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Genetic Diversity of Rice Varieties Cultivated in the Toamasina II District of Eastern Madagascar

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Abstract While the rich, endemic biodiversity of Madagascar is well studied, far less attention has been devoted to understanding the introduced crop diversity. Oryza sativa L. is the most widely consumed grain worldwide and constitutes 55% of the caloric diet of Malagasy peoples. A 2019 survey of rice varieties grown by 73 households around the Betampona Integral Nature Reserve in East Madagascar identified 81 locally named varieties (den Biggelaar et al., 2024). Rice varieties are named by farmers based on phenotypic and morphological characteristics (primarily of the seed and reproductive structures) and method of production (swamp/irrigated or upland/rainfed). Previous studies of Malagasy rice varieties using DNA analysis found a large number of synonyms and homonyms among studied individuals. This paper reports on the results of a microsatellite fingerprinting analysis used to determine the relatedness of named varieties in the villages around Betampona INR as well as three fokontany at different distances from the INR. Eighty-six fresh tissue samples were collected along with varietal names and specific collection coordinates. These samples were subjected to a modified CTAB extraction before conducting PCR using thirteen primers established by previous studies. Microsatellite bands were scored using a 50bp ladder on 3% agarose gels run for three hours at 50V. Results of our analysis show that within each fokontany, varieties are genetically distinct. Across the samples from the seven fokontany, we identified ten homonyms (35 samples) and sixteen synonyms. Using variety names only is, therefore, insufficient to study agrobiodiversity at larger scales, especially for a mostly self-pollinating crop such as rice, with farmers saving a portion of their crop as seed for the following crop season.

Keywords: *rice varieties, genetic diversity, DNA microsatellite analysis, Madagascar*

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1. Introduction

The loss of agrobiodiversity (i.e., the diversity of crops as well as the genetic diversity within them) may undermine agriculture’s long-term sustainability. The development of hybrid varieties of food crops has increased yields and production, but the loss of genetic diversity has made those crops more susceptible to pests, diseases, and environmental changes, increasing the dependence on human interventions and external inputs [1]. Local, traditional, and indigenous agroecosystems contain many of the genetic elements of sustainability, and we can learn from their example [1]. In-situ conservation is one approach to maintaining local varieties and genetic diversity and preserving local knowledge of these resources. Collaborating with farmers to reinforce local genetic resource management is, therefore, of vital importance for sustainable agriculture [2].

In Madagascar, farmers have selected many varieties of rice, the main staple food of Malagasy, for performance and adaption to local ecological conditions (weather, soils, insects and diseases), growing method (upland, swamp, irrigated), and social and cultural preferences (taste, grain color, stickiness, ease of hulling). In rural areas, improved (hybrid) varieties are difficult to find; even if available in the village, their price is often more than farmers can afford, more so as it requires them to buy new seed every season. Thus, farmers continue to grow their own local varieties even when not completely averse to trying new ones, as evidenced by the recuperation of unhulled grains from store-bought or food aid rice [3]. Before introducing new crop varieties, it is necessary to determine if there is a demand or need for them, that is, to learn more about existing local varieties, the desires of households, economic / ecological / socio-cultural constraints, characteristics of the new varieties to be introduced and the local capacity to manage them. To this end, an initial inventory of rice varieties cultivated by farmers around the Betampona Integral Nature Reserve (INR) in the Toamasina II District of Madagascar was made in the Fall of 2019, which resulted in the identification of 81 named varieties among the 73 households surveyed [3]. Results of a previous study of rice varieties cultivated by farmers in the Vakinakaratra region in the central highlands of Madagascar showed a great many homonyms and synonyms among the local varieties identified, as revealed by DNA analysis [4]. To determine whether that was also the case for the varieties we inventoried, this paper will report on the results of DNA analysis of most of the varieties initially identified.

2. Methods

Due to COVID travel restrictions, field collection of seed samples of the inventoried varieties was not possible until late Spring 2021, mostly after the rice harvest of the main growing season in May/June, and continued until the end of 2021. Not all varieties initially inventoried were still being cultivated, as farmers changed varieties, and some rice varieties may have been lost to cyclones, flooding, insects and diseases. Small seed samples were collected and germinated by Zafiroa at the MFG office in Toamasina in Spring 2022 until large enough to take small leaf samples that were stored in silica-powder-filled sample tubes and stored in a common chest freezer.

