

1 **DNA-validated parthenogenesis: first case in a captive female Cuban boa**

2 **(*Chilabothrus angulifer*)**

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15 **Short title: Parthenogenesis in a captive female Cuban boa**

16 **Abstract**

17 Parthenogenesis is a biological process of asexual reproduction. Recent studies have

18 highlighted the significance of this fascinating phenomenon in the vertebrate evolution.

19 Although parthenogenetic reproduction appears to be widespread among reptiles, a

20 restricted number of cases were reported in captivity and wild. Here, we studied and

21 reported an intriguing case of a 20-year old captive female Cuban boa (*Chilabothrus*

22 *angulifer*), from the Zoo da Maia (Maia, Portugal) collection, isolated from conspecifics

23 males, that gave birth twice in 4 years. The neonates from both deliveries, one fresh and
24 the other fixed in formalin, were submitted to histopathological and molecular genetic
25 analysis. Both neonates were homozygous for the loci analyzed, carrying only mother
26 alleles. Furthermore, morphological abnormalities (anophthalmia) were observed in the
27 second neonate. Our data support a pattern of parthenogenetic reproduction. This is the
28 first documented case of facultative parthenogenesis in a Cuban boa, which can be of
29 great interest for further research on ecology, evolution, captive breeding and
30 conservation of the species.

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32 Key words: parthenogenesis, snake, DNA, reptile, *Chilabothrus angulifer*

33

34 **Introduction**

35 Parthenogenesis is a natural form of asexual reproduction in which offspring is
36 produced from unfertilized eggs [1,2]. This uncommon reproductive strategy was
37 reported in less than 0.1% of vertebrate species, including a wide range of taxa (i.e.
38 fishes, amphibians, reptiles, birds and mammals), even in wild populations [1-5].
39 Parthenogenetic reproductive events have caught the attention of evolutionary and
40 conservation biologists, since the absence of genetic recombination accelerate the
41 accumulation of deleterious mutations in parthenogenetic individuals, which have
42 considerable implications for the management and conservation of the species [6-8].

43 Obligate parthenogenesis is a biological process where all individuals within a
44 species reproduce asexually [2,9]. This reproductive strategy is restricted to Squamate
45 reptiles, being reported in various lizards' species and one snake (*Indotyphlops*
46 *braminus*) [9-11]. The occasional occurrence of parthenogenesis in individuals of a

47 species that normally reproduce sexually (i.e. facultative parthenogenesis - FP) was first
48 mentioned in the late 1800s for birds [12]. FP have been reported in various species of
49 major vertebrate groups including reptiles, birds and elasmobranchs (sharks and rays)
50 [4,9,13,14]. Most FP events were documented from captive females after long periods
51 without contact to male conspecifics during their reproductive lifetime [8,9]. However,
52 parthenogenesis has more recently been reported in wild snake populations [3,15] and
53 females housed with males [16], suggesting that its occurrence may be more frequent
54 than previously thought in vertebrates. In addition, the reproductive viability of
55 parthenogenetic offspring was observed in some species, which highlights the
56 ecological and evolutionary significance of this reproductive strategy [15]. Nevertheless,
57 the biological basis and mechanisms underlying parthenogenesis remain mostly
58 unknown [4,9,17].

59 FP was described in at least six snake families, namely Boidae, Pythonidae,
60 Viperidae, Acrochordidae, Colubridae and Elapidae [5,9,17]. Among boid snakes
61 (Boidae family), parthenogenesis was recorded and validated by genetic analysis in *Boa*
62 *constrictor* and *Eunectes murinus*, as well as, in two species of the genus *Epicrates*
63 (*Epicrates maurus* and *Epicrates cenchria*) closely related to the genus *Chilabothrus*
64 [17-20]. FP confirmed by genetic analysis was also reported for Pythonidae (*Python*
65 *bivittatus*, *Python regius* and *Malayopython reticulatus*) [16,21], Viperidae
66 (*Agkistrodon contortrix*) [15,22], Colubridae (*Thamnophis marcianus* and *Thamnophis*
67 *couchii*) [23,24] and Elapidae (*Oxyuranus scutellatus* and *Acanthophis antarcticus*) [5].
68 The accurate identification and characterization of parthenogenesis in captive
69 individuals of non-model species may provide important data to understand the
70 frequency, causes, consequences and biological mechanisms of asexual reproduction
71 among vertebrates [1,25].

