Identifying Nile tilapia strains and their hybrids farmed in Brazil using microsatellite markers

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Abstract – The objective of this work was to evaluate a Bayesian model-based clustering method to identify the strains of Nile tilapia (*Oreochromis niloticus*) individuals from fish farms in Southern Brazil. Assignment methods using nine microsatellite loci were applied to differentiate individuals of five reference strains (GIFT, GST, Nilótica, Chitralada, and Saint Peter) and to identify individuals of unknown strains from fish farms near the Itaipu reservoir and in the Uruguay River basin. The procedure assigned the correct strain in more than 90% of the cases and was also able to detect hybrids between strains. The obtained results showed that several fish farms in Southern Brazil cultivate more than one tilapia strain and even interstrain hybrids. The proposed methodology is a reliable tool for the identification of the strain origin of Nile tilapia individuals.

Index terms: *Oreochromis niloticus*, aquaculture, strain certification.

Identificação de linhagens de tilápia-do-nilo e seus híbridos cultivados no Brasil por meio de marcadores microssatélites

Resumo – O objetivo deste trabalho foi avaliar uma metodologia de agrupamento baseada em modelo Bayesiano para identificação de linhagens de indivíduos de tilápia-do-nilo (*Oreochromis niloticus*) de pisciculturas no Sul do Brasil. Métodos de alocação com nove loci de marcadores microssatélites foram aplicados para diferenciar indivíduos de cinco linhagens-referência (GIFT, GST, Nilótica, Chitralada e Saint Peter) e para identificar indivíduos de linhagens desconhecidas coletados em pisciculturas ao redor do reservatório de Itaipu e na bacia do rio Uruguai. O procedimento atribuiu a linhagem correta em mais de 90% dos casos e pôde, inclusive, detectar híbridos entre linhagens. Os resultados obtidos mostraram que várias pisciculturas no Sul do Brasil cultivam mais de uma linhagem de tilápia-do-nilo e até mesmo híbridos entre as linhagens. A metodologia proposta é uma ferramenta confiável para a identificação das linhagens de origem de indivíduos de tilápia-do-nilo.

Termos para indexação: *Oreochromis niloticus*, aquicultura, certificação de linhagens.

Introduction

Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), is one of the most popular fish in global aquaculture and, therefore, has been a target species for numerous genetic improvement programs (Eknath & Hulata, 2009; Rodriguez-Rodriguez et al., 2013). As such, several strains have been developed and improved along the years. Specifically in Brazil, some of these strains have been officially imported and produced since 1971 (Moreira et al., 2007; Massago et al., 2010). A few notable examples include: the Chitralada strain from Thailand, imported in 1996 from the Asian Institute of Technology (Khlong Luang, Pathum Thani, Thailand); the GenoMar Supreme Tilapia (GST) strain from Norway, imported in 2002 from GenoMar (Oslo, Norway); and the Genetically Improved Farmed Tilapia (GIFT) strain from Malaysia, imported in 2005 from WorldFish (Bayan Lepas, Penang, Malaysia) (Lupchinski Júnior et al., 2008; Massago et al., 2010; Rodriguez-Rodriguez et al., 2013; Dias et al., 2016).

The identification of Nile tilapia at species and subspecies levels has been traditionally based on distinctive recognition features, such as reproduction traits, feeding habits, developmental differences, structural characters, and biogeography (Melo et al., 2008). However, due to considerable intraspecific variations, small interstrain differences, and the presence of hybrids, these characteristics are ineffective to discriminate some of those groups (Melo et al., 2008). In addition, the development of many strains has led to the coexistence of different varieties and

levels of hybridization among stocks, which clearly affects the accuracy of their identification. This many strains have different zootechnical characteristics, and the farming of interstrain hybrids may result in decreased productivity in fish farms (Sukmanomon et al., 2012a). Therefore, the accurate determination of species, subspecies, strains, and hybrids of farmed tilapia should be a priority to increase the production efficiency and reliability of Nile tilapia aquaculture.