Seeds of some varieties did not germinate, and some were lost to birds and rats. We ended up with 42 samples from the 4 *fokontany* neighboring the Betampona INR: Antaranarina (9), Ambodirafia (12), Analamangahazo (9), and Fontsimavo (7); and seven samples each from the *fokontany* of Ambonivato (near Parc Ivoloina) and Mahambo, a coastal town 80 km north of Toamasina in the Fenerive-Est district (Figure 1; approximate GPS coordinates are listed in the supplemental material).

Leaf samples of an additional 31 varieties and variety information were collected directly from farm fields by den Biggelaar in October/November 2022 in the *fokontany* around the Betampona INR (14 in Antaranarina, and four each in Analamangahazo and Ambodirafia) and five samples in the *fokontany* of Ampasina. Most of these 31 varieties were *horaka* (swamp/irrigated) varieties, as upland fields were still being prepared before the onset of the rains for the main growing season (December through May/June). Given lack of electricity in rural Madagascar and the remoteness of villages and fields (only reachable by foot, and further hiking to the rice fields), collected samples were kept in double Ziploc bags at ambient temperatures until they could be placed in a chest freezer at the MFG office in Toamasina several days later.

All 86 samples were brought to the US in December 2022 (USDA-APHIS permit PCIP-21-00036 and Madagascar Phytosanitary certificate 2205 / 22 / 52TM / RPS / MAG), and stored in an ultra-cold freezer (-80^{0}C) in a laboratory of the Department of Biology at Appalachian State University until DNA extraction and microsatellite fingerprinting.

2.1. Extraction of DNA

A single strand of rice leaf was ground into powder using a sterile sand and mortis-pestle system. A modified cetyl-trimethyl ammonium bromide (CTAB) extraction was used to extract the DNA [5]. Extracted DNA quality and concentration were determined using a NanoDrop 1000 spectrophotometer and a 1% tris-borate-EDTA (TBE) agarose gel. DNA extraction of eight samples was unsuccessful due to degradation or lack of sufficient tissue due to delayed transport during the pandemic.

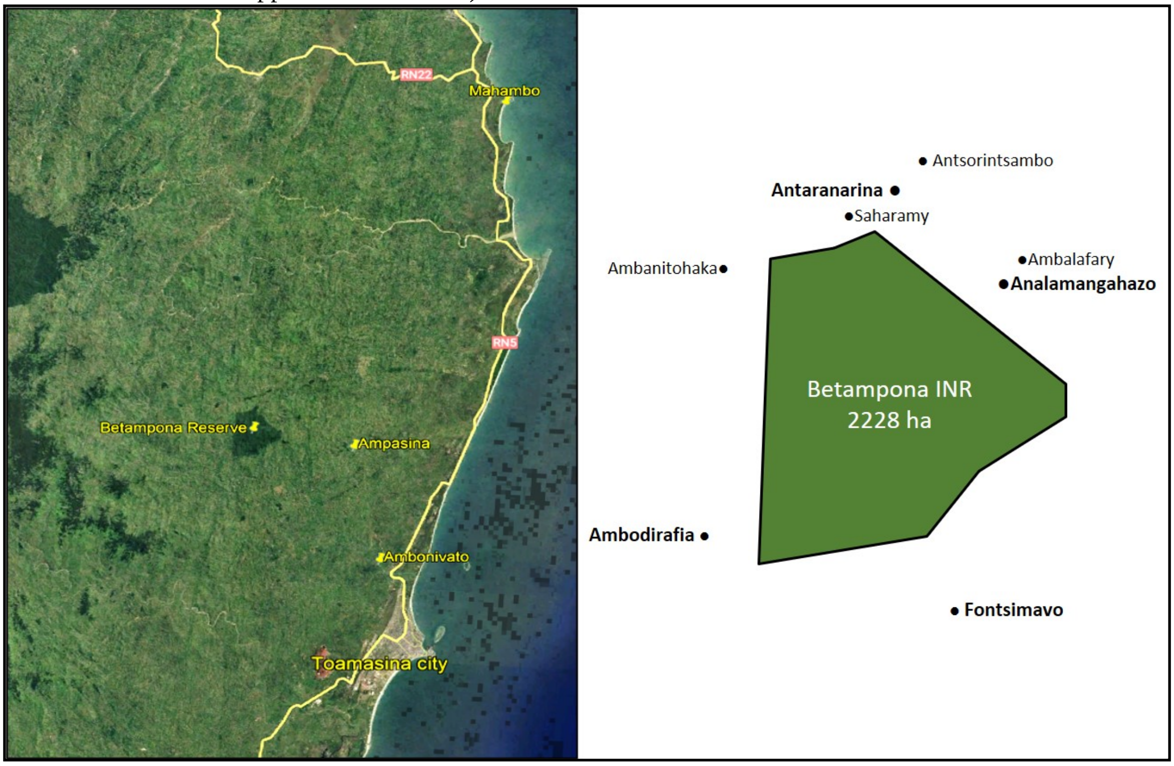

Figure 1. Map of the fokontany (names in bold) where seed and tissue samples were collected

