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## Article

# Mitochondrial Genetic Diversity among Farmed Stocks of *Oreochromis* spp. (Perciformes, Cichlidae) in Madagascar

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**Abstract:** The fast development of aquaculture over the past decades has made it the main source of fish protein and led to its integration into the global food system. Mostly originating from inland production systems, aquaculture has emerged as strategy to decrease malnutrition in low-income countries. The Nile tilapia (*Oreochromis niloticus*) was introduced to Madagascar in the 1950s, and is now produced nationally at various scales. Aquaculture mostly relies on fry harvested from wild populations and grow-out in ponds for decades. It has recently been diversified by the introduction of several fast-growing strains. Little is known how local genetic diversity compares to recently introduced strains, although high and comparable levels of genetic diversity have previously been observed for both wild populations and local stocks. Our study compares DNA barcode genetic diversity among eight farms and several strains belonging to three species sampled. DNA-based lineage delimitation methods were applied and resulted in the detection of six well differentiated and highly divergent lineages. A comparison of DNA barcode records to sequences on the Barcode of Life Data System (BOLD) helped to trace the origin of several of them. Both haplotype and nucleotide diversity indices highlight high levels of mitochondrial genetic diversity, with several local strains displaying higher diversity than recently introduced strains. This allows for multiple options to maintain high levels of genetic diversity in broodstock and provides more options for selective breeding programs.

**Keywords:** aquaculture; DNA barcoding; domestication; exotic species; management; tilapia



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## 1. Introduction

In the light of a global biodiversity decline and the wide-spread depletion of wild fish stocks [1–3], aquaculture has become an increasingly important source of animal protein in tropical countries [4,5]. With a global production of more than 160 million tons of fish (in 2015) aquaculture has become an important component of the global food system [5,6], with Asia contributing nearly 90% of the freshwater production. Globally it is now producing nearly twice as much as fisheries [4], mostly through inland aquaculture. It has been frequently integrated in national strategies to prevent malnutrition, particularly in low-income, tropical countries [7].

Inland aquaculture mostly relies on three species or species groups: carp (*Cyprinus carpio*) which is the most farmed fish worldwide with 13 million tons produced in 2017, followed by tilapia (*Oreochromis* spp.) and catfishes, such as *Pangasius* spp., with 5 million tons each produced in 2017 [5]. Their success lies mostly in the fast growth rate of these species and

the availability of protocols for farming and captive breeding. As a consequence, they were introduced in numerous countries outside their native distribution ranges [8].

In Madagascar, freshwater fish aquaculture has a long tradition. The first introductions of exotic species for aquaculture trace back to 1857 (giant goramy-*Osphronemus goramy*), 1861 (goldfish-*Carassius auratus*), 1912 (carp-*Cyprinus carpio*) [9], and the 1950s (nile tilapia-*Oreochromis niloticus*) [10,11]. The successful introduction of these species led to the establishment of multiple populations in the wild, further used as source of young fry for farming [12]. Nile tilapia and carp have been used in semi-intensive productions in association with rice cultivation in paddy fields since the earliest development of aquaculture in Madagascar [13]. Adults and fry are traditionally caught in the wild and introduced to paddy fields when rice plants are sown [12]. Aquaculture in ponds follows similar practices leading to similar genetic diversity found between farmed and wild tilapia populations [14]. More recently, multiple strains of *O. niloticus* and other *Oreochromis* species were introduced to upscale tilapia aquaculture by producing fry from imported strains known to have desirable properties, such as fast growth rates [15]. Some of these strains, such as the Genetically Improved Farmed Tilapia (GIFT) strain, are the result of multiple crossings between several farmed strains and wild populations, as well as selective breeding [16,17]. As a result, strains of multiple origins are currently maintained in farmed stocks of tilapia in Madagascar, but their genetic variability is not known.

The present study aims to estimate the genetic diversity of currently farmed strains of *Oreochromis* in Madagascar, which now include local and recently imported strains of *O. niloticus*, such as GIFT and JICA, as well as *O. mossambicus* and *O. macrochir* [14]. We opted for the use of a standardized mitochondrial marker, a 652 bp fragment of the cytochrome oxidase I gene known as DNA barcode [18,19], for a variety of reasons: (1) it can be easily retrieved thanks to the availability of universal primers for fish [20]; (2) a large number of DNA barcode sequences are available for *Oreochromis* (>1200 public records for most species) in the Barcode of Life Data System, BOLD [21]; (3) the mitochondrial diversity of farmed tilapia Madagascar is largely unknown.

