

**A peer-reviewed version of this preprint was published in PeerJ on 20 March 2018.**

[View the peer-reviewed version](https://peerj.com/articles/4470) (peerj.com/articles/4470), which is the preferred citable publication unless you specifically need to cite this preprint.

McGuire JA, Cotoras DD, O'Connell B, Lawalata SZS, Wang-Claypool CY, Stubbs A, Huang X, Wogan GOU, Hykin SM, Reilly SB, Bi K, Riyanto A, Arida E, Smith LL, Milne H, Streicher JW, Iskandar DT. 2018. Squeezing water from a stone: high-throughput sequencing from a 145-year old holotype resolves (barely) a cryptic species problem in flying lizards. PeerJ 6:e4470 <https://doi.org/10.7717/peerj.4470>

# Squeezing water from a stone: High-throughput sequencing from a 145-year old holotype resolves (barely) a cryptic species problem in flying lizards

Jimmy A McGuire<sup>Corresp., 1,2</sup>, Darko D Cotoras<sup>3,4</sup>, Brendan O'Connell<sup>5</sup>, Shobi Z S Lawalata<sup>1</sup>, Cynthia Y Wang-Claypool<sup>1,2</sup>, Alexander Stubbs<sup>1,2</sup>, Xiaoting Huang<sup>6</sup>, Guinevere O U Wogan<sup>7</sup>, Sarah M Hykin<sup>1,2</sup>, Sean B Reilly<sup>1,2</sup>, Ke Bi<sup>8</sup>, Awal Riyanto<sup>9</sup>, Evy Arida<sup>9</sup>, Lydia L Smith<sup>1</sup>, Heather Milne<sup>3</sup>, Jeffrey W Streicher<sup>10</sup>, Djoko T Iskandar<sup>11</sup>

<sup>1</sup> Museum of Vertebrate Zoology, University of California, Berkeley, Berkeley, California, United States

<sup>2</sup> Department of Integrative Biology, University of California, Berkeley, Berkeley, California, United States

<sup>3</sup> Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, Santa Cruz, California, United States

<sup>4</sup> Entomology Department, California Academy of Sciences, San Francisco, California, United States

<sup>5</sup> Department of Biomolecular Engineering and Bioinformatics, Baskin School of Engineering, University of California, Santa Cruz, Santa Cruz, California, United States

<sup>6</sup> Key Laboratory of Marine Genetics and Breeding, Ocean University of China, Qingdao, China

<sup>7</sup> Department of Environmental Science, Policy, and Management, University of California, Berkeley, Berkeley, California, United States

<sup>8</sup> Computational Genomics Resource Laboratory, California Institute for Quantitative Biosciences, University of California, Berkeley, Berkeley, California, United States

<sup>9</sup> Museum Zoologicum Bogoriense (MZB), Indonesia Institute of Sciences (LIPI), Cibinong, West Java, Indonesia

<sup>10</sup> Department of Life Sciences, The Natural History Museum, London, London, United Kingdom

<sup>11</sup> School of Life Sciences and Technology, Institute of Technology Bandung, Bandung, West Java, Indonesia

Corresponding Author: Jimmy A McGuire

Email address: mcguirej@berkeley.edu

We used Massively Parallel High-Throughput Sequencing to obtain genetic data from a 145-year old holotype specimen of the flying lizard, *Draco cristatellus*. Obtaining genetic data from this holotype was necessary to resolve an otherwise intractable taxonomic problem involving the status of this species relative to closely related sympatric *Draco* species that cannot otherwise be distinguished from one another on the basis of museum specimens. Initial analyses suggested that the DNA present in the holotype sample was so degraded as to be unusable for sequencing. However, we used a specialized extraction procedure developed for highly degraded ancient DNA samples and MiSeq shotgun sequencing to obtain just enough low-coverage mitochondrial DNA (547 base pairs) to conclusively resolve the species status of the holotype as well as a second known specimen of this species. The holotype was prepared before the advent of formalin-fixation and therefore was most likely originally fixed with ethanol and never exposed to formalin. Whereas conventional wisdom suggests that formalin-fixed samples should be the most challenging for DNA sequencing, we propose that evaporation during long-term alcohol storage and consequent water-exposure may subject older ethanol-fixed museum specimens to hydrolytic damage. If so, this may pose an even greater challenge for

sequencing efforts involving historical samples.