2.2. Identification of Appropriate Primers

Following the recommendations of [5,6], we chose primers indicated as effective in differentiating between subspecies of rice varieties within small geographic regions where differentiation might otherwise be difficult. These primers were cross-referenced with Gramene's panel of 50 standard markers for *Oryza* spp [7], resulting in a final set of 13 primers, eight from the work of [5] (RM-1, 11, 55, 105, 144, 215, 237, 287), and five indicated as effective by [6] (RM-338, 374, 484, 489 and 552).

2.3. Quantification and Identification of Microsatellite Bands

The 78 DNA samples and a negative control were arrayed in a 96-well PCR plate and amplified using the thirteen selected microsatellite markers. We then conducted gel electrophoresis on a 3% TBE agarose gel for 3 hours at 50V for each marker with a 50bp ladder (New England Biolabs, Inc) as a size standard. The separated PCR products were then imaged on an Alpha imager (Alpha Innotech) and hand scored to obtain genotypes for each marker.

The resulting genotype data was then formatted for analysis in GenAlEx 6.5 [8]. A genotype accumulation curve was constructed to ensure the microsatellite markers had the power to differentiate individuals. Genetic distance was calculated, and a principal coordinates analysis was performed to visualize the relationship between collected samples.

3. Results

The genotype accumulation curve demonstrated that only nine microsatellite markers were required to differentiate all individuals as unique multi-locus genotypes, also known as "fingerprints" (see supplemental material). This analysis utilized thirteen markers, suggesting we had ample power to differentiate all 78 samples.

Results of the Principal Component Analysis showed that variation in genetic distance explained by the first two axes is 13.33 and 9.30 % respectively, or 22.63% cumulatively. The third axis explains another 8.26%. Upland (*tanety*) varieties are primarily located on the left of Axis 1, the exceptions between Somotra and Ambidimamo (Figure 2). Varieties for swamp (horaka) and irrigated cultivation are mostly located on the right side of Axis 1, and more spread out along Axis 2.

Within a *fokontany* (the lowest administrative unit in Madagascar), our analysis shows that named varieties are genetically distinct, showing that farmers are able to distinguish varieties based on primarily phenotypic and morphological characteristics (Figure 2). This, however, is not the case across the *fokontany* where we collected our samples. Names only apply locally to distinguish varieties and are not 'universal' trademark names as in developed countries. Thus, identical names may not mean identical varieties. The practice leads to genetically similar varieties with different names (synonyms), or the use of names new in a particular village but already used elsewhere resulting in homonyms that are genetically different. To account for this phenomenon, we did collect multiple samples of several named varieties.

