Biota Neotropica 22(1): e20211241, 2022
www.scielo.br/bn

biotaneotropica
ISSN 1676-0611 (online edition)

Short Communication

Molecular confirmation of twinning in the West Indian Manatee (*Trichechus manatus*)

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MOREIRA, S., MEIRELLES, A.C.O., CARVALHO, V.L., RÊGO, P.S., ARARIPE, J. **Molecular confirmation of twinning in the West Indian Manatee (*Trichechus manatus*).** Biota Neotropica 22(1): e20211241. https://doi.org/10.1590/1676-0611-BN-2021-1241

Abstract: Few twinning events have been recorded in the West Indian manatee (*Trichechus manatus*, Sirenia: Trichechidae) and no previous published study has provided confirmation of this phenomenon based in molecular tools. Here we investigate a possible case of twinning in an endangered Brazilian population of *T. manatus* using molecular tools. We analyzed two male neonates found stranded in Ceará State, on the northeastern coast of Brazil. The DNA of both individuals was isolated, and 10 microsatellite loci were amplified and genotyped. Following the identification of the alleles, the probabilities of identity by descent (Δ_7 and Δ_8) and relatedness (r_{xy}) were calculated using estimators that evaluate inbreeding. The two individuals shared most of the alleles, with differences in the genotypes being identified in only two loci. All the estimators identified a level of relatedness compatible with that found between siblings (selfed or outbred), indicating they were dizygotic twins. This is the first confirmed case of fraternal twins in free-ranging West Indian manatees in South America. The recognition of this type of twinning provides elements to improve actions for the rehabilitation of stranded animals and their subsequent release to the environment.
Keywords: *Sirenians; relatedness; microsatellite; stranding; dizygotic twins.*

Confirmação molecular de gemelaridade em peixes-boi marinho (*Trichechus manatus*)

Resumo: Poucos eventos de gemelaridade foram registrados para o peixe-boi marinho (*Trichechus manatus*, Sirenia: Trichechidae) e nenhum estudo previamente publicado confirmou esse fenômeno com base em ferramentas moleculares. Aqui investigamos um possível caso de gemelaridade em uma ameaçada população brasileira de *T. manatus* utilizando ferramentas moleculares. Foram analisados dois neonatos machos encontrados encalhados no Ceará, costa nordeste do Brasil. O DNA dos indivíduos foi isolado e 10 loci microsatélites foram amplificados e genotipados. Após a identificação dos alelos, as probabilidades de identidade por descendência (Δ_7 e Δ_8) e relação (r_{xy}) foram calculadas usando estimadores que avaliam endogamia. Os dois indivíduos partilharam a maioria dos alelos, com diferenças nos genótipos sendo identificadas em apenas dois loci. Todos os estimadores identificaram um nível de parentesco compatível com o encontrado entre irmãos (com e sem endogamia), o que aponta para o fato de serem gêmeos dizigóticos. Este é o primeiro caso confirmado de gêmeos fraternais em peixes-boi marinho de vida livre na América do Sul. O reconhecimento deste tipo de gemelaridade fornece elementos para aprimorar ações que visem a reabilitação de animais encalhados e sua posterior soltura ao ambiente.
Palavras-chave: *Sirênios; parentesco; microssatélites; encalhe; gêmeos dizigóticos.*

Introduction

The West Indian manatee (*Trichechus manatus* Linnaeus, 1758) is an aquatic mammal which is considered vulnerable to extinction due to the natural processes and anthropogenic impacts that have affected its populations, primarily over the past two centuries (Deutsch et al. 2008). The traditional classification of the species includes two subspecies, one of which is restricted to the Florida peninsula (*Trichechus manatus latirostris*), and the other to the Caribbean and South America (*Trichechus manatus manatus*). A recent study found this classification inadequate, pointing to the existence of two Evolutionary Significant Units - ESUs (Ryder 1986), one in the Atlantic (on the Brazilian coast), and the other in the Caribbean, between Venezuela and Florida (Lima et al. 2021). The Brazilian and Guiana populations should be of the highest priority for conservation due to its isolation and high level of differentiation (Garcia-Rodriguez et al. 1998, Vianna et al. 2006, Barros et al. 2017, Lima et al. 2021). Reproductive rates in *T. manatus* are low, with females reaching sexual maturity from around three years of age, and only begin breeding between five and eight years of age (Marmontel 1995, O`shea & Hartley 1995). Births are almost invariably of singletons, following a gestation of 12-14 months (Marmontel 1995, Rathbun et al. 1995). The females typically select a sheltered environment, such as estuaries, to give birth (Silva et al. 2016). However, the unregulated human occupation of these environments (Borges et al. 2007, Silva et al. 2016) has been the principal factor leading to the high rate of neonate strandings on the northeastern coast of Brazil (Meirelles 2008, Balensiefer et al. 2017).

