that may affect the ecosystem's functioning and services (Linders et al. 2019; Pathak et al. 2021).

Even though Java Island has a rapid habitat degradation and fragmentation rate, Java is still occupied by a vulnerable leaf-eating colobine monkey, the ebony langur, *Trachypithecus auratus* (Nijman 2000; Estrada et al. 2018). Formerly, the langur (Figure 1) inhabited the primary and secondary forest canopy from the lowland to the mountain forest areas in Java, Bali, and Lombok Island (Nijman 2013). As the human primate population began to invade the lowland forest, this colobine monkey became restricted to the mountain forest (Estrada et al. 2018). Furthermore, the langur must adapt to the changing forest habitat with continuing upland forest degradation. The failure to adapt to the new environment may decrease the langur population size, causing the low population's viability to exist and extinction (Cavada et al. 2016).

The langur's diet in the fragmented and degraded forest may provide information on the impact of the previously mentioned degradation sources. The research on the diet composition of the ebony langur will importantly reveal ecological data related to its feeding adaptations and conservation, particularly in fragmented areas. There are two possibilities for foraging adaptation of the langur. First, the langur may adapt to consume the new kind of vegetation available in the forest. Second, the new floristic composition may negatively affect the food preference as the langur may ultimately depend on the specific food source. We scrutinized the linkage between habitat degradation, foraging choice, and conservation efforts by

referring to the three representative examples of research conducted by Rivera and Calmé (2005), Dunham (2011), and Silva et al. (2018). The study conducted by Rivera and Calmé (2005) in Calakmul forest in Mexico found that the fragmented habitat has caused some preferred trees for feeding to be insufficiently available to support the Black Howler Monkey *Alouatta pigra.* As this species was not successfully adapting to the changing environment, the study suggested performing vital tree restoration in the conservation program. Silva et al. (2018) demonstrated that the feeding behavior of two frugivorous primates, *Sapajus flavius* and *Alouatta belzebul*, in the fragmented area of the Atlantic Forest was correlated to a particular plant community providing fruit and seed for the diet. In addition to these two cases, a fragmented forest does not always correspond to an unsuccessful adaptation story. Dunham (2011) showed that the leaf-eating colobine *Colobus angolensis palliatus* could adapt and persist in the fragmented habitat in East Sagara Forest, Tanzania. The monkey showed behavioral changes in its activity budgets and feeding efforts to cope with the fragmented habitat.

Thus far, research related to the diet of *T. auratus* stands only on the visual observation of their feeding behavior and choices (Kool 1992, 1993; Tsuji et al. 2019). Whereas useful, this observation-based research has a low resolution and limitation as the researcher cannot follow complete foraging activities in all sites visited by the langur (Rytkönen et al. 2019). Indeed, visual observation methods may fail in reporting the actual dietary diversity. Fecal analysis through DNA barcoding technique may be an effective noninvasive approach for studying the diet of the Ebony Javan langur (Pompanon et al. 2012). By employing the DNA barcoding method, fecal sequencing using NGS can provide a large amount of sequence data on the dietary composition of the langur (Quéméré et al. 2013). Therefore, this study examines the frequent plant composition eaten by the Ebony Javan Langur from the fecal using the NGS analysis. We focused the discussion on the frequent plants eaten by the langur and the implications for the conservation program of the ebony langur.

MATERIALS AND METHODS

Study area

We carried out our study at Mount Merbabu, one of the inactive stratovolcanoes in Central Java, Indonesia. Mount Merbabu (Figure 2) is located at 7º28'0"- 7º28'40" S and 110º27'0" - 110º27'50" E. Our survey was conducted from 1997-2229 MASL in the protected area managed by Gunung Merbabu National Park. This protected area covers three types of dry forests; primary, secondary, and plantation forests. The small size of primary and secondary dry forests of the northern part of Mount Merbabu are the remained habitat for the ebony langur and a site where the study is conducted. These two kinds of forests have suffered from forest degradation and fragmentation (Dewi 2009), causing a low diversity value of the vegetation community. In addition to degradation and fragmentation, a rapid rate of alien species invasion may change the indigenous vegetational structure (Padmanaba et al. 2017), that in turn, may negatively affect the langur's dietary preferences as they may depend ultimately on a particular vegetation structure and phenology that existed before the disturbances (Kool 1992; Tsuji et al. 2019).

Procedures

General outline

Figure 3 illustrates a general workflow outline for the dietary analysis of the ebony langur examined in this study. We performed NGS DNA mini barcoding to identify plant food eaten by the langur since this new method has effectively and efficiently revealed the diet of animals (Pompanon et al. 2012; Ingala et al. 2021). However, since DNA from feces is quickly degraded, and the NGS analyses produce only a tiny fragment of DNA, the NGS can cause a bias in the actual diet composition (Gerwing et al. 2016). Therefore, we conducted the vegetation analysis towards the study site to develop the plant species database to provide a reference for the results of the NGS analysis.

Study site, developing the database, and fecal sampling

We collected six individuals' fecal pellets immediately after defecation during the dry season sampling from June to September 2020. We collected these six samples in the day and the afternoon after the langur performed a period hour of foraging activities. Therefore, we can expect that the plant DNA found in all fecal samples is an appropriate proxy for the plant eaten and ingested in the several hours preceding the sampling (Kool 1993). We handled the feces with sterile gloves, placed them in a marked 15 ml centrifuge tube with screw caps, and sealed the tube to prevent leakage and contamination. We preserved all fecal samples in 96% ethanol following Yang et al. (2020) and stored the sample at -4ºC before the DNA extraction. The entire sample was stored for ten days after being collected.