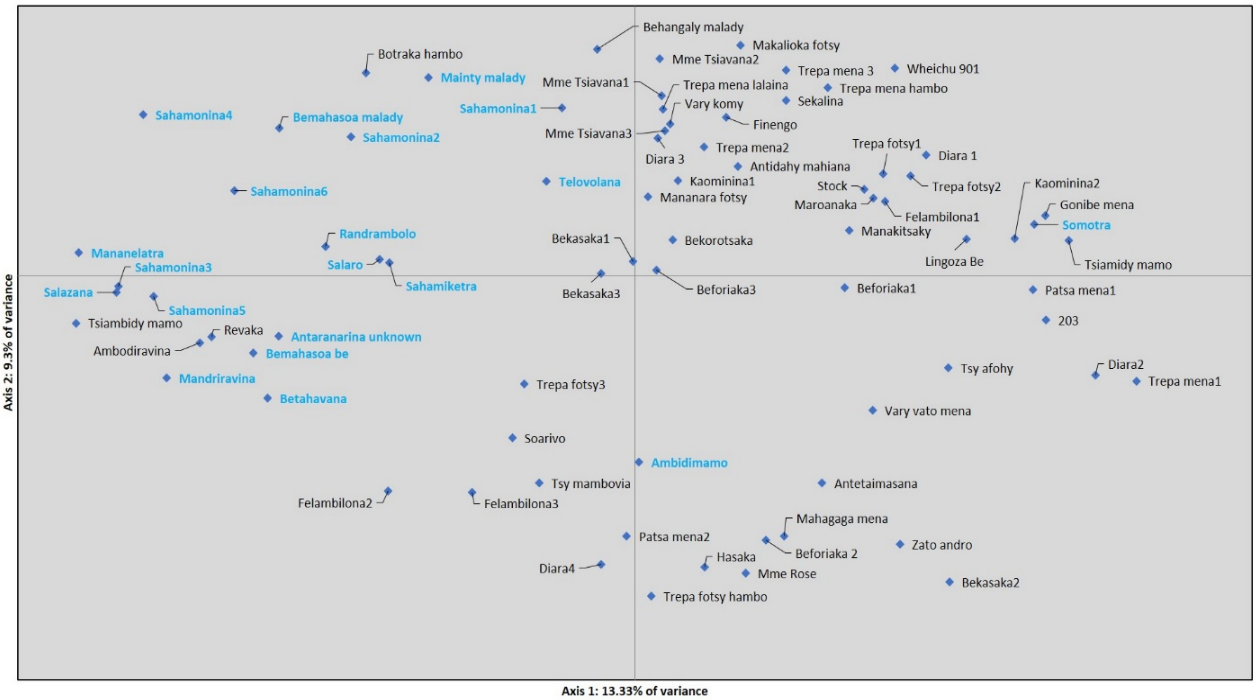

Figure 2. Principal coordinate analysis of 78 rice varieties; upland, rain-fed varieties in blue

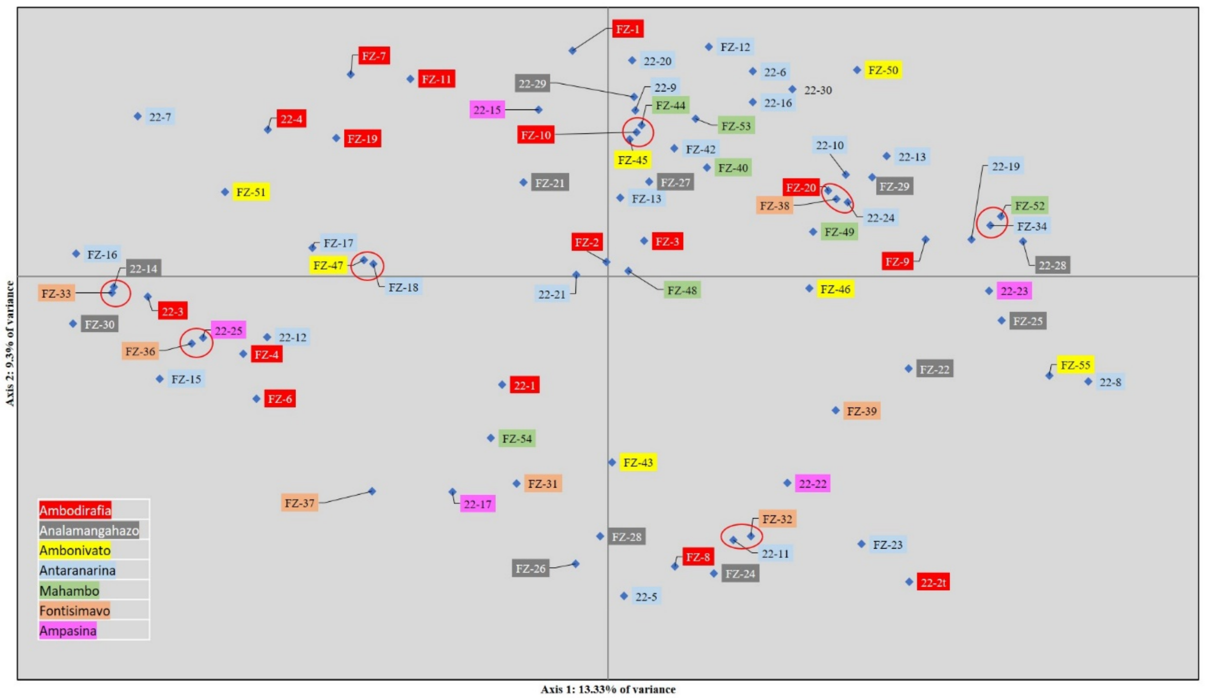

Figure 3. Principal coordinate analysis of varieties by fokontany of collection. Red circles indicate possible synonyms