Molecular markers are widely used to identify diverse taxa, irrespectively of their life stage, allowing a limited amount of tissue sampling. Given these attributes, such markers might represent a suitable and reliable tool to overcome the problems inherent in determining the identity of cultured Nile tilapia strains. Previous attempts to discriminate these strains were based on random amplification of polymorphic DNA (RAPD) (Bardakci & Skibinski, 1994) and on 5S rDNA (Alves-Costa et al., 2006). However, the problems related with RAPD replicability (Freeland et al., 2011) and the uncontrolled introgression of genes in Nile tilapia (Sukmanomon et al., 2012b) have rendered these techniques less applicable than other modern multilocus molecular markers. For instance, Van Bers et al. (2012) described a pool of 384 single nucleotide polymorphisms (SNP) that could be used to differentiate individuals from different species and strains of Nile tilapia. Likewise, microsatellite markers can also be adopted for this purpose. The latter method was previously used to test genetic changes in strains of Nile tilapia (Sukmanomon et al., 2012a; Li et al., 2015) and to assess genetic introgression on feral populations of the species from Thailand (Sukmanomon et al., 2012b), Fiji (McKinna et al., 2010), and southern Africa (D'Amato et al., 2007).

Although the outcomes from the aforementioned studies indicate that this method is promising as a tool to identify the strain of origin of Nile tilapia individuals, there is no known study that performs such analysis.

The objective of this work was to evaluate a Bayesian model-based clustering method to identify the strains of Nile tilapia individuals from fish farms in South Brazil.

Materials and Methods

To create a baseline dataset of genotypes, a total of 99 samples were collected from reference strains of Nile tilapia obtained from five commercial hatcheries that maintain their own brood stock. These samples included: 20 specimens of the Saint Peter strain, from Piscicultura Dal Bosco (Toledo, PR, Brazil); 20 specimens of the Nilótica strain, from Aquacultura Tupi (Guaíra, PR, Brazil); 20 specimens of the Chitralada strain, from Aquabel Piscicultura (Rolândia, PR, Brazil); 20 specimens of the GST strain, also from Aquabel Piscicultura (Rolândia, PR, Brazil); and 19 specimens of the GIFT strain, from Universidade Estadual de Maringá (Maringá, PR, Brazil). It should be noted that the Chitralada, GST, and GIFT strains farmed by these commercial hatcheries are direct descendants of the original stocks from the Asian Institute of Technology, GenoMar, and WorldFish, respectively, whereas the reference specimens for the two remaining strains, Nilótica and Saint Peter, do not represent pure individuals. However, this apparent limitation should not hinder further analyses because the methodology employed in the present study for strain discrimination is based on the detection of shared genetic profiles of individuals, in spite of the amount of mixing with other strains, which can also be estimated with this protocol.

To test the effectiveness of the method, four individuals were collected from each of the eight fish farms near the Itaipu reservoir, in the state of Paraná, and from ten fish farms in the Uruguay river basin, in the state of Rio Grande do Sul (Table 1). Both reference individuals and with unknown strains were sampled in 2010.

Fin clips from each individual were removed, preserved in a saline EDTA-DMSO buffer (Seutin et al., 1991), and stored at -20°C. Total genomic DNA was extracted with the iPrep ChargeSwitch kit using an automated liquid-handling robot (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's specifications. All individuals were genotyped for nine microsatellite loci of different linkage groups, which are frequently used to access the genetic diversity of Nile tilapia species and strains, using the following fluorescent-labeled primers, with the GenBank accession number between parentheses: UNH104 (G12257.1), UNH118 (G12271.1), UNH146 (G12298.1), UNH160 (G12312.1), UNH169 (G12321.1), UNH178 (G12330.1), UNH208 (G12359.1), UNH211 (G12362.1), and UNH222 (G12373.1). Ten μL PCR reactions for each locus included the following final concentrations: 1.5 μmol L^{-1} fluorescent-labeled forward primer, 1.5 μmol L^{-1} unlabeled reverse primer,

0.2 mmol L^{-1} dNTP, 0.03 U *Taq* Platinum, 1x buffer, 1.5 mmol L^{-1} MgCl, and 0.5 ng µL^{-1} DNA template. PCR conditions for all loci included initial denaturation for 3 min at 94ºC, followed by 35 cycles of denaturation for 30 s at 95ºC, annealing temperature for 60 s at 48ºC (UNH146 at 56ºC), extension for 60 s at 70ºC, and final extension for 1 hour at 70ºC. Genotyping was performed on the ABI 3130 sequencer (Applied Biosystems, Thermo Fisher Scientific Waltham, MA, USA), and fragment analyses were conducted using the GeneMarker software, version 1.6 (SoftGenetics, State College, PA, USA).

The presence of null alleles and scoring errors was assessed using the Micro-Checker software, version 2.2.3 (van Oosterhout et al., 2004). Diversity, Hardy-Weinberg equilibrium, and F$_{ST}$ analyses were

implemented in the Arlequin software, version 3.5 (Excoffier & Lischer, 2010). In all tests in which multiple comparisons were made, the significance levels were corrected by Bonferroni's method.