72 Two interesting reproductive events were recorded for a captive female Cuban
73 boa (*Chilabothrus angulifer*) isolated from males for eleven years. These occurrences
74 could be explained by two hypotheses: (i) long-term sperm storage from the last mating
75 or (ii) parthenogenetic reproduction. Here, we applied molecular and histopathological
76 methodologies to evaluate these hypotheses, providing the first evidences of facultative
77 parthenogenesis in a Cuban boa.

78

79 **Material and methods**

80 **Specimen history and sampling**

81 On 20 September 2017 a 20-year old captive female Cuban boa (*Chilabothrus angulifer*
82 or *Epicrates angulifer*) from the Zoo da Maia (Maia, Portugal) collection gave birth to a
83 stillborn and multiple non-embryonated eggs. This female, purchased to the zoological
84 collection on 1999, had no contact with a male since 2006, when the conspecific male
85 died. Previously, in 2013, this same female delivered a yellowish mass of non-
86 developing eggs and a dead neonate that has been preserved on 10% buffered formalin.
87 The offspring from these two deliveries, one fresh and another formalin fixed, were
88 analysed in the Histology and Anatomical Pathology Laboratory of Trás-os-Montes e
89 Alto Douro University (UTAD). Tissues samples were processed for histopathology
90 according routine technique for light microscopy and staining with haematoxylin and
91 eosin (HE).

92

93 **DNA extraction and microsatellite genotyping**

94 The DNA isolation from the formalin-fixed specimen (neonate 2013) was carried out
95 using the Quick-DNA Miniprep Plus Kit (Zymo Research) according to manufacturer's

96 protocol, with some additional steps before sample digestion. Briefly, a mixture of
97 different tissues (liver, lung, gut and skin) was sliced into small pieces with a scalpel.
98 Then, the tissues were washed with PBS during 24 h (the buffer was replaced twice).
99 The DNA extraction from muscle tissues of the neonate borne at 2017 was performed
100 using the NZY Tissue gDNA Isolation kit (Nzytech). The mother's DNA was isolated
101 from blood using the NZY Blood gDNA Isolation kit (Nzytech). Both extractions were
102 performed following the standard protocols recommended by the manufacturer.
103 Thirteen microsatellite markers previously characterized for bovid species were analysed:
104 μ sat 1, μ sat 10, μ sat 13, μ sat 24, μ sat 32, μ sat 36, Ci25, Ci34, Ci35, Ci36, Ci37,
105 55HDZ554 and 55HDZ617 [19,26-28]. The pre-screening of microsatellite variations
106 among mother and offspring samples were performed using high-resolution melting
107 (HRM) analysis [29,30]. PCR amplification and melting acquisition were carried out
108 using a QuantStudio 3 Real-Time PCR System (Applied Biosystems). The reaction
109 mixture was prepared in a 20 μ l final volume containing 10 μ l of MeltDoctor HRM
110 Master Mix (Applied Biosystems), 5 pmol of each primer and 5 ng of genomic DNA.
111 All PCR reactions were performed in duplicate.
112 The amplification protocol was run as follows: 1 cycle of 95 °C for 10 min; 40 cycles of
113 95 °C for 15 s, 60 °C for 1 min (fluorescence signal was captured at the end of each
114 cycle); 1 cycle of 95 °C for 15 s, 60 °C for 1 min and then sequential temperature
115 increments of 0.025 °C/s with temperature ranging from 60 °C to 95 °C, with continuous
116 fluorescence measurements. The melting curve data were analysed with the
117 QuantStudio Design & Analysis software v.1.4 (Applied Biosystems) and High
118 Resolution Melting (HRM) Software v.3.0.1 (Thermo Fisher Scientific), assessing
119 differences in melting curve shapes to characterize microsatellite allelic variability.
120 Forward primers of the microsatellites with variations among samples were labelled

121 with 6-FAM to determine the genotypes using capillary electrophoresis. PCR
122 amplifications were performed in a total volume of 20 μ l containing 10 μ l of 2x MyTaq
123 HS Mix (Bioline), 5 pmol of each primer and 5 ng DNA. PCR thermal conditions were
124 as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30
125 s, 60 °C for 1 min, 72 °C for 30 s and a final extension at 60 °C for 10 min. Amplified
126 fragments were electrophoresed on an ABI PRISM 3130xl Genetic Analyzer (Applied
127 Biosystems) using the GeneScan 500 LIZ size standard. Allele sizes were determined
128 using Peak Scanner v.3.0.2 (Thermo Fisher Cloud).