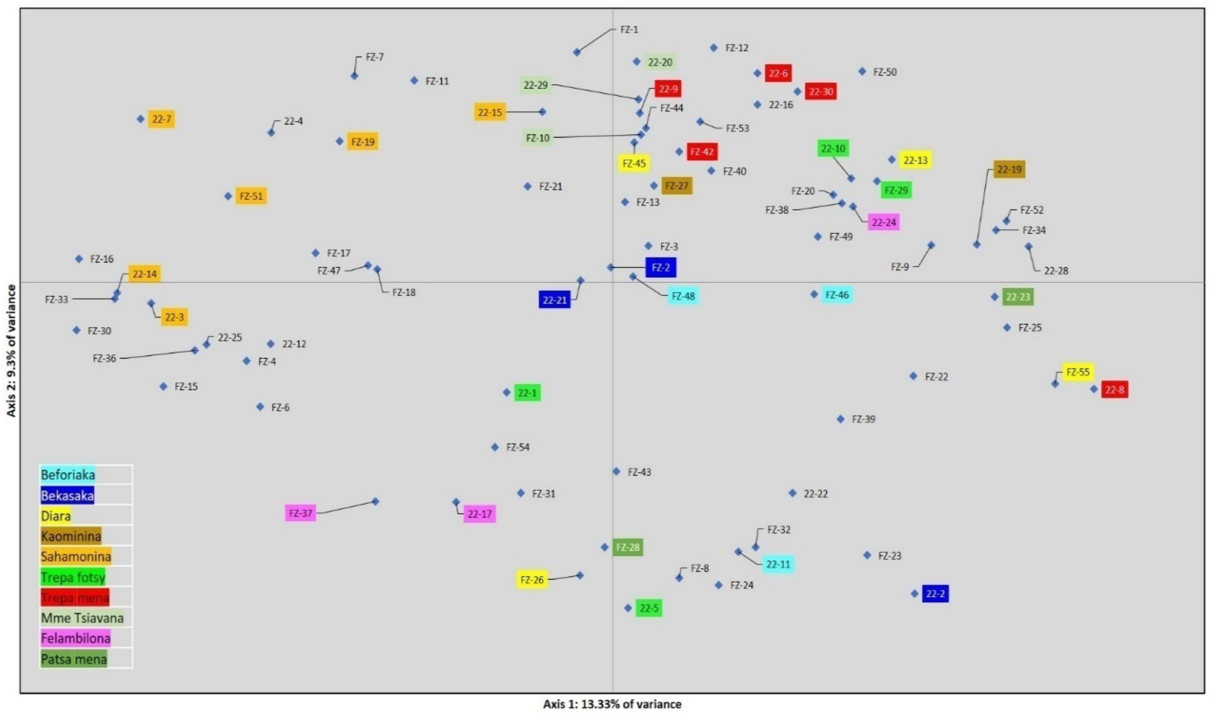

Figure 4. Principal coordinates of varieties with multiple samples with the same name (homonyms)

Results show sixteen varieties with one or more synonyms, i.e., have different names but appear genetically close (indicated with red circles in Figure 3), resulting in seven 'unique' varieties (Table 1). As could be expected, no synonym varieties were found within a fokontany. Geographic distance between fokontany where samples were collected is the major explanation for the existence of synonyms, as names do not usually travel with the seed. A variety name may already be in use within a fokontany, so a new name is given to distinguish it from already existing ones in cultivation.

The results show ten varieties with the same name (homonyms) which appear to be genetically different (Figure 4 and Table 2). The only variety that is logically explained as a homonym is Sahamonina, as the name means "local to the land/valley/community" and nearly every village has a variety called Sahamonina. It refers to a variety that has been cultivated in a specific valley or community for a long time passed on from generation to generation. The only occurrences of this variety that are genetically close are 22-14 and 22-3 from the *fokontany* of Ambodirafia and Analamangahazo, respectively, which are located on opposite sides of the Betampona Reserve. Sahamonina samples 22-3 and FZ-19 are both from the fokontany of Ambodirafia, but genetically very different as the villages where they were collected are about a 3 to 4-hour hike distant from each other.