Figure 1. The vulnerable ebony langur *Trachypithecus auratus* in the study site. Photo courtesy of Jarot Wahyudi, forest officer at the Mount Merbabu National park, Central Java, Indonesia

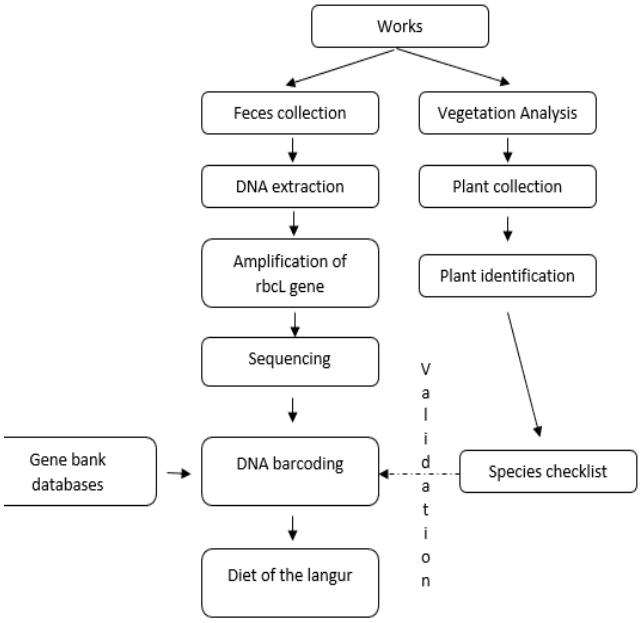

Figure 3. The general workflow for revealing the diet of the ebony langur

Because having a small sequence fragment can result in ambiguity during BLAST, some previous studies used several techniques to confirm the result of the NGS. Zhong et al. (2019) performed a micro-histological analysis solving the bias of the OTU analysis resulting from the NGS. In addition to the micro-histological approach, Ando et al. (2013) used a sequence reference database developed preceding the research. Due to the relatively small forest area of the study site (318 acres), we performed a cheaper but effective method by developing a species-list database consisting of a floristic checklist of the trees and lower crop species through a vegetation survey from June to September 2020. We applied the point-centered quarter method to survey the tree stratum and the quadrate method to assess the ground community, as these two methods are appropriately used for vegetation analysis in the tropical mountain forest survey (Jafari et al. 2013). Using a local species-list database is considered cost-effective as the relatively small site with low diversity of floristic composition sufficiently provides an accurate species database. The results of the vegetation survey found 26 tree species, 23 genera, 20 families, and 15 orders. We identified 98 species, 87 genera, 45 families, and 32 orders for the lower crop community. The floristic list is given in Tables 1 and 2.

DNA extraction

We extracted the DNA from all fecal samples using ZymoBIOMICS DNA Microprep Kit (D4300) from ZYMO Research, Inc. We added each collected fecal sample to a ZR Bashingbead lysis tube (0.1 and 0.5 mm) and added 750-µL ZymoBIOMICS lysis solution to the tube. Further steps followed the manufacturer's protocol until the filtered DNA was suitable for the downstream application.

This research used a mini barcode approach in the PCR to amplify the plant DNA extracted from previous steps. The small-sized barcode primer in this research is the ribulose-biphosphate carboxylase gene (*rbcl*) plant DNA barcode region since this primer has been accepted and widely implemented as a standard primer used in *rbcl* research (Pompanon et al. 2012). In this study, we employed forward locus-specific primer sequence 5'-CTTACCAGYCTTGATCGTTACAAAGG-3' and the reverse primer sequence 5'-GTAAAATCAAGTCCACC RCG-3' following (Erickson et al. 2017). These two primers are considered appropriate as the amplicon size from the primer pair constantly produces a 379 base pair across the alignment of the 500 *rbcl* (Erickson et al. 2017).

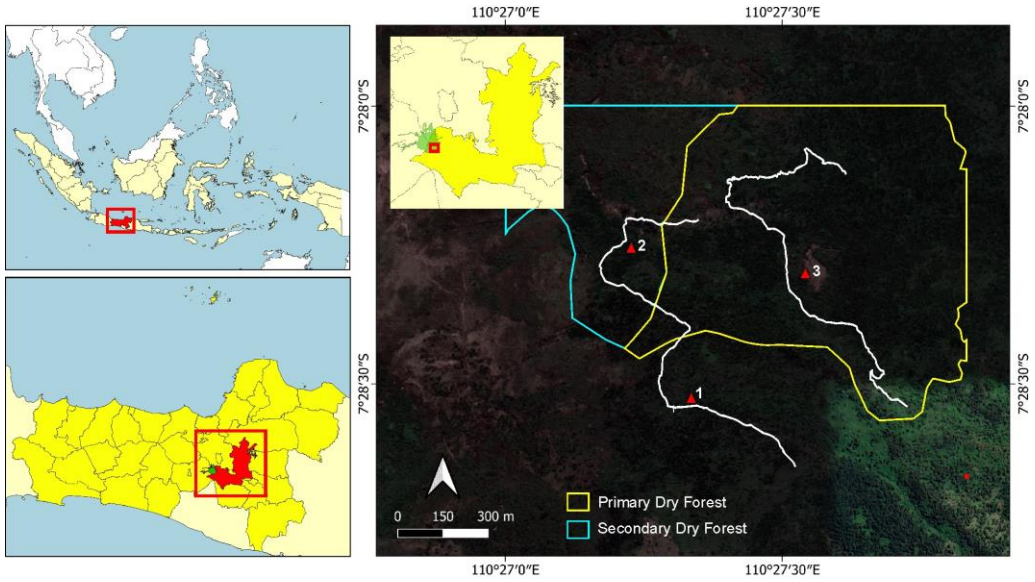

Figure 2. The study site in Mount Merbabu, Central Java, Indonesia. Point 1, 2, and 3 are the point of the fecal collection. We collected two fecal samples from different individuals from each point during the sampling

Table 1. The list of the lower crop community in the primary and secondary forest in Mount Merbabu, Central Java, Indonesia