We generated DNA barcodes for currently farmed strains of tilapia, and applied DNA-based delimitation methods to detect mitochondrial lineages among them and to estimate their diversity. By using new DNA barcode records and published data from BOLD, potential evolutionary origins of revealed lineages are discussed.

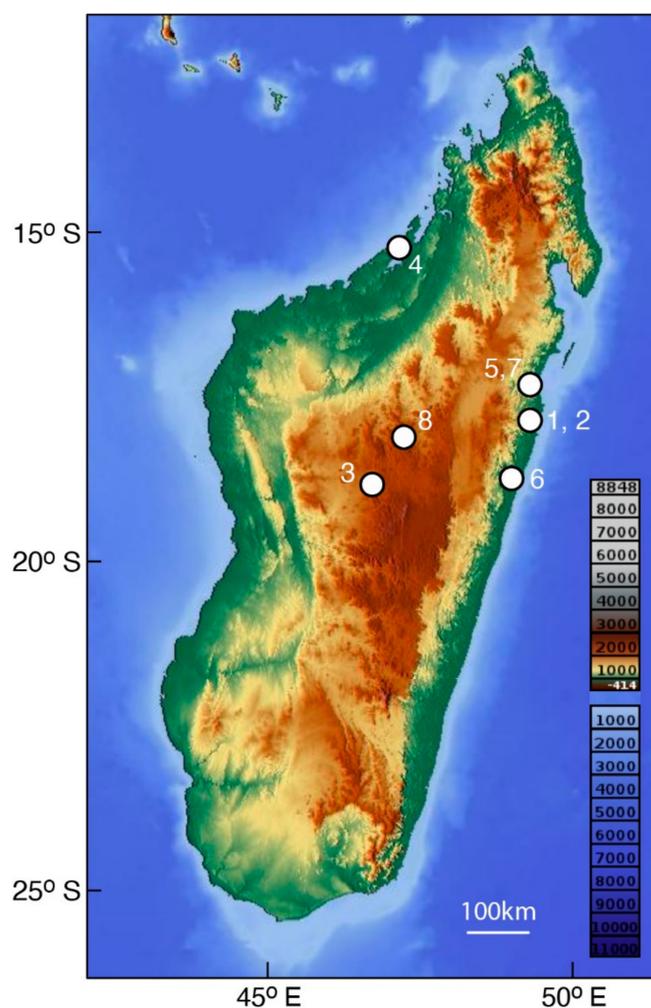
## 2. Materials and Methods

### 2.1. Sampling, Sequencing and International Repositories

In February 2016, a total of 262 specimens was collected at eight sites maintaining breeding stocks (Figure 1). Collected information, including geocoordinates, are included in the dataset DS-TILMADA ([dx.doi.org/10.5883/DS-TILMADA](https://dx.doi.org/10.5883/DS-TILMADA)) on BOLD. Specimens were identified to the species level based on the information of the farmers. Specimens were further photographed, individually labeled, and voucher specimens were preserved in a 5% formalin solution. A fin clip or a muscle biopsy were taken from each specimen and fixed in a 96% ethanol solution for further genetic analyses.

Genomic DNA was extracted from fin clip samples using a Qiagen DNeasy 96 tissue extraction kit following manufacturer's specifications. A 652-bp segment from the 5' region of the cytochrome oxidase I gene (COI) was amplified using the primer cocktail C\_FishF1t1/C\_FishR1t1 [20]. PCR amplifications were done on a Veriti 96-well Fast thermocycler (ABI-AppliedBiosystems) with a final volume of 10.0 µL containing 5.0 µL Buffer 2X, 3.3 µL ultrapure water, 1.0 µL each primer (10 µM), 0.2 µL enzyme Phire Hot Start II DNA polymerase (5 U), and 0.5 µL of DNA template (~50 ng). The following thermocycler regime was used: initial denaturation at 98 °C for 5 min followed by 30 cycles denaturation at 98 °C for 5 s, annealing at 56 °C for 20 s, and extension at 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min. PCR products were purified with ExoSap-IT (USB Corporation, Cleveland, OH, USA) and sequenced in both directions. Sequencing reactions were performed at the Centre for Biodiversity Genomics, University of Guelph, Canada,