Table 1. Varieties that may be synonyms

Sample # and variety	Fokontany	Sample # and variety	Fokontany
FZ-33 Salazana 22-14 Sahamonina3	Fontsimavo Analamangahazo	22-25 Revaka FZ-36 Ambodiravina	Ampasina Fontsimavo
FZ-10 Mme Tsiavana3 FZ-44 Vary Komy FZ-45 Diara3	Ambodirafia Mahambo Ambonivato	22-24 Felambilona1 FZ-20 Stock FZ-38 Maroanaka	Antaranarina Ambodirafia Fontsimavo
FZ-34 Somotra FZ-52 Gonybe mena	Antaranarina Mahambo	22-11 Beforiaka2 FZ-32 Mahagaga mena	Antaranarina Fontsimavo
FZ-18 Sahamiketra FZ-47 Salaro	Antaranarina Ambonivato		

Table 2. Homonym varieties that show genetic difference

Variety	Sample #	Fokontany	Village
Mme Tsiavana	22-20 22-29 FZ-10	Antaranarina Analamangahazo Ambodirafia	*Antsoritsambo*
Bekasaka	22-2 22-21 FZ-2	Ambodirafia Antaranarina Ambodirafia	*Ambanitohaka* *Ambodirafia*
Beforiaka	22-11 FZ-46 FZ-48	Antaranarina Ambonivato Mahambo	*Antoritsambo*
Trepa fotsy Trepa fotsy hambo	22-1 22-10 FZ-29 22-5	Ambodirafia Antaranarina Analamangahazo Antaranarina	
Trepa mena Trepa mena hambo Trepa mena lalaina	22-6 22-8 FZ-42 22-30 22-9	Antaranarina Antaranarina Antaranarina Analamangahazo Antaranarina	*Antaranarina* *Antaranarina* *Antaranarina*
Patsa	22-23 FZ-28	Ampasina Analamangahazo	*Sahasina*
Felambilona	22-17 22-24 FZ-37	Ampasina Antaranarina Fontsimavo	*Sahasina* *Antsoritsambo*
Diara	22-13 FZ-26 FZ-45 FZ-55	Antaranarina Analamangahazo Ambonivato Ambonivato	*Antsoritsambo*
Kaominina	22-19 FZ-27	Antaranarina Analamangahazo	*Antsoritsambo*
Sahamonina	22-3 22-7 22-14 22-15 FZ-19 FZ-51	Ambodirafia Antaranarina Analamangahazo Ampasina Ambodirafia Ambonivato	*Ambanitohaka* *Antsoritsambo* *Analamangahazo* *Ambodibonara* *Ambodirafia*

All four samples of Diara are genetically distant from each other, including the two samples (FZ-45 and FZ-55) from the *fokontany* of Ambonivato.

The three samples of Mme Tsiavana ("madame does not have to weed") have a similar position on Axis 1, but vary on Axis 2. As noted above, Diara3 and Vary Komy are genetically close to Mme Tsiavana3, and these three varieties may be synonyms.

Bekasaka (an improved variety) and Beforiaka (a local variety) were recommended after multilocational trials for the Tamatave region in 1966 and are still cultivated at present. Bekasaka is especially appreciated by farmers for its productiveness and taste. The three Beforiaka samples are genetically very different; Bekasaka 22-21 and FZ-2 are relatively close on Axis 2, but differ on Axis 1 and both of these differ from 22-2.

Felambilo samples FZ-37 and 22-17 have a similar position in Axis 2, but differ on Axis 1; both of these are very dissimilar to Felambilo 22-24.

The two samples of Patsa are very different on both axes.

Trepa is a dwarf variety, likely a more recently introduced hybrid given a local name that has become popular for swamp/irrigated production. Farmers appear to use the name Trepa in a more general sense for any short-stem rice as shown by the widely scattered genetic distance between the red and white variants and their subtypes. Trepa comes in white (*fotsy*) and red grain (*mena*) varieties, color being obviously genetically determined. In some cases, farmers added the additional adjectives of *hambo* (slightly taller), *fohy* (short, dwarfish), which could be due to soil fertility differences and/or weather rather than genetics. The one instance of the addition of the adjective *lalaina* (to be held dear) points to its value as a productive variety and for household food security.

Kaominina is the improved variety X265, a high-yielding indica cultivar released in 1986; the variety was developed to be resilient to climate change, able to produce well across a range of rainfall, with a short growing cycle and weaker photoperiod sensitivity than traditional varieties [10]. In 2018, seed of X265 was donated to and distributed by the major of the commune of Ambodiriana after cyclone Ava made landfall near Toamasina and caused major damage in the city and the Ivoloina and Ifontsy valleys, causing many farmers to lose their rice crops (the cyclone struck land on January 5, 2018, a few weeks after the start of the main December to May/June growing season). Farmers renamed it Kaominina (=commune), a name easier to remember than X265. The two samples of Kaominina (F27 and 22-10) are, however, genetically dissimilar; the farmer from whom we collected 22-10 stated that he obtained the seed in the commune of Mangabe and it may, therefore, not be X265.