Order	Family		Species
Alismatales	Araceae	1 genera, 1 species	*Scindapsus* sp.
Apiales	Apiaceae	1 genera, 1 species	*Centella asiatica,*
	Araliaceae	2 genera, 2 species	*Aralia laevis, Hydrocotyle ranunculoides*
Arales	Araceae	1 genera, 2 species	*Arisaema triphyllum, Arisaema tortuosum*
Asparagales	Orchidaceae	1 genera, 1 species	*Oberonia similis*
Asterales	Asteraceae	13 genera, 13 species	*Ambrosia acanthicarpa, Ageratina riparia, Erigeron sumatrensis, Crassocephalum crepidiodes, Anaphalis javanica, Galinsoga parviflora, Emilia sonchifolia, Gamochaeta calviceps, Sonchus palustris, Ageratum conyzoides, Gnaphalium* sp., *Chromolaena odorata, Acmella uliginosa*
Caryophyllales	Amarantahceae	2 genera, 2 species	*Achyranthes aspera, Alternanthera* sp.
	Phytolaccaeae	1 genera, 1 species	*Phytolacca icosandra-purpurascens*
Cucurbitales	Cucurbitaceae	1 genera, 1 species	*Gynostemma pentaphyllum*
Cyatheales	Cyaheaceae	1 genera, 1 species	*Cyathea* sp.
Cyperales	Cyperaceae	2 genera, 3 species	*Cyperus odoratus, Cyperus rotundus, Kyllinga monocephala*
Equisetales	Equisetaceae	1 genera, 1 species	*Equisetum hyemale*
Fabales	Fabaceae	2 genera, 2 species	*Parochetus communis, Desmodium repandum*
Gentianales	Rubiaceae	2 genera, 2 species	*Richardia brasiliensis, Rubia cordifolia*
Geraniales	-	-	
Gleicheniales	Gleicheniaceae	1 genera, 1 species	*Dicranopteris linearis*
Lamiales	Lamiaceae	4 genera, 4 species	*Scutellaria serrata, Solenostemon scutellarioides, Clinopodium vulgare, Buddleja davidii*
Liliales	Colcicaceae	1 genera, 1 species	*Disporum cantoniense*
	Smilacaceae	1 genera, 1 species	*Smilax anceps*
Lycopodiales	Lycopodiaceae	1 genera, 1 species	*Huperzia selago*
Malphigiales	Violaceae	1 genera, 1 species	*Viola reichenbachiana*
Myrtales	Melatomataceae	2 genera, 3 species	*Melastoma* sp., *Melastoma malabathricum, Clidemia hirta*
Myricales	Myricaceae	1 genera, 1 species	*Morella cerifera*
Piperales	Piperaceae	1 genera, 1 species	*Peperomia rotundifolia*
Plantaginales	Plantaginaceae	1 genera, 1 species	*Plantago major*
Poales	Poaceae	14 genera, 15 species	*Oplismenus hirtellus, Digitaria sanguinalis, Panicum effusum, Imperata cylindrica, Pennisetum purpureum, Isachne globosa, Themeda quadrivalvis, Zoysia matrella, Briza minor, Poa annua, Setaria pumila, Setaria parviflora, Arthraxon hispidus, Brachypodium sylvaticum, Pennisetum purpureum, Microlaena stipoides*
	Cyperaceae	1 genera, 1 species	*Carex baccans*
Polygonales	Polygonaceae	3 genera, 4 species	*Persicaria chinensis, Polygonum nepalense, Polygonum chinensis, Rumex obtusifolius*
Primulales	Myrsinaceae	1 genera, 1 species	*Lysimachia* sp.
Polypodiales	Aspleniceae	1 genera, 1 species	*Pteridium aquilinum*
	Dennstaedtiaceae	1 genera, 1 species	*Polystichum braunii, Polystichum semifertile*
	Dryopteridaceae	1 genera, 2 species	*Asplenium pruemorium*
	Pteridaceae	1 genera, 1 species	*Adiantum caudatum*
	Lindsaeaceae	1 genera, 1 species	*Odontosoria chinensis*
	Polypodiaceae	2 genera, 2 species	*Lepisorus sublinearis, Leptochilus decurrens*
Ranunculales	Ranunculaceae	3 genera, 4 species	*Actaea rubra, Clematis cirrhosa*
	Berberidaceae	1 genera, 1 species	*Ranunculus lanuginosus, Ranunculus repens*
Rosales	Rhamnaceae	1 genera, 1 species	*Rhamnus alaternus*
	Rosaceae	4 genera. 8 species	*Rubus rosaefolia, Rubus niveus, Rubus moluccanus, Rubus fraxinifolius, Rubus lineatus, Agrimonia procera, Parietaria Judaica, Potentilla tabernaemontani*
	Urticaceae	1 genera, 1 species	*Debregeasia longifolia*
Sapindales	Rutaceae	1 genera, 1 species	*Triphasia trifolia*
Selaginellales	Selaginellaceae	1 genera, 1 species	*Selaginella saginata*
Solanales	Convolvulaceae	1 genera, 1 species	*Ipomea violaceae*
	Solanaceae	1 genera, 1 species	*Solanum nigrum*
Vitales	Vitaceae	1 genera, 1 species	*Cayratia japonica*
30 orders	**45 families**	**87 genera**	**98 species**

Table 2. The list of the tree community in the primary and secondary forest in Mount Merbabu, Central Java, Indonesia

Order	Family		Species
Apiales	Araliaceae	1 genera, 1 species	*Macropanax dispermus*
	Pittosporaceae	1 genera, 1 species	*Pittosporum moluccanum*
Casuarinales	Casuarinaceae	1 genera, 1 species	*Casuarina junghuhniana*
Ericales	Ericaceae	1 genera, 1 species	*Rhododendron javanicum*
	Theaceae	1 genera, 2 species	*Schima norhoe, Schima wallichii*
Euphorbiales	Phyllanthaceae	1 genera, 1 species	*Glochidion kollmannianum*
Fabales	Fabaceae	2 genera, 2 species	*Acacia decurrens, Paraserianthes lophantha*
Fagales	Juglandaceae	1 genera, 1 species	*Engelhardia spicata*
	Fagaceae	1 genera, 1 species	*Lithocarpus* sp.
Gentianales	Rubiaceae	1 genera, 1 species	*Cinchona* sp.
Laurales	Laurceae	1 genera, 2 species	*Cinnamomum parthenoxylon, Cinnamomum verum*
Malpighiales	Euphorbiaceae	1 genera, 1 species	*Homalanthus giganteus*
	Pandaceae	1 genera, 1 species	*Galearia filiformis*
Malvales	Thymelaeaceae	1 genera, 1 species	*Daphnopsis americana*
Oxalidales	Elaeocarpaceae	1 genera, 1 species	*Elaeocarpus stipularis*
Pinales	Cupressaceae	2 genera, 3 species	*Cupressus arizonica, Cupressus lusitanica, Pinus merkusii*
Sapindales	Meliaceae	2 genera, 2 species	*Aglaia odoratissima, Chisocheton pentandrus*
	Sapindaceae	1 genera, 1 species	*Dodonaea viscosa*
Theales	Actinidiaceae	1 genera, 1 species	*Saurauia bracteosa*
Urticales	Moraceae	1 genera, 1 species	*Ficus fistulosa*
13 orders	**21 families**	**23 genera**	**26 species**