using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit following standard protocols. Sequencing was performed on an ABI 3730xl capillary sequencer (Applied Biosystems). Sequences and collateral information were deposited on BOLD [21], and are available as a public dataset ([dx.doi.org/10.5883/DS-TILMADA](https://dx.doi.org/10.5883/DS-TILMADA), Table S1).



**Figure 1.** Sampling sites in Madagascar for the 262 DNA barcode records of *Oreochromis* spp. analyzed in this study. Site 1, *O. niloticus* GIFT strain at Matera (−18.0433, 49.3682); Site 2, *O. niloticus* GIFT strain at Tamatave (−18.449, 49.3987); Site 3, *O. niloticus* local strain at Ampefy (−19.0408, 46.7363); Site 4, *O. niloticus* JICA strain at Mahajunga (−15.6932, 46.3193); Site 5, *O. niloticus* local strain at Fenerive (−17.3666, 49.3961); Site 6, *O. niloticus* local strain at Brickaville (−18.8172, 49.0674); Site 7, *O. mossambicus* local strain at Fenerive (−17.3632, 49.3992); Site 8, *O. macrochir* local strain at Milaso (−18.2224, 47.1461).

## 2.2. Mitochondrial Lineage Delimitation and Genetic Diversity

Several methods for species delineation based on DNA sequences have been proposed [22–26]. Each of these have different properties, particularly when dealing with singletons (i.e., lineages represented by a single sequence) or heterogeneous speciation rates among lineages [27]. A combination of different approaches is increasingly used to overcome potential pitfalls arising from uneven sampling [28–32]. We used six different sequence-based methods of species delimitation to identify Molecular Operational Taxonomic Units (MOTU): (1) Refined Single Linkage (RESL) as implemented in BOLD and used to generate Barcode Index Numbers (BIN) [25]; (2) Automatic Barcode Gap Discovery (ABGD) [24]; (3) Poisson Tree Process (PTP) in its single (sPTP) and multiple rates version (mPTP) as implemented in the stand-alone software mptp\_0.2.3 [26,33]; (4) General Mixed

Yule-Coalescent (GMYC) in its single (sGMYC) and multiple threshold version (mGMYC) as implemented in the R package Splits 1.0–19 [34].

The mPTP algorithm and the GMYC both use phylogenetic trees as input file. We reconstructed a maximum likelihood (ML) tree for the former using RAxML [35] with a GTR + I +  $\Gamma$  substitution model. For the GMYC algorithm we calculated an ultrametric, fully resolved tree using the Bayesian approach implemented in BEAST 2.6.2 [36]. Sequences were collapsed into haplotypes prior to reconstructing the ultrametric tree using RAxML, and Bayesian reconstruction was based on a strict-clock prior of 1.2% per million year [37]. Two Markov chains of 20 million each were ran independently using Yule pure birth and GTR + I +  $\Gamma$  substitution models, other tree priors were used as default. Stability (ESS > 200) and convergence of Markov chains was verified using Tracer 1.7.1 [36]. Trees were sampled every 5000 states, after an initial burn in period of 5 million states. Both runs were combined with trees re-sampled every 20,000 states using LogCombiner 2.6.2, and the maximum credibility tree was constructed using TreeAnnotator 2.6.2 [36].

A final COI gene tree was reconstructed using the SpeciesTreeUCLN algorithm of the StarBEAST2 package [38]. This approach implements a mixed-model including a coalescent component within species and a diversification component between species that allows accounting for variations of substitution rates within and between species [39]. SpeciesTreeUCLN jointly reconstructs gene trees and species trees, and, as such, requires the designation of species, which were determined using the consensus of our species delimitation analyses. The SpeciesTreeUCLN analysis was performed with the same parameters as mentioned above.