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132 **Results**

133 Macro and microscopically, both reptiles correspond to fully develop stillborn snakes
134 that showed no morphological alterations except in the 2017 specimen (#2) that present
135 bilateral anophthalmia (Fig 1). Microscopic examination of organs showed no
136 alterations and the presence of the reproductive system confirmed both to be female.

137 Of the 13 microsatellite loci screened, two markers (μ sat 13 and Ci34) did not
138 amplify or generated non-specific PCR products. The high-resolution melting (HRM)
139 analysis allowed the identification of four microsatellite loci (μ sat 10, μ sat 24, Ci36 and
140 Ci37) with allelic variability among mother and offspring samples (Fig 2). No evidence
141 of allelic variability was detected in the remaining loci for the samples analysed (Fig 2).
142 These results were validated using capillary electrophoresis to determine allele sizes for
143 all polymorphic markers and two non-polymorphic loci (Table 1). Maternal
144 heterozygosity was observed for polymorphic loci and a homozygosity pattern was

145 obtained for the non-polymorphic loci analysed (Table 1). The offspring was
146 homozygous for all microsatellite loci, always carrying an allele present in the mother
147 (Table 1). The locus Ci37 is a potential null allele in the neonate of 2013, since the
148 amplification failed using different DNA samples and PCR conditions.

149

150

151 **Figure captions**

152 **Fig 1.** Neonates of Cuban boa (*Chilabothrus angulifer*): (a) Neonate of 2013 with a
153 normal head; (b) Neonate of 2017 with bilateral anophthalmia.

154

155 **Fig 2.** Melting curve profiles obtained in the pre-screening of the microsatellite loci
156 using HRM analysis. The fluorescence differences in four loci (μ sat 10, μ sat 24, Ci36
157 and Ci37) allowed the accurate differentiation of the mother and offspring genotypes
158 (red and blue curves). No significant fluorescence variations were detected in loci with
159 same genotype in mother and offspring (Ci35 and 55HDZ617).

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163 **Table 1. Genotypes of the mother and offspring characterized for the potentially**
164 **parthenogenic Cuban boa (*Chilabothrus angulifer*)**

Individual	μ sat 10	μ sat 24	Ci35	Ci36	Ci37	55HDZ617
Mother	401/409	215/217	318/318	220/228	278/298	185/185
Neonate 2013	409/409	217/217	318/318	220/220	-	185/185
Neonate 2017	409/409	215/215	318/318	228/228	298/298	185/185

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168 **Discussion**

169 Our results support the first evidence of FP in the Cuban boa (*Chilabothrus angulifer*).

170 The allele homozygosity in the offspring, which carries only alleles present in the

171 mother, are similar to previous cases described of FP reported for boid species [17-20].

172 In Boidae family (for genera Boa and Epicrates), the parthenogenesis was initially

173 detected in all-female litters [18,19]. Similarly, the two stillborn analysed in this study

174 were both females. Accidental FP in captive individuals generally occurs after long

175 periods of isolation from mates [9]. The female in this study had no contact with a

176 conspecific male for 13 years now. Prolonged sperm storage has been documented in

177 various snake species, but the longest time period of suspected sperm storage reported

178 for a snake was seven years and six months [31-33]. In this case the molecular analysis

179 demonstrated the lack of male genetic contribution to the offspring excluding prolonged

180 sperm storage. The high levels of homozygosity detected in the offspring is a

181 characteristic of the parthenogenetic mode explained by terminal fusion automixis

182 [18,19] as recently inferred for long-term captive copperhead (*Agkistrodon contortrix*)

183 and cottonmouth (*Agkistrodon piscivorus*) [5,17].