Reports of twins in free-ranging West Indian manatees are scant, and monitoring data indicate that multiple births occur extremely infrequently (Marmontel 1995, Rathbun et al. 1995). Records of twinning events are often restricted to unpublished reports and graduate thesis, which limits the potential for a more systematic analysis of the phenomenon, since the data is of limited access. In the present study, we investigate a rare report of possible case of twinning in two neonate West Indian manatees found stranded in Ceará State, on the northeastern coast of Brazil, contributing to a broader understanding of this phenomenon and proposing the use of an applicable methodology for future similar reports.

Material and Methods

On October 15th 2014, two male West Indian manatees (Aquasis registers #02S0111/65 and #02S0111/66, here denominated Tma65 and Tma66, respectively) were found stranded on Agulhas beach (04º23’13.4’’ S, 37º49’50.0’’ W) in the municipality of Fortim, on the eastern coast of Ceará State. The two individuals were found 10 meters apart, and both had umbilical stumps and fetal folds, and were similar in size and weight (Tma65 = 1.16 m and 27.3 kg; Tma66 = 1.21 m and 30.1 kg), all indicatives that they were newborn. The animals were rescued by the Association for Research and the Preservation of Aquatic Ecosystems (Aquasis) and sent for rehabilitation in the Marine Mammal Rehabilitation Center (CRMM) in Caucaia, Ceará State.

During the rescue and handling of these animals, skin samples were obtained from each individual tail under local anesthetic (lidocaine 2%) using the protocol described by Bonde et al (2012). The samples were stored in ethanol 90% at -20 ºC at the Genetic and Conservation Laboratory, Federal University of Pará (UFPA). The total DNA was isolated using the Wizard Genomic DNA protocol (PROMEGA), with a final concentration of 712 ng/µl for Tma65 and 251 ng/µl for Tma66. Ten microsatellite loci extensively using with *Trichechus* (*TmaE08*, *TmaE11*, *TmaA09* - Garcia-Rodriguez et al. 2000, and *TmaE1, TmaSC5*, *TmaJ02*, *TmaKb60*, *TmaSC13, TmaE14*, *TmaE07* - Pause et al. 2007) were amplified by PCR. The amplified products were genotyped in an ABI 3500XL (Applied Biosystems) fragment analyzer and the alleles were identified using GeneMapper 4.1, with the default parameters and autobins, and inspected visually to confirm the reads.

To estimate the relatedness of the individuals, the genotypes of two other rescued manatees from the coast of Ceará State (#02S0112/69 and #02S0111/72) were incorporated into the database. These four samples were analyzed as part of a bank of 55 genotyped West Indian manatees (data not shown) to check for possible genotyping errors using the Micro-Checker software (Van Oosterhout et al. 2004), and none of the estimated errors were evident. Prior the analysis, a simulation was run in the Coancestry program (Wang 2011) to determine the best estimator of relatedness for the data. This simulation included two estimators of probability (TrioML and DyadML), which consider inbreeding, and two moment estimators (Wang and LynchLi), which are appropriate for samples of small size (Wang 2011). A total of 10,000 genotypes were simulated based on the observed allele frequencies, with 1,000 dyads (pairs of individuals) being analyzed for each of the five categories of relatedness (unrelated, half siblings, full siblings, parent-offspring, and monozygotic twins), as defined by Bonin et al. (2012). The probabilities of identity by descent (Δ_7 and Δ_8) and relatedness (r_{xy}) were calculated to estimate the degree of relatedness between the two manatee calves, with the values being compared with those of each category of relatedness defined by Wang (2011).

Results and Discussion

Alleles of all 10 microsatellites were identified in both individuals, which shared the same genotypes for almost all the loci, except for *TmaE14* and *TmaE08*, for which Tma65 was homozygous in both cases and Tma66 was heterozygous, also in both cases (Figure 1). The signal intensity for the *TmaE14* locus was 24.739 RFU for individual Tma65 (allele A), and 8.916 RFU and 6.996 RFU for individual Tma66 (alleles A and B, respectively). For the *TmaE08* locus, the intensities were 31.815 RFU for individual Tma65 (allele C), and 22.611 RFU and 15.705 RFU for individual Tma66 (alleles C and D respectively). The genotypes of these two markers were confirmed by repeating the genotyping, with a new amplified product (additional amplification by PCR), and the nucleotide sequencing of the alleles of the two samples.