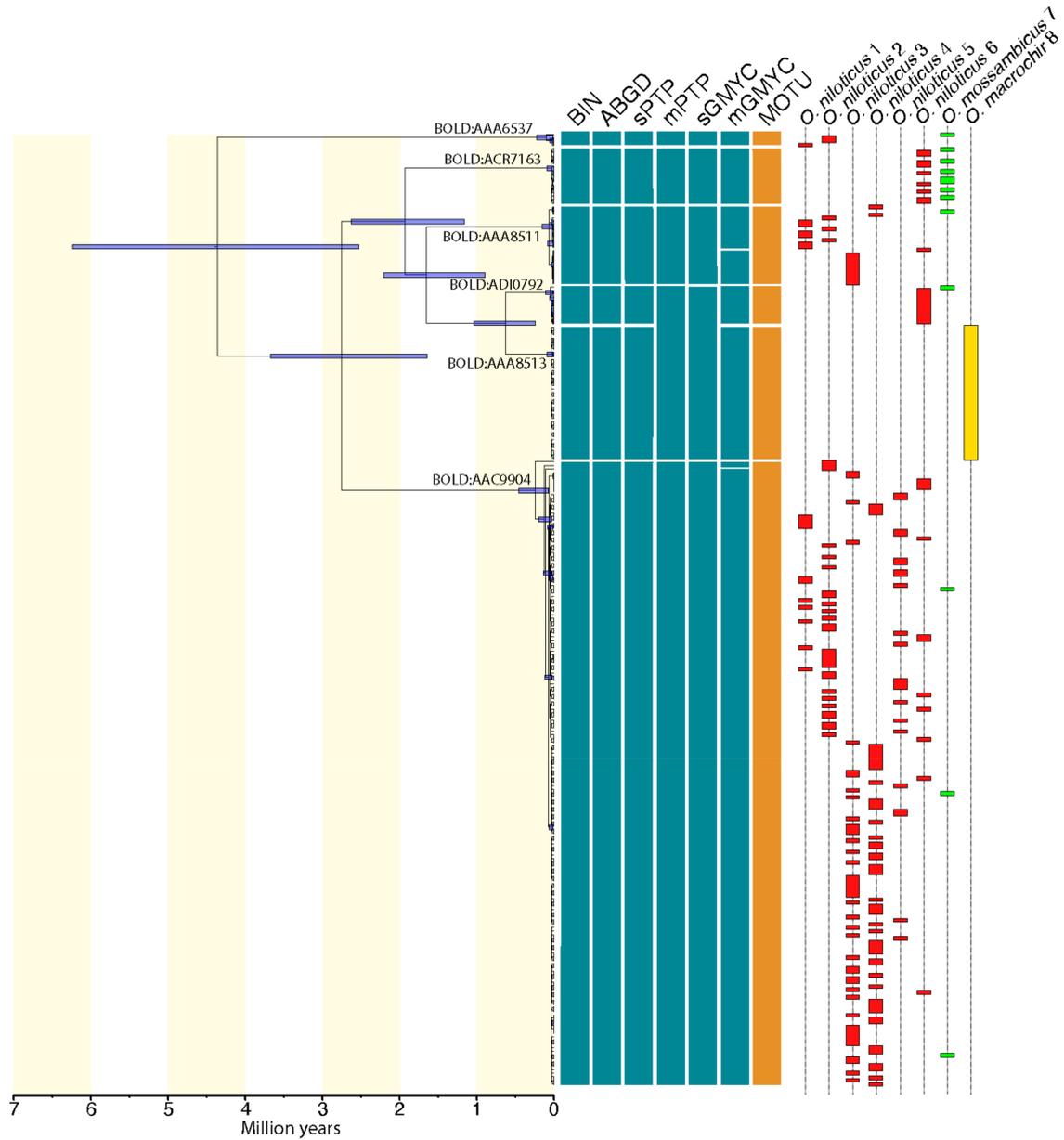
Several parameters of genetic diversity were estimated using the R package pegas 1.0 [40], including the number of haplotypes ( $h$ ), haplotype diversity ( $Hd$ ) [41], nucleotide diversity ( $\pi$ ) [42], genetic diversity based on the number of segregating sites ( $\theta$ ) [43], and Tajima's  $D$  test of neutrality [44]. Kimura 2-parameter (K2P) [45] pairwise genetic distances were calculated using the R package Ape 5.4 [46]. Maximum intraspecific and nearest neighbor genetic distances were calculated from the pairwise K2P distance matrix using the R package Spider 1.5 [47].