4. Conclusion

The term "variety" is legally defined in various national laws such as the American Plant Variety Protection Act (APVA - US Code Title 7 Chapter 57 Sub II.D §2401) [11], and international agreements such as the International Union for the Protection of New Varieties of Plants (UPOV). A variety must be (1) distinct, that is, easily distinguishable through certain characteristics from any other known variety (protected or otherwise); and (2) uniform and stable, meaning that individual plants of the new variety must show no more variation in the relevant characteristics than one would naturally expect to see, and that future generations of the variety through various propagation means must continue to show the relevant distinguishing characteristics. APVA (§2401.b(5)) states that "the distinctness of one variety from another may be based on one or more identifiable morphological, physiological, or other characteristics (ex., processing or product characteristics) with respect to which a difference in genealogy may contribute evidence."

This is largely similar to the way Malagasy farmers recognize rice varieties (perhaps more correctly termed landraces), but with names in most cases more directly describing those characteristics then the alpha numeric numbering used by breeders and seed companies in developed countries. For practical purposes, variety names do not have to be unique beyond the immediate area (village and *fokontany*), as seed is not traded on a commercial basis beyond that area. Our results indicate that within a *fokontany*, varieties do appear to be genetically distinct, but not across *fokontany* or commune. Although variety names may be similar, one cannot assume that they are genetically the same; variety names apply only locally, not regionally or nationally.

As a mostly self-pollinating crop, the part of the harvest set aside as seed for next-year's crop can, therefore, be considered "a unit with regard to the suitability of the plant grouping for being propagated unchanged (§2401.a(10))." Some cross-pollination may occur, especially since some upland fields may be planted with more than one variety, and individual parcels for swamp/irrigated rice in valleys shared by several households can each be planted with different varieties as well. Rice is sold unprocessed in rural markets (located in each rural commune), where other farmers may purchase it for consumption or for use as seed without knowing what variety it is. If, upon producing a crop from that seed, it is different from already grown varieties, it is sufficient to provide it a new name to differentiate it from existing varieties in their village or *fokontany*. The modern conception of a named variety having a unique genotype and phenotype that can be trademarked or patented, therefore, does not apply. As a result, this as well as other studies on rice varietal diversity in Madagascar, found a large number of homonyms and synonyms among the samples tested; however, homonyms may 'hide' true genetic diversity whereas synonyms decrease it. On a regional scale, using local variety names alone is, therefore, not sufficient to assess true genetic diversity. In Madagascar, where most farmers save a share of the harvest as seed, crop genetic diversity is best studied on finer scales (fokontany, village), as at this level farmers are able to distinguish varieties on phenotypic characteristics alone, avoiding homonyms and synonyms that complicate analysis.

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Supplemental Material

Sample codes, variety names and collection location

Upland/rainfed varieties in italics; varieties have red grain except when followed by [w] (=white grain)