Assignment of individuals of unknown strains to their putative source populations, as well as the evaluation of the accuracy of the method, was carried out using a Bayesian model-based clustering approach in the Structure software, version 2.3.1 (Pritchard et al., 2000). First, the genetic structure of the reference populations was assessed to determine the number of clusters (K) that best differentiated the strains, using no admixture model. Once the best number of clusters was identified for the reference strains, an assignment analysis was performed using a model without admixture, including the individuals of unknown strains sourced from fish farms. The calculations were performed for each K between 1 and 8, with 10 runs of 100,000 burn-in and 1,000,000 replicates per run. Individuals that presented 0.1< q <0.9 were considered hybrids (Vähä & Primmer, 2006).

Table 1. Geographical location of fish farms in the Uruguay River basin and near the Itaipu reservoir where individuals of unknown strains of Nile tilapia (*Oreochromis niloticus*) were sampled.

Region	Municipality	Geographical coordinates
	Campos Novos	27°33'51.72"S 51°18'41.68"W
	Alto Bela Vista	27°25'6.33"S 51°55'1.43"W
		27°28'14.03"S 51°57'27.05"W
Uruguay River basin	Campinas do Sul	27°42'42.32"S 52°36'20.34"W
		27°46'32.00"S 52°36'29.00"W
		27°46'56.39"S 52°36'18.90"W
	Ijuí	28°19'22.74"S 53°36'2.75"W
	Entre-Ijuís	28°22'41.82"S 54°15'12.32"W
	Frederico Westphalen	27°22'52.48"S 53°25'40.20"W
	Santa Terezinha de Itaipu	25°27'28,3"S 54°25'48,7"W
		25°26'20,8"S 54°25'42,2"W
Itaipu reservoir	Itaipulândia	25°07'16,9"S 54°16'59,6"W
		25°09'19,7"S 54°15'03,4"W
	Pato Bragado	24°36'44,6"S 54°13'10,5"W
		24°31'45,5"S 54°16'27,8"W
	Guaíra	24°05'48,2"S 54°13'55,9"W
		24°15'18,8"S 54°15'25,8"W

Results and Discussion

All nine loci evaluated in the present study presented a high number of alleles and levels of heterozygosity in the reference samples (Table 2), similar to those found by Rutten et al. (2004), Melo et al. (2006), Moreira et al. (2007), and Petersen et al. (2012). The presence of null alleles, scoring errors, linkage, and Hardy-Weinberg disequilibrium was not recurrent among stocks and among loci. Pairwise F$_{ST}$ revealed significant genetic differentiation among all reference strains (Table 3), presenting values that ranged from 0.056 (p=0.004), between Chitralada and GST strains, to 0.597 (p<0.001), between GST and Nilótica strains.

In the assignment test, the best estimate for K was 4. In this analysis, all strains were well differentiated from

Table 2. Number of individuals genotyped (N), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficient (Fis), and number of alleles of each loci.

Strain	N	Ho	He	Fis	UNH104	UNH118	UNH146	UNH160	UNH169	UNH178	UNH208	UNH211	UNH222	Mean
Nilótica	20	0.469	0.533	0.123	2*	5	4	1	3	5	5	6	4	4.2
Saint Peter	20	0.750	0.771	0.028	9	9	4	8	12	8	9	7	7	8.1
GIFT	19	0.731	0.702	-0.042	9	6	4	7	9	5	5	7	3	6.1
Chitralada	20	0.700	0.692	-0.010	7	8	3	5	10	8	8	10	5	7.1
GST	20	0.761	0.737	-0.033	11	8	2	8	8	9	8	10	3	7.4

*Significant Hardy-Weinberg disequilibrium after Bonferroni correction. UNH, primer.

each other, with the exception of GIFT and GST that were assigned to the same cluster, thereafter named GIFT-GST (Table 4). The inaccuracy of the proposed protocol to distinguish individuals of the GIFT and GST strains likely reflects their origins. The genetic improvement of both strains was based on the same genetic profile. GenoMar acquired all marketing rights of GIFT in 1999 and developed the GenoMar Supreme Tilapia strain (GST) from Generation 10 (Gupta & Acosta, 2004). However, it is possible that a greater number of loci may be more effective in differentiating these strains.

When only individuals of reference strains were included in the assignment test, the total percentage of correct assignment was 91%. Individuals of the Nilótica, Saint Peter, and GIFT strains were correctly assigned in 100% of the cases, whereas individuals from the Chitralada and GST strains were correctly assigned in 90 and 60% of them, respectively (Figure 1). The percentage of correct assignment increased to 94% of reference strain individuals and to 80% in the analysis of the GST strain in the analysis comprising individuals of unknow strains.