From the DNA samples to the final data, each step, including sample test, PCR, library preparation, and sequencing, influences the quality of the data, and data quality directly impacts the analysis results (Bokulich et al. 2013). We check the reliability of the data by doing quality control (QC) that was performed at each step of the procedure. The QC methods for the DNA samples include testing DNA purity using Nanodrop, testing DNA degradation and potential contamination using agarose gel electrophoresis, and quantifying DNA concentration using a Qubit 2.0 fluorometer (Invitrogen, Inc.).

PCR was performed based on the *rbcl* primers following Erickson et al. (2017). Although a combination of *rbcl* and matK provides a better discrimination power and reliability in identifying land plants, this research considered using *rbcl* primer as a mini barcode for plant identification since it has been widely used as a standard for plant identification with a high level of taxonomic resolution (Pompanon et al. 2012; Bell et al. 2017; Kress 2017). We conducted PCR to amplify the *rbcl* mini barcode towards the entire extracted DNA. All DNA samples were put through identical PCR conditions. We performed the reaction consisting of 12.5 µL of 2× EmeraldAmp MAX PCR Master Mix, 0.1 µL each of 100 µM forward and reverse primer, 1 µL of DNA, and 11.3 µL of ultrapure water. Thermocycling was conducted in an ABI 2720 with one cycle of 95°C for 4 min; 35 cycles of 94°C for 20 s, 55°C for 30 s, 72°C for 1 min; 1 final extension of 72°C for 5 min; and hold at 9°C until the reaction was removed. Each DNA sample had its PCR reaction repeated once, and the two reactions were pooled in equal concentration as measured using a Qubit fluorometer prior to PCR cleanup. The negative control was used in this protocol to check how laboratory processing affected the results.

The amplicons resulting from the *rbc*l PCR were verified through gel electrophoresis using the Agilent TapeStation machine and purified with Ampure beads (0.8× volume of beads to PCR volume) washed with 80% Ethanol. DNA was eluted from beads by mixing 52.5 µL of 10 mM Tris (pH 8.5) buffer with the beads and recovering 50 µL of clean DNA solution following the separation of the beads with a magnet. Clean-up was performed on a plate using a magnet plate from Ambion. The amplicons resulting from the *rbcl* PCR were verified through gel electrophoresis using the Agilent TapeStation machine and purified with Ampure beads (0.8× volume of beads to PCR volume) and washed with 80% Ethanol twice. DNA was eluted from beads by mixing 52.5 µL of 10 mM Tris (pH 8.5) buffer with the beads and recovering 50 µL of clean DNA solution following the separation of the beads with a magnet. Clean-up was performed on a plate using a magnet plate from Ambion. The cleaned PCR product was then used in a second PCR using a unique combination of Nextera XT indexes (Illumina catalog number FC-131-1002) for each sample. For each index PCR amplification, we used: 5 µL of cleaned PCR product, 5 µL each of the Nextera XT index primers (S5XX and N7XX, respectively), 25 µL of 2× EmeraldAmp MAX PCR Master Mix, and 10 µL of H_2O. Each reaction was conducted on an ABI 2720 using the program: one cycle of 95°C 3 min; 8 cycles of 95°C 30 s, 55°C 30 s, 72°C 30 s; 1 final extension of 72°C 5 min; followed by a hold at 9°C until the reaction was removed. The indexed PCR was cleaned using a 1.12× volume of Ampure beads (56 µL of Ampure beads mixed with 50 µL PCR) and washed twice with 80% Ethanol while on a magnet stand. Clean index PCR was eluted from beads using 27.5 µL of 10 mM Tris (pH 8.5) buffer, with 25 µL of solution recovered.

Libraries were performed on a paired-end Illumina platform to generate 2x250 bp paired-end raw reads using

NovaSeqTM 6000 v1.5 Reagent Kit. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence (Schwartz et al. 2011). The paired-end reads were merged using FLASH version 1.2.7 (Magoč and Salzberg 2011). Quality filtering on the raw tags was performed under specific filtering conditions to obtain high-quality clean tags. The primer sequence was truncated, and the reads were filtered based on the expected error value using the -fastq_filter command from USEARCH version 7 (Edgar et al. 2011). The amplicon was performed on a paired-end Illumina platform to generate 250bp paired-end raw reads (Raw PE), and then assembled and pretreated to obtain Clean Tags. The chimeric sequences on Clean Tags are detected and removed to obtain the effective tags finally (Edgar et al. 2011). The original data obtained from the high throughput sequencing platforms are transformed into sequenced reads by base calling. Raw data are recorded in a fast file containing sequenced reads and corresponding sequencing quality information (Frampton and Houlston 2012). The flowchart for generating the OTUs (OTUs clustering) is summarized in Figure 4. We filtered the reads to remove the low-quality reads with an expected error > 1. The unique reads were used to build OTUs using a *de novo* clustering approach with 97% similarity.

Data analysis

All OTUs have been identified against the gene bank data at https://www.ncbi.nlm.nih.gov/. However, the small amount of mitochondrial DNA fragments may result in an appropriate identification (Pompanon et al. 2012). Therefore, validation should be made to verify the taxa resulting from the gene bank. This paper verified the output of the blast analysis by manually screening the OTU's against our floristic list facilitated by Microsoft Excel. First, we arrange all the OTUs and taxa gathered from GenBank according to the OTUs' abundance from the highest to the lowest. We removed any zero abundance OTUs, considering this value implies a taxonomic unit that the langur does not eat. Finally, we confirmed all OTUs against the species list that resulted from the floristic survey.