Sample Code	Variety name	Fokontany	Village
FZ 01	*Behangaly malady*	Ambodirafia	
FZ 02	Bekasaka1	Ambodirafia	
FZ 03	Bekorotsaka	Ambodirafia	
FZ 04	*Bemahasoa be*	Ambodirafia	
FZ 06	*Betahavana*	Ambodirafia	
FZ 07	Botraka hambo	Ambodirafia	
FZ 08	Hasaka	Ambodirafia	
FZ 09	Lingoza Be	Ambodirafia	
FZ 10	Mme Tsiavana3	Ambodirafia	
FZ 11	*Mainty malady*	Ambodirafia	
FZ 12	Makalioka fotsy [w]	Antaranarina	
FZ 13	Mananara fotsy [w]	Antaranarina	
FZ 15	*Mandriravina*	Antaranarina	
FZ 16	*Mananelatra*	Antaranarina	
FZ 17	*Randrambolo*	Antaranarina	
FZ 18	*Sahamiketra*	Antaranarina	
FZ 19	*Sahamonina2*	Ambodirafia	
FZ 20	Stock	Ambodirafia	
FZ 21	*Telovolana*	Analamangahazo	
FZ 22	Tsy Afohy	Analamangahazo	
FZ 23	Zato Andro	Antaranarina	
FZ 24	Mme Rose	Analamangahazo	
FZ 25	203 [w]	Analamangahazo	
FZ 26	Diara4	Analamangahazo	
FZ 27	Kaominina1	Analamangahazo	
FZ 28	Patsa mena2	Analamangahazo	
FZ 29	Trepa fotsy2 fohy [w]	Analamangahazo	
FZ 30	Tsy Ambidy mamo	Analamangahazo	
FZ 31	Tsy Mambovia	Fontsimavo	
FZ 32	Mahagaga mena	Fontsimavo	
FZ 33	*Salazana*	Fontsimavo	
FZ 34	*Somotra*	Antaranarina	
FZ 36	Ambodiravina	Fontsimavo	
FZ 37	Felambilona2	Fontsimavo	
FZ 38	Maroanaka	Fontsimavo	
FZ 39	Varyvato mena	Fontsimavo	
FZ 40	Antidahy Mahiana	Mahambo	
FZ 42	Trepa mena	Antaranarina	
FZ 43	*Ambedimamo*	Ivoloina	
FZ 44	Vary Komy	Mahambo	
FZ 45	Diara3	Ambonivato	
FZ 46	Beforiaka1	Ambonivato	
FZ 47	*Salaro*	Mahambo	
FZ 48	Beforiaka3	Mahambo	
FZ 49	Manakitsaky	Ambonivato	
FZ 50	Wheichu 901 (hybrid) [w]	Ambonivato	
FZ 51	*Sahamonina6*	Ambonivato	
FZ 52	Gonibe mena	Mahambo	
FZ 53	Finengo	Mahambo	
FZ 54	Soarivo	Mahambo	
FZ 55	Diara2	Ambonivato	
22-01	Trepa fotsy3 [w]	Ambodirafia	*Ambodirafia*
22-02	Bekasaka2	Ambodirafia	*Ambanitohaka*
22-03	Sahamonina5	Ambodirafia	*Ambanitohaka*
22-04	Bemahasoa malady [w]	Ambodirafia	*Ambanitohaka*
22-05	Trepa fotsy hambo [w]	Antaranarina	*Antaranarina*
22-06	Trepa mena3	Antaranarina	*Antaranarina*
22-07	Sahamonina4	Antaranarina	*Antaranarina*
22-08	Trepa mena1	Antaranarina	*Antaranarina*
22-09	Trepa mena lalaina	Antaranarina	*Antaranarina*
22-10	Trepa fotsy1 [w]	Antaranarina	*Antsoritsambo*
22-11	Beforiaka2 [w]	Antaranarina	*Antsoritsambo*
22-12	unknown	Antaranarina	*Antsoritsambo*
22-13	Diara1	Antaranarina	*Antsoritsambo*
22-14	Sahamonina3	Analamangahazo	*Analamangahazo*
22-15	Sahamonina1	Ampasina	*Ambodibonara*
22-16	Sekaline	Antaranarina	*Antsoritsambo*
22-17	Felambilona3	Ampasina	*Sahasina*
22-19	Kaominina2	Antaranarina	*Antsoritsambo*
22-20	Mme Tsiavana2	Antaranarina	*Antsoritsambo*
22-21	Bekasaka3	Antaranarina	*Antsoritsambo*
22-22	Antetaimasana	Ampasina	*Sahasina*

22-23	Patsa mena1	Ampasina	*Sahasina*
22-24	Felambilona1	Antaranarina	*Antsoritsambo*
22-25	Revaka	Ampasina	*Ampasina*
22-28	Tsiamidy mamy	Analamangahazo	*Analamangahazo*
22-29	Mme Tsiavana1	Analamangahazo	*Analamangahazo*
22-30	Trepa mena hambo	Analamangahazo	*Analamangahazo*

Notes: *fotsy*=white; *mena*=red; *hambo*=slightly taller; *fohy*= dwarfish, short; *lalaina*=to be held dear/valuable.

Approximate GPS coordinates of the fokontany and villages within each fokontany from where samples were collected.

Fokontany	*Village*	coordinates
Ambodirafia		-17.928081 49.181598
	Ambodirafia	-17.868843 49.188684
Antaranarina	*Ambanitohaka*	-17.860782 49.223628
	Antaranarina	-17.854070 49.239250
Analamangahazo	*Antsoritsambo*	-17.880790 49.258752
		-17.878346 49.254936
Fontsimavo	*Ambalafary*	-17.943297 49.226881
Ampasina		-17.912110 49.338549
	Ampasina	-17.906949 49.321262
	Ambodibonara	-17.919555 49.323950
Ambonivato	*Sahasina*	-18.045722 49.358349
Mahambo		-17.493457 49.452700

Genotype accumulation curve

Thirteen markers were used to produce multi-locus genotypes, but only nine markers were required to separate individuals effectively.