The inability to fully distinguish between individuals from the Chitralada and GST strains is not unprecedented. Sukmanomon et al. (2012a) found similar introgression between Chitralada and GIFT strains, when using 14 microsatellite loci and 28 individuals of the GIFT strain (reference population), 80 individuals of the Chitralada strain (reference population), and 50 individuals from each of the two Chitralada-derived populations. This recurrence suggests that this introgression is real, instead of a potential shortcoming of the method.

Despite the inability of the method to separate GIFT and GST strains, the present protocol showed high efficiency (91–94% correct assignments) in identifying the strains of Nile tilapia individuals. The addition to the analysis of individuals of unknown strains from fish farms near the Itaipu reservoir and in the Uruguay River basin improved the resolution of the assignment to the GST strain, that is, the percentage of correct assignment. This result indicates that using more individuals may increase the efficiency of the identifications. Therefore, the results of the present study suggest that at least 20 individuals from each strain should be used to create the baseline dataset, comprising the reference populations. However, a more robust framework is most likely to be reached using around 30 individuals from each known strain. This number is approximately the mean value of the sum of the number of individuals assigned within each strain, which was of 31.6 in the analysis that used the whole dataset (i.e., individuals from reference strains jointly with the assigned individuals from the fish farms) and that correctly determined the strain of 94% of the individuals of the reference populations.

High levels of assignment of the individuals from the reference strains were maintained only when the following loci were removed: UNH178 (92%); UNH104 and UNH178 (91%); and UNH104, UNH178, UNH 208, and UNH211 (91%). When only the UNH118, UNH146, UNH160, and UNH169 loci were analyzed, accuracy was reduced to 85%. In other words, although each reference strain was assigned correctly in the analyses with five to nine loci, analyses using a lower number of loci resulted in a less accurate assignment of individuals from the reference populations. Therefore, at least five loci should be used, in this case, UNH118, UNH146, UNH160, UNH169, and UNH222, but

Table 3. F_{ST} values (above diagonal) among five different strains of Nile tilapia (*Oreochromis niloticus*) and their respective p-values (below diagonal)[1].

Strain	Nilótica	Saint Peter	GIFT	Chitralada	GST
Nilótica		0.129	0.496	0.561	0.597
Saint Peter	0.001		0.248	0.316	0.354
GIFT	0.000	0.000		0.095	0.063
Chitralada	0.000	0.000	0.001		0.056
GST	0.000	0.000	0.002	0.004	

[1]Significant differences at 0.5% probability after Bonferroni correction.

Table 4. Proportion of the genotypes from each strain (lines) belonging to each of the four clusters (columns) in the analyses using only reference strains of Nile tilapia (*Oreochromis niloticus*) (before bar) and using both reference and unknown strains (after bar).

Strain/cluster	Nilótica	Saint Peter	GIFT-GST	Chitralada
Nilótica	0.999 / 0.999*	0.001 / 0.001	0.000 / 0.000	0.000 / 0.000
Saint Peter	0.004 / 0.005	0.996 / 0.995*	0.000 / 0.000	0.000 / 0.000
GIFT	0.000 / 0.000	0.000 / 0.000	1.000 / 1.000*	0.000 / 0.000
Chitralada	0.000 / 0.000	0.000 / 0.000	0.059 / 0.107	0.941 / 0.893*
GST	0.000 / 0.000	0.000 / 0.000	0.651 / 0.819*	0.349 / 0.181

*Highest proportion of membership of each strain.

using seven (plus UNH208 and UNH211) or eight (plus UNH104, UNH208, and UNH211) may be advantageous in order to improve the assignment accuracy of strains, probably even of those that were not examined in the present study.