RESULTS AND DISCUSSION

Results

The *rbcl* primer was successfully sequenced for 238 operational taxonomic units (OTUs). Of the 238 OTUs, a total of 130 sequences, or 54.6%, were unidentified, according to the BLAST against the genetic databases at https://www.ncbi.nlm.nih.gov/. The remaining OTUs belongs to 38 order, 48 families, 79 genera, and 75 species (Table 3). Considering that our ecological survey gained 43 orders, 66 families, 100 genera, and 125 species, we assumed that the floristic list resulting from our survey is appropriate to explain the OTUs data resulting from the NGS analysis. We used R and R Studio to make a crona visualization of the entire taxonomic unit resulting from the NGS. The crona visualization is given in Figure 5.

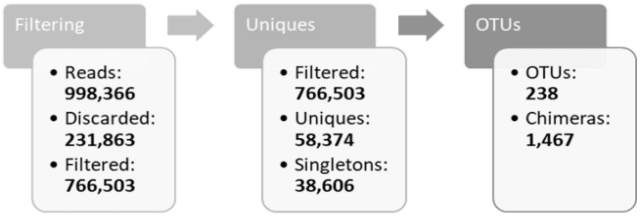

Figure 4. The general outline of the works for revealing the diet of the Ebony Javan Langur

After a series of confirmations of the OTUs, we still have one unidentified OTUs (with 2169 OTUs' abundance), six OTUs that are not matched with our species list, and five unicellular OTUs that unconfirmed by our floristic database. We used our database to ignore the six OTUs that are not matched with our database and confirm the one-starred OTUs by replacing them with the closest relative according to our floristic list. The results are 15 tree taxa in 13 families and 26 taxa in 22 families of lower vegetation communities (Table 4). The actual diet composition, of course, will be more than the results yielded in our study. Since the *rbcl* primer used in this research cannot reveal a more detailed taxonomic unit, this study was focused on the frequently eaten dietary composition of the ebony Javan Langur. Therefore, we analyzed the diet of the langur based on the OTUs abundance data that resulted from the NGS analysis.

Discussion

Ebony Langur's diet

While the NGS produced 42 taxa, our visual observation of the langur's diet in the fragmented primary and secondary mountain forest in Merbabu yielded only six taxa. Hence, the result of this study shows the effectiveness of performing the NGS approach to complement the visual observation. Despite the power of NGS, however, the tiny fragment of DNA may yield inappropriate results during the BLAST. The *rbcl* has a low ability to identify specific orders, families, genera, and species. For example, OTU 2 in Table 3 proves the low discrimination rate of the NGS in differing within the Apiales. The DNA barcoding ended with Apiales, whereas our vegetation study identified two families (Araliaceae and Pittosporaceae) and even species within the families in the Apiales (*Macropanax dispermus* in the Araliaceae and *Pittosporum moluccanum* in the Pittosporaceae). Therefore, we replaced the Apiales resulting from the NGS with Araliaceae and Pittosporaceae (Table 4). The failure to identify the species category is also shown, for example, in the OTU 4 (Table 3; *Debregeasia saeneb*) that we confirmed with *Debregeasia longifolia* in Table 4. The low discrimination power of the *rbcl* may be enhanced by applying the *matK* as a second marker complementing *rbcl*. However, it may be inappropriate for diet metabarcoding analysis since we cannot easily match *matK* and *rbcl* sequences from the same plant cells in a mixed diet. Hence, we used a vegetation survey producing a floristic list database to confirm the data from our single primer-NGS analysis. The

vegetation survey is considered fast and effective in confirming the plant eaten by the ebony langur. This effectiveness is because of the low degree of species richness of the vegetation in the fragmented forest in Mount Merbabu, making the vegetation survey provide a fast and more accurate confirmation against the results of the NGS.

Our research yielded 13 trees and 22 lower vegetation families of the ebony langur consumed in the fragmented forest. It is smaller than the two previous studies conducted by Kool (1993) that gained 16 tree families, and Tsuji et al. (2019), that observed 23 tree families and three lower vegetation families. These two previous studies were conducted in the lowland forest (100 masl), where the vegetation is more diverse than in the mountain forest. Refers to Kool (1993) and Tsuji et al. (2019), we ascertained five tree families that are constantly consumed by *T. auratus*, i.e., Euphorbiaceae, Fabaceae, Lauraceae, Rubiaceae, and Sapindaceae; and five lower vegetation families, i.e., Cucurbitaceae, Lamiaceae, Rubiaceae, Rutaceae, and Urticaceae. These plant families are consumed in the lowland and the mountain forest; hence, they probably indicated an evolutionary preferred food plant family for *T. auratus.* According to our floristic database, in Table 5, we ascertained these tree and lower vegetation communities by specifying these taxa into species based on Table 1 and Table 2. Personal observation by the visual survey showed frequent foraging activities of the langur on the native plant *Homalanthus giganteus* (Euphorbiaceae), *Acacia decurrens*, and *Paraserianthes lophantha* in the Fabaceae. Even though not of the best OTUs abundance, the relatively high abundance of *Acacia decurrens* and *Paraserianthes lophantha* (Fabaceae) in the forest show that these two introduced species were consumed by the langur and hence, indicating an adaptive ability of the langur to consume a new food source.

Surprisingly, our results indicate that the langur is likely to penetrate the lower vegetation stratum. The langur usually spends most of its time living in the canopy (Nijman 2013). Penetrating the lower vegetation stratum at the forest floor or an opened space may risk being eaten by the predator. However, diet evidence proves that the langur went down to the lower stratum and consumed more than half of its diet, as shown in Figure 6. This penetration is probably related to the low diversity of the food substrate in the fragmented forest or the less abundant preferred food plant phenology (Tsuji et al. 2019). These two causes have forced the langur to forage beyond its typical habit.