The DyadML and TrioML probability estimator were the most adequate for the dataset, given that they returned the lowest variances (Table 1), although all four estimators provided highly similar values for the three parameters analyzed, with values of Δ_7 and r_{xy} of around 0.5, and those of Δ_8 equal to zero. The comparison of these values with the reference values defined by Wang (2011) allows us to reject the hypotheses that the two animals are monozygotic twins (r_{xy}=1.0) or unrelated (r_{xy}=0). The most adequate category for the observed r_{xy} value was sibs, with and without inbreeding (selfed outbred sibs and full-sibs, respectively), or between parent-offspring. The latter relationship was discarded, given that both individuals were newborn. The Δ_7 and Δ_8 values indicate the occurrence of inbreeding in the population. It is important to note here that the relatedness probability values for dizygotic twins are the same as those for full siblings, given that the latter also originate from distinct gametes produced by the same parents.

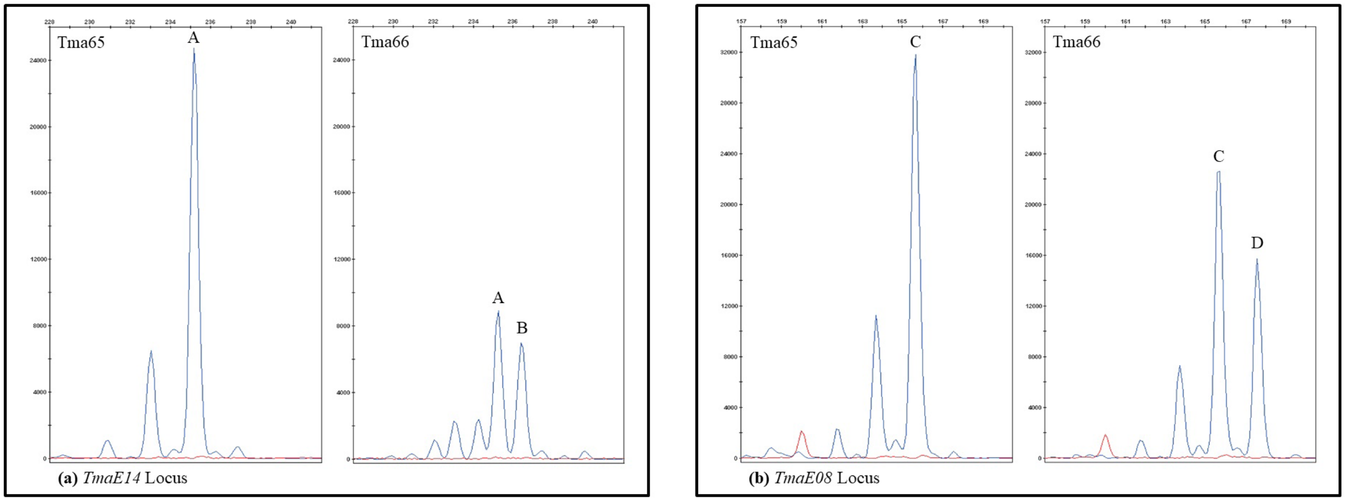

Figure 1. Electropherograms showing the alleles (A, B, C and D) of the loci *TmaE14* (box a) and *TmaE08* (box b). The x-axis indicates the position while the y-axis shows the signal intensity of the identified alleles.

Table 1. Variance recorded in the simulation of 1.000 dyads for each of the five categories of relatedness, and the probabilities of identity by descent (Δ_7 and Δ_8) and relatedness (r_{xy}) between Tma65 and Tma66 recorded by each estimator. *The LynchLi estimator does not provide probabilities of identity by descent (Δ_7 and Δ_8).

Estimator	Variance	Δ_7	Δ_8	r_{xy}
DyadML	0.11441	0.5260	0.0000	0.4332
TrioML	0.11474	0.5393	0.0000	0.5393
LynchLi	0.21732	*	*	0.6854
Wang	0.21858	0.4895	0.0000	0.7160

Overall, then, these findings, together with the morphometric data and the estimated ages of the individuals at the timing of their stranding, indicate that the two manatee calves are dizygotic (fraternal) twins.