The molecular protocol tested in the present study also has an outstanding potential to evaluate the degree of hybridization among strains of Nile tilapia. Therefore, it may be instrumental in assessing the management of captive systems. Interstrain hybrids were found in fish farms from both analyzed areas (Figure 2). However, despite the lower number of sampled fish farms and genotyped individuals, hybrids were more prevalent in the farms near the Itaipu reservoir. Of these fish farms, only one cultivated single-strain tilapia (GIFT-GST), while seven (88%) presented more than one strain, and two (25%) interstrain hybrids. In fish farms from the Uruguay River basin, only the farm fish in Entre-Ijuís cultivated hybrid specimens. All reference strains were detected in this region, with a prevalence of the Chitralada strain in 50% (n = 4) of the studied fish farms. Individuals from fish farms in Frederico Westphalen (Figure 2 H–J), assigned as Chitralada and GIFT strains, and from the reference strains of the database of the present study were obtained from the same suppliers – Aquabel GenoMar and Universidade
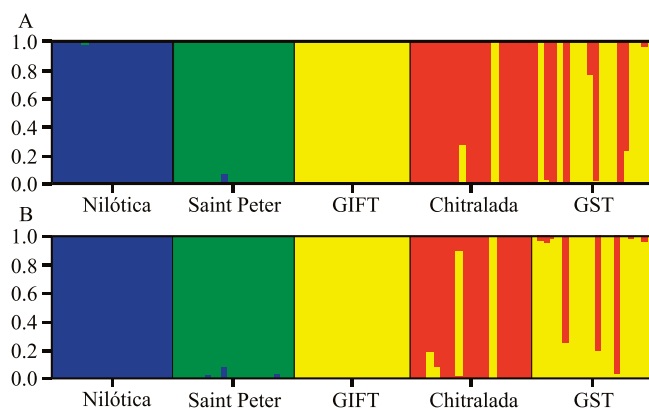
Estadual de Maringá, respectively –, and the analyses assigned them correctly.

The presence of multiple strains produced in the same fish farm, often in the same tank or pond, along with the detection of interstrain hybrids is a matter of concern. The different strains have distinct zootechnical characteristics, and the choice of an appropriate strain for a set of conditions could increase farming efficiency (Eknath & Hulata, 2009). It should be highlighted that the farming of interstrain hybrids is undesirable and could lead to a decrease in productivity (Sukmanomon et al., 2012a).

The identification of individuals of unknown strains is important to certify Nile tilapia larvae and fry. Oftentimes, producers purchase but do not receive fry from the expected strain of tilapia; this occurs due to lack of knowledge of the buyer but also of the producers of the larvae (Chammas, 2008). This method can help producers to ensure the identity of the strains that they are producing and trading, with the associated benefits of culturing a known strain. The choice of strains with specific best performance, the avoidance of crossbreeds among stocks, and strain certification of
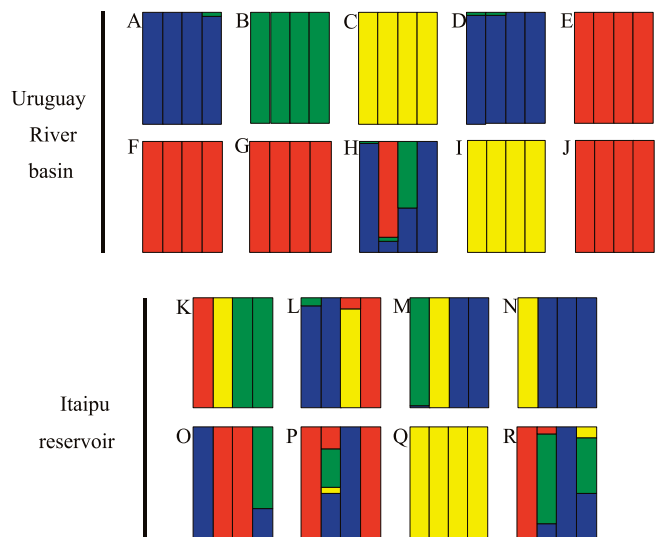

Figure 2. Relative assignment of Nile tilapia (*Oreochromis niloticus*) individuals derived from unknown strains sourced from ten fish farms in the Uruguay River basin (A–J) and from eight fish farms near the Itaipu reservoir (K–R). Single bars represent an individual. Colors represent the posterior probability that an individual belongs to each cluster: blue, Nilótica cluster; green, Saint Peter cluster; yellow, GIFT-GST cluster; and red, Chitralada cluster.

Figure 1. Relative assignment of Nile tilapia (*Oreochromis niloticus*) individuals in analyses using only reference strains (A) (K = 4) and using both reference and unknown strains (B) (K = 4). Single bars represent an individual. Colors represent the posterior probability that an individual belongs to each cluster: blue, Nilótica cluster; green, Saint Peter cluster; yellow, GIFT-GST cluster; and Red, Chitralada cluster. K, number of clusters.

fry are essential stages towards the professionalization of aquaculture in Brazil (Chammas, 2008).

Conclusion

The Bayesian model-based clustering method implemented with microsatellite data presents high accuracy and, therefore, can be used to distinguish among all tested strains from farmed and traded Nile tilapia (*Oreochromis niloticus*) in Brazil.

Acknowledgments

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