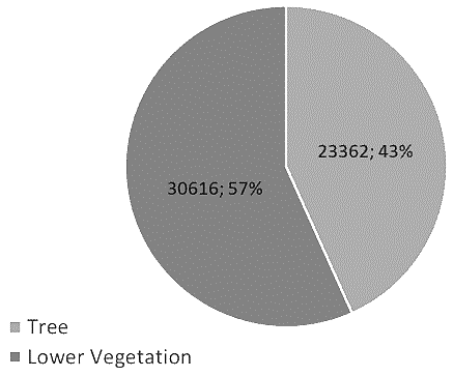

Figure 6. Diet composition of the Ebony Javan Langur in the fragmented forest according to the plant strata. The OTUs abundance for the lower vegetation (3061 indicated that more than 50% of the diet belongs to the lower vegetation community

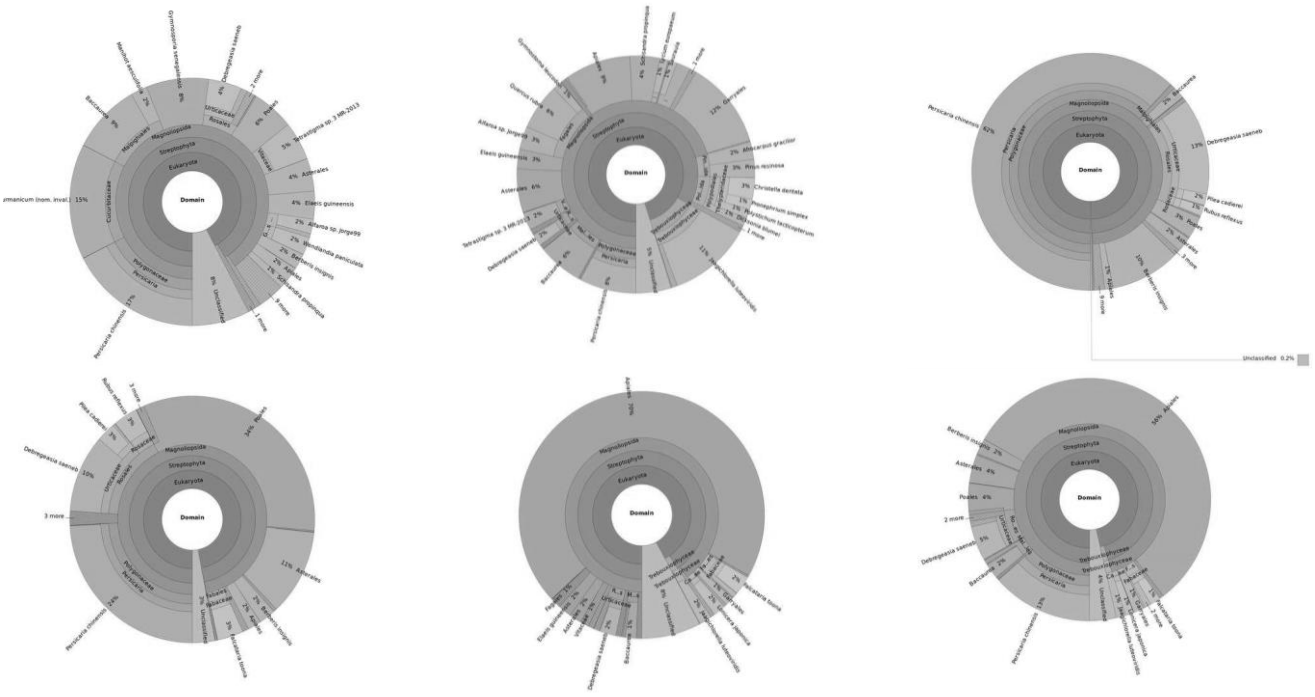

Figure 5. Crona visualization of all taxonomic units of plant eaten by the langur for the six samples. The visualizations show the taxonomic unit along with its percentage

According to Kool (1992), *T. auratus* primarily consumes young leaves since it has a high protein ratio versus acid detergent fiber. This food source is preferred due to its high protein, low fiber, and yet high digestibility value. Indeed, *T. auratus* is likely to be a young-leaf eating colobine that eats young leaves slightly less than 50% of its diet (Kool 1992). *Engelhardtia spicata* in the family Juglandaceae (533 OTUs abundance) is a unique native tree in the study site. This species drops all of its leaves in the rainy season and begins to sprout in the dry season, and yet, it may serve as a young leaf and bud provider for the ebony langur during the dry periods.

Table 3. The raw data of the OTUs and the taxonomic categories identified against the gene bank. We signed with * for the order or family or the smallest taxonomic unit that is considered different compared to our floristic database. Several OTUs that signed with ** belongs to the unicellular microalgae organism, yet these were excluded from our validation. The taxa below were sequenced during the NGS as they have similar *rbcl* gene segments in their mitochondrial DNA