184 The genome wide homozygosity of parthenogenetic offspring may be related to

185 the development of malformations [3]. Embryos and stillborn offspring with

186 developmental abnormalities (e.g. anophthalmia, microphthalmia, encephalocoele and

187 head foreshortening) has been associated with parthenogenetic events in reptiles

188 [5,9,34]. The morphological evaluation of the Cuban boa neonate born in 2017

189 evidenced bilateral anophthalmia, a malformation found in parthenogenetic offspring of

190 other reptile species [34].

191 In conclusion, we characterized the first record of FP in the Cuban boa
192 supported by specimen history, histological analysis and molecular markers. This may
193 have important ecological and evolutionary implications, being interesting to
194 understand the frequency of this reproductive strategy in captivity, and maybe in the
195 wild, as recorded for some species [3,15,35]. The increasing number of reports on
196 parthenogenic births in a wide range of snakes and other vertebrates also evidences the
197 evolutionary significance of this reproductive phenomena still poorly understood, being
198 a research field with high potential [8,36]. Therefore, we incentive zoo workers,
199 veterinarians, curators, wildlife managers and researchers to pay attention to evidences
200 of abnormal births in this species and related taxa, since these events can be easily
201 neglected.

202

203

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208

209 **Conflict of Interest Statement**

210 The authors declare that the research was conducted in the absence of any commercial or
211 financial relationships that could be construed as a potential conflict of interest.

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215 **Author Contributions**

216 All authors listed have made a substantial, direct and intellectual contribution to the
217 work, and approved it for publication:

218 Fernanda Seixas: Conceptualization, Investigation, Validation, Writing – Original Draft
219 Preparation

220 Francisco Morinha: Formal Analysis, Investigation, Validation, Writing – Original
221 Draft Preparation

222 Claudia Luis, Nuno Alvura: Resources, Writing – Original Draft Preparation

223 Maria dos Anjos Pires: Funding Acquisition, Supervision, Writing – Review & Editing

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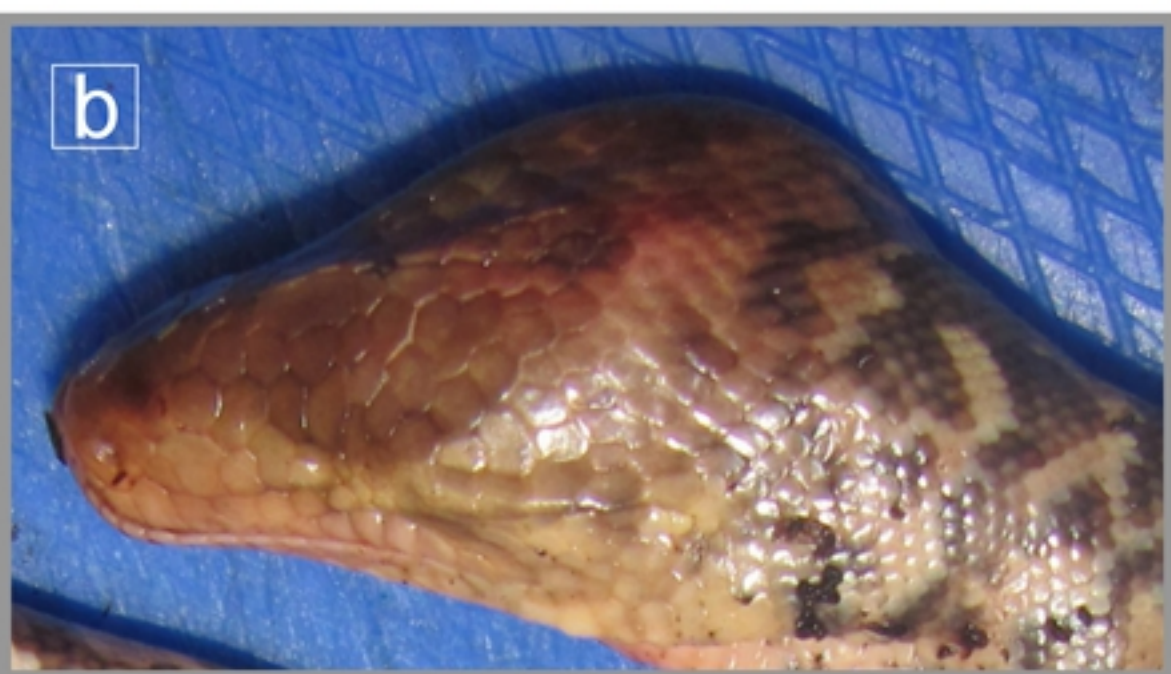