### 3. Results

A total of 263 COI sequences were generated from six sites for *O. niloticus*, and one site for *O. mossambicus* and *O. macrochir*. Sequences consisted of mostly full-length sequences (652 bp) and no stop codons were detected, suggesting that the sequences collected represent functional coding regions. DNA-based species delimitation methods resulted in congruent delimitation schemes with 6 MOTUs for ABGD, RESL, and sPTP, 5 MOTUs for mPTP and sGMYC, and 8 MOTUs for mGMYC (Figure 2, Table S1). The final consensus consisted of 6 MOTUs (Figure 2). Maximum distances within MOTU ranged from 0 (BOLD:AAA8513, BOLD:ACR7163, BOLD:ADI0792) to 0.007 (BOLD:AAC9904), and minimum distances between MOTUs ranged from 0.016 (BOLD:AAA8513, BOLD:ADI0792) to 0.065 for BOLD:AAA6537 (Table 1). K2P distances were 7-fold higher on average between than within MOTUs. None of the MOTUs were restricted to a single strain, excepting BOLD:AAA8513 restricted to *O. macrochir* (Figure 2). The five remaining MOTUs were shared between strains of *O. niloticus* and *O. mossambicus* (Figure 2, Table 1).

Shared MOTUs among species of *Oreochromis* were found on BOLD for several BINs (Table 1). Records associated to: (1) BOLD:AAA6537 members mostly belong to *O. niloticus*; (2) BOLD:AAA8511 members mostly belong to *O. niloticus* and *O. mossambicus*; (3) BOLD:AAA8513 members belong to *O. macrochir*; (4) BOLD:AAC9904 members mostly belong to *O. niloticus*; (5) BOLD:ACR7163 members mostly belong to *O. urolepis*; and (6) BOLD:ADI0792 members mostly belong to *O. mossambicus*. A substantial proportion of *Oreochromis*, BOLD records have not been identified to species particularly within the BINs BOLD:AAA6537 and BOLD:AAA8513, resulting in a low frequency of sequences named. Two discrepancies in the proportion of species per MOTU were detected between BOLD records and sequences generated for the present study. BOLD:ACR7163 mostly contains

records assigned to *O. urolepis* in BOLD although this species was never reported from Madagascar. BOLD:ADI0792, mostly represented by *O. niloticus*, is a new occurrence of this lineage for this species.



**Figure 2.** Mitochondrial gene tree for the 262 DNA barcodes of *Oreochromis* spp. inferred with SpeciesTreeUCLN, including 95% HPD interval for node age estimates, genetic lineage delimitation results for five methods (mGMYC discarded) and their 50% consensus, BOLD Barcode Index Numbers (BIN) for each MOTU, and distribution of farmed strain individuals.

**Table 1.** Summary of genetic distances and species composition of each MOTUs including their BOLD Barcode Index Number (BIN), number of individuals in present study, number of publicly available records in BOLD (not including newly generated records here), maximum intraspecific and minimum interspecific K2P genetic distances, and relative proportions of species within MOTUs according to the present study and in publicly available BOLD records.

BIN	N		K2P Distance		<i>O. niloticus</i> (Freq.)		<i>O. mossambicus</i> (Freq.)		<i>O. macrochir</i> (Freq.)		<i>O. aureus</i> (Freq.)		<i>O. urolepis</i> (Freq.)	
	Present Study	BOLD	Within (Max)	Between (Min)	Present Study	BOLD	Present Study	BOLD	Present Study	BOLD	Present Study	BOLD	Present Study	BOLD
BOLD:AAA6537	4	304	0.004	0.065	0.75	0.33	0.25	0.0001	-	-	-	0.3	-	-
BOLD:AAA8511	22	234	0.004	0.035	0.9995	0.14	0.0005	0.52	-	-	-	-	-	-
BOLD:AAA8513	37	18	0	0.016	-	-	-	-	1	0.17	-	-	-	-
BOLD:AAC9904	172	417	0.007	0.050	0.9998	0.73	0.0002	0.0001	-	-	-	-	-	-
BOLD:ACR7163	16	24	0	0.036	0.56	-	0.44	0.01	-	-	-	-	-	0.55
BOLD:ADI0792	11	28	0	0.016	0.9	-	0.1	0.29	-	-	-	-	-	-