OTUs	Order/Family	Smallest taxon unit	OTUs	Order/Family	Smallest taxon unit
OTU2	Apiales	**Unidentified**	OTU52	Malphigiales	**Unidentified**
OTU168	Apiales	*Dendropanax trifidus**	OTU37	Malvales	*Aquilaria* sp. *
OTU29	Araucariales*	*Afrocarpus gracilior**	OTU66	Myrtales*	Unidentified*
OTU9	Arecales*	*Elaeis guineensis**	OTU27	Pinales	*Pinus resinosa**
OTU73	Asparagales	*Habenaria intermedia**	OTU3, 12	Poales	Unidentified
OTU5	Asterales	**Unidentified**	OTU39	Poales	*Schizachyrium scoparium **
OTU192	Asterales	*Melanthera nivea**	OTU156	Poales	*Setaria pumila*
OTU227	Asterales	*Ageratina luciae**	OTU228	Poales	*Prionium serratum **
OTU16	Austrobaileyales*	*Schisandra propinqua**	OTU229	Poales	*Carex bicolor **
OTU92	Boraginales*	*Mertensia paniculata**	OTU233	Poales	*Phalaris arundinacea **
OTU203	Polygonales	*Brunnichia ovata**	OTU238	Poales	*Triplachne nitens **
OTU1, 216, 169	Polygonales	*Persicaria chinensis*	OTU215	Poales	*Carex pulicaris**
OTU188	Polygonales	*Symmeria paniculata**	OTU225	Poales	*Typha angustifolia**
OTU10	Celastrales*	*Gymnosporia senegalensis**	OTU226	Poales	*Carex fuscula**
OTU8, 33	Chlorophyta**	*Jaagichlorella luteoviridis **	OTU235	Poales	*Khaosokia caricoides **
OTU59	Chlorophyta **	*Kalinella apyrenoidosa***	OTU31	Polypodiales	*Christella dentata**
OTU77, 109, 129	Chlorophyta **	*Coccomyxa* sp. **	OTU35	Polypodiales	*Pronephrium simplex*
OTU89	Chlorophyta **	*Dictyochloropsis* sp.**	OTU47	Polypodiales	*Polystichum tacticopterum**
OTU93	Chlorophyta **	*Parachlorella kessleri***	OTU7	Ranunculales	*Berberis insignis**
OTU98. 128	Chlorophyta **	Dictyochloropsis sp.**	OTU123	Rosales	*Rubus phoenicolasius**
OTU185	Cornales*	*Alangium javanicum **	OTU175	Rosales	*Boehmeria calophleba **
OTU48	Crossomatales*	*Turpinia pentandra**	OTU202	Rosales	*Chamabainia cuspidata **
OTU63	Cucurbitales	*Cucumis sativus**	OTU214	Rosales	*Rubus reflexus**
OTU95	Cucurbitales	*Gynostemma burmanicum**	OTU4	Rosales	*Debregeasia saeneb**
OTU199	Cucurbitales	*Hemsley* sp.*	OTU21	Rosales	*Pilea cadierei**
OTU38	Cyatheales	*Dicksonia blumei*	OTU25	Rosales	*Rubus reflexus**
OTU136	Dipsacales*	*Lonicera japonica **	OTU53	Rosales	*Parasponia parviflora **
OTU204	Dipsacales *	*Valeriana jatamansi **	OTU64	Rosales	**Unidentified**
OTU206	Dipsacales *	*Lonicera japonica **	OTU119	Rosales	*Pilea cadierei **
OTU26	Ericales	Ericaceae*	OTU177	Rosales	*Rosa carolina**
OTU43	Ericales	*Saurauia**	OTU178	Rosales	*Pilea cadierei **
OTU19, 224	Fabales	*Falcataria toona**	OTU190	Rosales	*Myriocarpa longipes **
OTU44	Fabales	*Campylotropis griffithii**	OTU201	Rosales	*Rosa minutifolia **
OTU194	Fabales	**Unidentified**	OTU213	Rosales	*Ziziphus pubescens **
OTU208, 217	Fabales	**Unidentified**	OTU184	Santales*	*Lepionurus sylvestris **
OTU236	Fabales	*Hoffmannseggia watsonii**	OTU62	Sapindales	*Pachycormus discolor **
OTU14	Fagales	*Quercus rubra**	OTU94	Rosales	*Acer kweilinense **
OTU18	Fagales	*Alfaroa* sp.*	OTU30	Solanales	*Lycium europaeum **
OTU36	Fagales	*Gymnostoma leucodon**	OTU67	Solanales	*Ipomea trifida**
OTU57	Fagales	*Morella faya**	OTU42	Splachnales* *	**Unidentified****
OTU61, 114, 15, 17, 32, 40	Garryales *	**Unidentified**	OTU147	Chlorophyta**	*Dictyochloropsis splendida*
OTU210	Gentianales	*Strychnos ericsonii**	OTU11	Vitales	*Tetrastigma* sp.*
OTU22	Gentianales	*Wendlandia paniculata*	OTU146	Vitales	*Vitis vinifera**
OTU54	Gleicheniales	*Dicranopteris linearis*	OTU197	Vitales	*Cayratia geniculata**
OTU139	Gleicheniales	*Diplopterygium rufum **			
OTU132	Hypnales**	**Unidentified**			
OTU124	Lamiales	*Vitex cofassus **			
OTU231	Laurales	*Ediandra* sp. *			
OTU6	Malphigiales	*Baccaurea**			
OTU20	Malphigiales	*Manihot aesculifolia **			

Table 4. The OTUs abundance and the plant taxa eaten by the ebony Javan langur. We signed with * for the order, family, or species that we replaced confirming our species list database

Tree	OTUs' abundance	Family	Lowe crop community	OTUs' abundance	Family
Apiales (Apiaceae, Araliaceae)*	14008	Apiaceae, Araliaceae	*Persicaria chinensis*	12602	Polygonaceae
*Glochidion kollmaniannum**	2195	Phyllanthaceae	Poales	4598	Poaceae, Cyperaceae
Fabaceae/Acacia/Passerianthes*	1079	Fabaceae	*Debregeasia longifolia**	4180	Urticaceae
Lithocarpus sp.*	768	Fagaceae	Asteraceae*	2895	Asteraceae
*Engelhardtia spicata**	533	Juglandaceae	*Gynostemma pentaphyllum**	1723	Cucurbitaceae
*Saurauia bracteosa**	382	Actinidiaceae	*Berberis julianae**	1662	Berberidaceae
*Homalanthus giganteus**	281	Euphorbiaceae	*Cayratia japonica**	842	Vitaceae
*Pinus merkusii**	279	Pinaceae	*Rubus**	680	Rosaceae
Wendlandia paniculata	247	Rubiaceae	*Solanum nigrum**	307	Solanaceae
*Daphnopsis americana**	98	Malvales	Polypodiales*	296	Polypodiaceae
*Macropanax dispermus**	39	Apiaceae	Polypodiaceae*	148	Polypodiaceae
Sapindaceae*	38	Sapindaceae	Poaceae	127	Poaceae
*Dodonaea viscosa**	8	Sapindaceae	Polysthicum sp*	123	Dryopteridaceae
Cinnamomum sp.*	2	Lauraceae	*Dicksonia blumei*	120	Cyatheaceae
			Rosaceae	98	Ramnaceae, Rosaceae, Urticaceae
			Dicranopteris linearis	39	Gleicheniaceae
			Setaria pumila	37	Poaceae
			Rosales*	35	Ramnaceae, Rosaceae, Urticaceae
			*Carex baccans**	33	Cyperaceae
			*Oberonia similis**	29	Orchidaceae
			Melastoma sp*	28	Melastomaceae
			*Ipomoea violacea**	24	Convolvulaceae
			*Ageratina riparia**	4	Asteraceae
			Polygonaceae	3	Polygonaceae
			Rubiaceae*	3	Rubiaceae
			Lamiaceae	1	Lamiaceae
16 taxa in 13 families			26 taxa in 22 families		