This is the first published report of the use of molecular tools to confirm twinning in the West Indian manatee as well as to determine the type of twins observed. In addition to the fact that multiple births are extremely rare in this species (Rathbun et al. 1995), the simultaneous stranding of two infants is an even rarer event, which reinforces the exceptional nature of the record presented here. Data on possible twinning events in *T. manatus* are very scant in general, and many cases may only be available in unpublished reports, which limits attempts to comprehend the broader scenario. Example of this are the theses of Bonde (2009) and Luna (2013), which presented evidence of twinning events in free-living manatees in Florida and in a rehabilitation center in northeastern Brazil, respectively. While the relatedness of the twins was confirmed using molecular tools (microsatellites, in both cases), these data have yet to be published. Other evidence confirming twin gestations and multiple births based on carcass analyses were published in Florida more than two decades ago (Marmontel 1995, Rathbun et al. 1995).

The confirmation of twinning in free-ranging aquatic mammals based on monitoring and necropsies, while relatively frequent, is nevertheless occasional and often imprecise (IJsseldijk et al. 2014, Davison et al. 2016). Estimates of twinning rates in sirenians are invariably low, with 1.4-4.0% of the pregnant female *T. m. latirostris* carcasses necropsied in Florida bearing twins (Marmontel 1995, Rathbun et al. 1995). In the dugong (*Dugong dugong*), the only record is of a free-ranging female being accompanied by two calves (Lanyon et al. 2009), although it was not known whether they were in fact siblings. Any estimates based on observations are obviously vulnerable to behavioral phenomena, such as adoptions (involving abandoned or orphaned infants), as well as stochastic events, such as the death of one of the fetuses or neonates (Meirelles et al. 2016). In the Antarctic fur seal (*Arctocephalus gazella*), for example, the molecular analysis of supposed twins confirmed cases of fraternal twinning, but also revealed adoptions and even heteropaternity (Bonin et al. 2012, Hoffman & Forcada 2009). Adoption cases have already been recorded in captive West Indian manatees in Brazil (Luna & Passavante 2010). This reinforces the need for molecular analyses, like that presented here, associated with long-term monitoring, to confirm the occurrence of twinning events and provide estimates of their frequency, which will be important for the better understanding of the social structure and behavior of the species.

The present study has also validated the viability of the use of genetic tools of biparental inheritance and manatee rescue data to confirm the occurrence of twinning in these mammals, as well as identifying the type of twins. The application of this molecular approach at the population level makes it possible to clarify the relatedness between individuals and also allows the quantification of inbreeding rates. These findings will provide valuable insights into the reproductive and demographic parameters of the endangered Brazilian population of West Indian manatees, which is genetically (Lima et al. 2021), cytogenetically, and morphologically (Barros et al. 2017) distinct from the other populations, which will be important for the development of adequate management strategies. The reliable determination of the relatedness of manatees in rehabilitation will be essential for the adequate planning of ongoing release strategies on the northeastern coast of Brazil (Luna et al. 2012). As the stranding of neonates is one of the principal threats to the manatee population of the Brazilian coast (Meirelles 2008, Balensiefer et al. 2017), the recognition of twins in rescued individuals may also contribute to a better understanding of the factors driving calf strandings process, such as the inexperience or incapacity of the mother during the puerperium, especially following multiple births.

Acknowledgments

This study was supported by Petrobras through the Manati Project - Petrobras Socioambiental. The authors are grateful to the Brazilian National Council for Scientific and Technological Development (CNPq: JA – processes 312404/2019-0 and 439040/2018-3, and PSR – process 311539/2019-0), and the Coordination for Higher Education Personnel Training (CAPES: PSR – process 88881.337398/2019-01). We also would like to thank the anonymous reviewers for their helpful input and Dr. Stephen Ferrari and Dr. Gabriel Melo-Santos for their careful review of this manuscript.

Associate Editor

Diego Astúa

Author Contributions

Sávia Moreira: Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content.

Ana Carolina O. de Meirelles: Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to critical revision, adding intellectual content

Vitor Luz Carvalho: Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to critical revision, adding intellectual content

Péricles Sena do Rêgo: Substantial contribution in the concept and design of the study; Contribution to data analysis and interpretation; Contribution to critical revision, adding intellectual content

Juliana Araripe: Substantial contribution in the concept and design of the study, Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content

Conflicts of Interest

The authors declares that they have no conflict of interest related to the publication of this manuscript.

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Received: 25/05/2021
Accepted: 03/02/2022
Published online: 11/03/2022