Figure 1

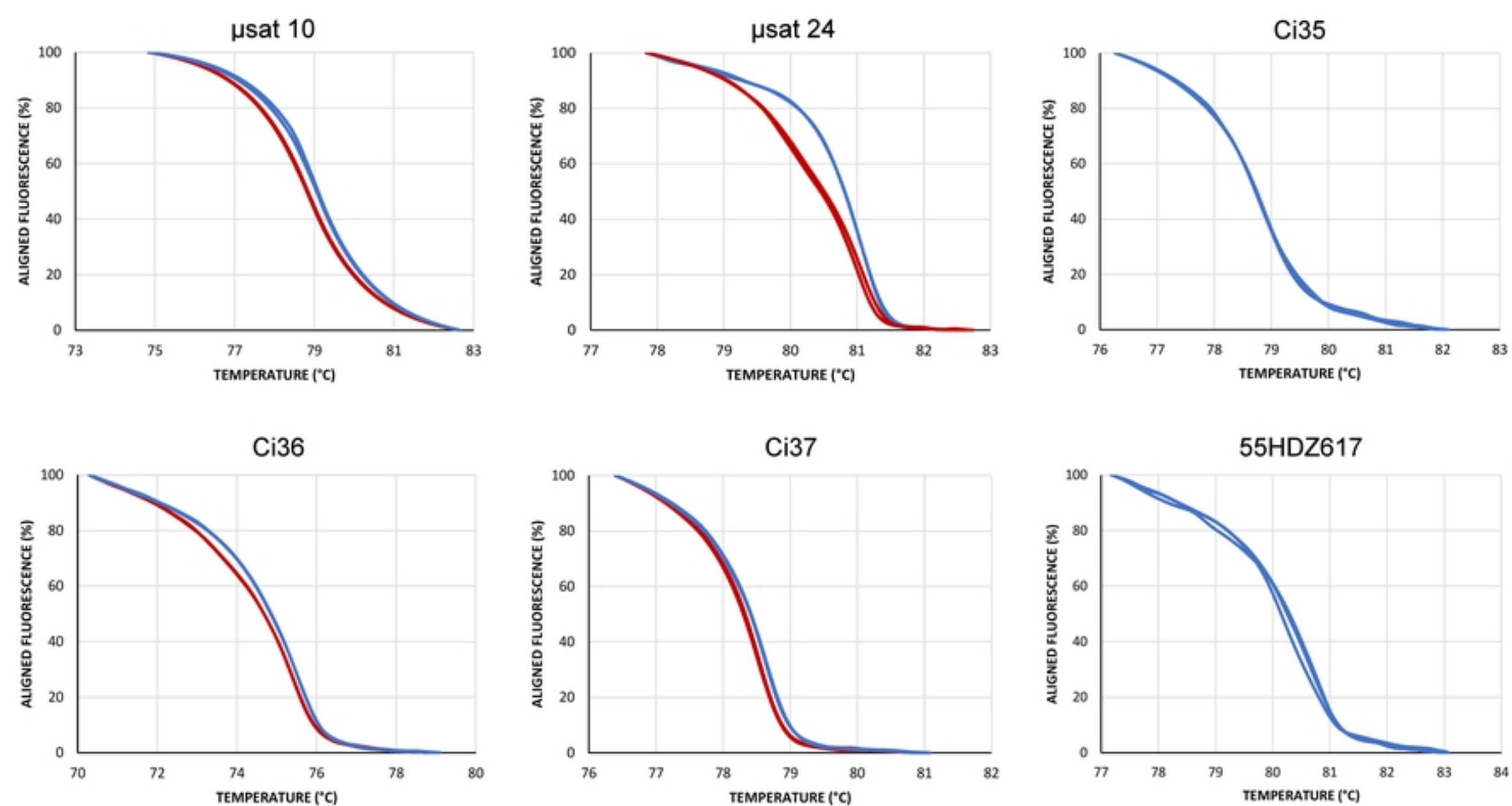


Figure 2