As a result of the presence of multiple mitochondrial lineages in breeding stocks, which are estimated to have diverged between 0.6 and 2.7 million years ago (Figure 2), the nucleic diversity in most stocks is high, with  $\pi$  and  $\theta w$  above 0.015 and 8, respectively (Table 2). However, the JICA stock of *O. niloticus* (Site 4, Table 2) and *O. macrochir* at Milaso (Site 8, Table 2) display much lower nucleic diversity. The high diversity estimates of nucleic diversity are accompanied by high haplotype diversity at sites 1, 2, 3, 6, and 7, due to the occurrence of a high number of haplotypes, peaking at 8 in site 2 (Table 2). None of the Tajima's *D* tests were significant, indicating that diversity was mostly balanced for the eight breeding stocks, despite some negative values at site 2 (*O. niloticus* GIFT at Tamatave) and site 4 (*O. niloticus* JICA at Mahajunga) indicative of a deficit of rare haplotypes.

**Table 2.** Summary statistics of genetic diversity per MOTU. *N*, number of individuals; *h*, number of haplotypes; *Hd*, haplotypic diversity;  $\pi$ , nucleotide diversity;  $\theta w$ , theta; Tajima's *D* test including *D* value and significance of the test (\* significant at 0.025 threshold, none of the tests were significant).

Species	Strain	Locality	Site	<i>N</i>	<i>h</i>	<i>Hd</i>	$\pi$	$\theta w$	<i>D</i>	<i>p</i> -Value
<i>O. niloticus</i>	GIFT	Matera	1	16	3	0.575	0.031	18.082	0.434	0.664
<i>O. niloticus</i>	GIFT	Tamatave	2	31	8	0.539	0.018	15.519	−1.262	0.207
<i>O. niloticus</i>	Local	Ampefy	3	58	7	0.622	0.016	8.209	0.554	0.579
<i>O. niloticus</i>	JICA	Mahajunga	4	46	4	0.243	<0.001	0.455	−1.481	0.127
<i>O. niloticus</i>	Local	Fenerive	5	30	3	0.393	0.008	8.582	−1.719	0.086
<i>O. niloticus</i>	Local	Brickaville	6	31	6	0.785	0.037	14.268	2.086	0.037
<i>O. mossambicus</i>	Local	Fenerive	7	13	6	0.718	0.037	23.52	0.067	0.947
<i>O. macrochir</i>	Local	Milaso	8	37	2	0.054	<0.001	0.240	−1.131	0.258

#### 4. Discussion

The present study highlights high levels of mitochondrial genetic diversity, both at nucleotide and haplotype level, across most *Oreochromis* stocks assessed. Such high levels of genetic diversity originate from the co-occurrence of multiple, highly divergent MOTUs within most stocks, for both *O. niloticus* and *O. mossambicus*. However, high haplotype diversity also indicates the presence of substantial genetic diversity within each MOTU. These likely have multiple origins. In their native range distribution, *Oreochromis* species are known to easily hybridize after secondary contacts and can result in discordant evolutionary histories between genomes [48]. Multiple cases of introgression of both mitochondrial and nuclear genomes have been reported [49–53]. This likely accounts for the evolutionary success of the group. Hybrids with higher fitness than parental lineages, living outside of the ecological range of their parental species, have been detected in cichlids [54,55] and other perciformes [56]. In the context of fish farming, mitochondrial genomes resulting from ancient introgression events are likely introduced during the building of brood stock or through introductions into the wild. The occurrence of BOLD:ACR7163 and BOLD:AAA6537, mostly assigned to *O. urolepis* and *O. aureus* records on BOLD, respectively, seems to support the assumption that heterospecific mitochondrial genomes resulting from ancient introgression events were inadvertently introduced into Madagascar stocks of *O. niloticus*, as *O. urolepis* and *O. aureus* have never been reported as introduced species for Madagascar [57]. Alternatively, inadvertent and unrecorded introductions of *O. urolepis* and *O. aureus* individuals within imported batches of *O. niloticus* cannot be discarded, particularly if young, immature, and morphologically indistinguishable individuals were introduced.