Table 5. Plant families that probably become an ultimate preferred food selected by *Trachypithecus auratus.* These families are presented along with their species according to the local floristic database

Tree			Lower vegetation community		
Family	**Species**	**Local name**	**Family**	**Species**	**Local name**
Euphorbiaceae	*Homalanthus giganteus*	*Krembi*	Cucurbitaceae	*Gynostemma pentaphyllum*	Jiagulan
Fabaceae	*Acacia decurrens, Paraserianthes lophantha*	*Akasia, Kemlandingan gunung*	Lamiaceae	*Scutellaria serrata*	Mint
Lauraceae	*Cinnamomum verum, Cinnamomum parthenoxylon*	*Kayu manis*	Rubiaceae	*Richardia brasiliensis*	Goletrak
Rubiaceae	*Cinchona* sp.	*Kina*	Rutaceae	*Tripasia tifolia*	Kingkit
Sapindaceae	*Dodonaea viscosa*	*Tesek*	Urticaceae	*Debregeasia longifolia*	Urang urang

This species is visually observed as one plant eaten by *T. auratus* in the primary and secondary forests during the observation. This species also serves as a sleeping tree and the playing ground for the langur as it is considered a big tree with a dense and interlocking canopy. The unique species *Engelhardtia spicata* has avoided the ebony langur from food scarcity in the dry season when food may be restrictedly available.

The value of the OTUs abundance in the Table 4 implies that higher OTU abundance may reflect the plant sequence with relatively higher abundance in the feces. This abundance implies a semi-quantitative measurement of the plants eaten mainly by the langur. The tree eaten mainly by the langur is in the order Apiales. As the short sequence of the *rbcl* in the Apiales cannot recognize the taxa specifically, we compared the results of the NGS to our floristic database and direct foraging observation. Our confirmation suggested that the native tree *Macropanax dispermus*, locally known as '*Pampung*' (Figure 7), is the specific taxon identity for the Apiales resulting from the NGS analysis. This kind of tree is abundant in primary and secondary forests and provides a rich-water tree for *T. auratus*, considered a less-drinking primate (Nijman 2000). This tree may reach considerable size, yet it may serve as a preferred food for the langur to spend most of its time foraging and doing many other non-foraging activities. The second best is *Glochidion kollmannianum* or 'Dempul' (2195 OTUs Abundance).

Figure 7. *Macropanax dispermus* (Apiales: Apiaceae), the highest OTUs abundance in the feces, indicating the amont of tree eaten by *Trachypithecus auratus* in the fragmented upland forest in Mount Merbabu. The tree's leaf is herbaceous; it provides the ebony langur with much water during foraging

According to the OTU, the ebony langur also consumed *Persicaria chinensis* (12602 OTUs abundance) in the lower vegetation community. This invasive species is highly abundant on the forest floor in all areas of study. The evidence of consuming this lower crop vegetation species by the langur indicates the penetration of the langur above ground to seek other alternatives. This penetration also tells the ecological importance of the invasive species as an alternative food substrate for *T. auratus.*

Implication for conservation

Our finding suggests that *T. auratus* living in a fragmented forest in Mount Merbabu has developed local foraging adaptation to choose both tree species and plants of lower communities in the forest ground of the mountain forest of Mount Merbabu. In addition, a result comparison between our study and previous reports conducted by Kool (1992, 1993) and (Tsuji et al. 2019) shows that the langur's diet comprises a vast taxonomic diversity of vegetation. By this premise, many plant taxa in tropical forests and lower crop communities may serve as a food substrate for the langur; hence, the ebony langur may not have a severe problem with food stocks and availability. Indeed, the ability of the langur to select many alternative plant species is supported by its unique digestive structure and microfloral composition that allow this primate to consume a large variety of plant resources even in the fragmented forest (Kool 1993). By this uniqueness, the langur may become the generalist primate taxon that potentially has more opportunities to survive and exist in the degraded and fragmented forest. Despite our study that reported an adaptation of the ebony langur on a new floristic composition in the upland forest, further research on nutritional sufficiency is still needed to ascertain the health status of the ebony langur. Garcia-Feria et al. (2017) found that even though the howler monkey (*Alouatta pigra*) adapted to foraging a new food resource, they can suffer from protein and carbohydrate deficiencies that physiologically affect the health status of the primate.

The ebony langur's habitat in Mount Merbabu is prone to habitat destruction, harming their foraging substrate and habitat preference. According to Dewi (2009), the primary and secondary forests of the langur habitat in Mount Merbabu are always vulnerable to the forest fire. The immense forest burning in 2019 has caused significant damage to the main home for the ebony langur. This forest damage has decreased the tree and forest ground diversity, which, in turn, caused the food availability and variability for the langur to be restricted. As indicated in the diet composition, penetrating the ground cover by the ebony langur is probably due to the fast-growing ground vegetation compared to the tree stratum. The ebony langur penetrated the ground cover for alternative food, which is limited in availability after the forest burning.

In conclusion, we compare the NGS'results to the data of floristics reference from the vegetation survey preceding the fecal analysis. The NGS found 238 OTUs that belong to 32 taxa. Most of the langur's diet belongs to the lower crop community. The results suggested that the dietary composition of the Ebony Javan Langur reflects an adaptation to the new floristic composition. The stakeholders must perform appropriate home-building-based habitat management practices. Ecological intervention to create a habitat that serves the langur with ground and foraging substrate is essential. The intervention should involve habitat restoration and plant diversity management through replanting with an appropriate kind of fast-growing tree that temporarily provides a ground and food substrate for the langur (for example, the *Acacia decurrens*) while also focusing on restoring the local tree in the longer-term program. Even though the alien species that massively invade the forest may serve as an alternative food source for the ebony langur, controlling such invasive species is necessary to improve the species diversity of the local floristic types.

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