The high mitochondrial diversity and multiple occurrences of MOTUs among species and strains might further reflect local breeding strategies and stock maintenance. Brood-stock are known to display similar levels of genetic diversity in comparison with wild populations, which is the result of introductions in the 1950s [14]. Our study suggests tilapia production in Madagascar relies mostly on natural populations, with fry being collected in rivers and grow-out occurrence in ponds. Alternatively, fry are produced in ponds and broodstock genetic diversity is maintained by the regular addition of wild

adults [14,58]. Relationships between broodstocks and wild populations depend on the scale of the fish farms. Oswald et al. [58] identified at least three type of fish farms in Madagascar: (1) small-scale, artisanal farms consisting of a single pond for fry production and grow-out with production mostly devoted to local consumption; (2) medium-scale farms where fry production and grow-out are managed in different ponds, and fry production is partly disseminated to small-scale farms for grow-out; and (3) large-scale farms devoted to fry production and distribution, constituting the main source of fry for aquaculture. Fry distribution can occur over large distances within Madagascar, and most small farms have no particular strategy to maintain strains. The similar mitochondrial composition of sampled stocks, and multiple occurrences of MOTUs in *O. niloticus* and *O. mossambicus* strains confirms that stocks used to be mixed in the past, and further contributed to increase strain mitochondrial genetic diversity. However, management of the most recently introduced strains seems to depart from this trend as the *O. macrochir* strain hosts a single and private MOTU. Observed haplotype and nucleotide diversity among imported (GIFT, JICA) and local tilapia strains are mostly similar, however, several local strains display higher mitochondrial genetic diversity. This trend suggests that the initial genetic pool of *O. niloticus* introduced in the 1950s was already diverse. Its successful introduction likely accounts for the persistence of high mitochondrial genetic diversity in farmed strains, due to their historical reliance on wild populations. Several studies have shown a relationship between individual genetic diversity and fitness in fishes [59–61]. As such, this high mitochondrial genetic diversity, also previously reported using nuclear markers [14], suggests local strains constitute good candidates for selective breeding, with genetically diverse wild populations available for maintaining high levels of genetic diversity.

## 5. Conclusions

The present study shows that tilapia stocks in Madagascar have genetically diverse mitochondrial genomes, likely resulting from an intricate history of ancient introgressions, introduction of genetically diverse populations in the 1950s, and local practices. The detection of higher mitochondrial genetic diversity in several local strains in comparison to introduced strains of *O. niloticus*, suggests local practices are beneficial in maintaining a high level of genetic diversity. In the context of fast development of modern aquaculture, this high genetic diversity, also previously observed for nuclear markers [14], certainly constitutes an asset. With genetically diverse wild populations, multiple options are available for maintaining high levels of genetic diversity in broodstock and several strategies are further available for selective breeding programs.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/d13070281/s1>. Table S1: Results of the genetic species delimitation, including the majority-rule consensus.

**Author Contributions:** Conceptualization, E.P., H.d.V. and N.H.; methodology, E.P., H.d.V. and N.H.; software, E.P., H.d.V. and N.H.; validation, J.-M.M., E.P., D.S., D.E.A.-M., H.d.V. and N.H.; formal analysis, N.H.; investigation, J.-M.M., E.P., D.S., D.E.A.-M., H.d.V. and N.H.; resources, E.P., D.E.A.-M., H.d.V., D.S. and N.H.; data curation, E.P. and N.H.; writing—original draft preparation, N.H., E.P., J.-M.M. and H.d.V.; writing—review and editing, D.E.A.-M. and D.S.; visualization, J.-M.M., E.P., D.S., D.E.A.-M., H.d.V. and N.H.; supervision, E.P. and N.H.; project administration, E.P.; funding acquisition, E.P., D.S. and N.H. All authors have read and agreed to the published version of the manuscript.

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