1 **Squeezing water from a stone: High-Throughput Sequencing from a 145-year old holotype**  
2 **resolves (barely) a cryptic species problem in flying lizards**

3

4 Jimmy A. McGuire<sup>1,2</sup>, Darko D. Cotoras<sup>3,4</sup>, Brendan O'Connell<sup>5</sup>, Shobi Z. S. Lawalata<sup>1,2,6</sup>,  
5 Cynthia Y. Wang-Claypool<sup>1,2</sup>, Alexander Stubbs<sup>1,2</sup>, Xiaoting Huang<sup>7</sup>, Guinevere O. U. Wogan<sup>8</sup>,  
6 Sarah M. Hykin<sup>1,2</sup>, Sean B. Reilly<sup>1,2</sup>, Ke Bi<sup>9</sup>, Awal Riyanto<sup>10</sup>, Evy Arida<sup>10</sup>, Lydia L. Smith<sup>1</sup>,  
7 Heather Milne<sup>3</sup>, Jeffrey W. Streicher<sup>11</sup>, and Djoko T. Iskandar<sup>12</sup>.

8

9 <sup>1</sup> Museum of Vertebrate Zoology, 3101 VLSB, University of California, Berkeley, CA 94720, USA

10 <sup>2</sup> Department of Integrative Biology, University of California, Berkeley, CA 94720, USA

11 <sup>3</sup> Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, 1156 High  
12 Street, Santa Cruz, CA 95064, USA

13 <sup>4</sup> Entomology Department, California Academy of Sciences, 55 Music Concourse Dr., Golden Gate Park,  
14 San Francisco, CA 94118, USA

15 <sup>5</sup> Department of Biomolecular Engineering and Bioinformatics, Baskin School of Engineering, University  
16 of California, Santa Cruz, 1156 High Street, Santa Cruz, CA 95064, USA

17 <sup>6</sup> United in Diversity Foundation, Jalan Hayam Wuruk No. 28, Jakarta 10220, Indonesia

18 <sup>7</sup> Key Laboratory of Marine Genetics and Breeding, Ocean University of China, Ministry of Education, 5  
19 Yushan Road, Qingdao 266003, China

20 <sup>8</sup> Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA  
21 94720, USA

22 <sup>9</sup> Computational Genomics Resource Laboratory, California Institute for Quantitative Biosciences, 238  
23 Koshland Hall, University of California, Berkeley, CA 94720, USA

24 <sup>10</sup> Museum Zoologicum Bogoriense (MZB), Indonesia Institute of Sciences (LIPI), Jalan Raya Bogor-  
25 Jakarta KM 46, Cibinong 16911, Indonesia

26 <sup>11</sup> Department of Life Sciences, The Natural History Museum, London SW7 5BD, United Kingdom

27 <sup>12</sup> School of Life Sciences and Technology, Institut Teknologi Bandung, Labtek XI Building, 10 Jalan  
28 Ganesa, Bandung 40132, Indonesia

29

30 Corresponding Author: Jimmy A. McGuire

31

32 Email address: mcguirej@berkeley.edu

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50 **ABSTRACT**

51

52 We used Massively Parallel High-Throughput Sequencing to obtain genetic data from a 145-  
53 year old holotype specimen of the flying lizard, *Draco cristatellus*. Obtaining genetic data from  
54 this holotype was necessary to resolve an otherwise intractable taxonomic problem involving the  
55 status of this species relative to closely related sympatric *Draco* species that cannot otherwise be  
56 distinguished from one another on the basis of museum specimens. Initial analyses suggested  
57 that the DNA present in the holotype sample was so degraded as to be unusable for sequencing.  
58 However, we used a specialized extraction procedure developed for highly degraded ancient  
59 DNA samples and MiSeq shotgun sequencing to obtain just enough low-coverage mitochondrial  
60 DNA (547 base pairs) to conclusively resolve the species status of the holotype as well as a  
61 second known specimen of this species. The holotype was prepared before the advent of  
62 formalin-fixation and therefore was most likely originally fixed with ethanol and never exposed  
63 to formalin. Whereas conventional wisdom suggests that formalin-fixed samples should be the  
64 most challenging for DNA sequencing, we propose that evaporation during long-term alcohol  
65 storage and consequent water-exposure may subject older ethanol-fixed museum specimens to  
66 hydrolytic damage. If so, this may pose an even greater challenge for sequencing efforts  
67 involving historical samples.

68

69

70 **INTRODUCTION**

71

72 The advent of Massively Parallel High-Throughput Sequencing (HTS) has dramatically altered  
73 the manner in which geneticists conduct their research. This is certainly true for molecular  
74 phylogeneticists and population geneticists, who now routinely have access to large multilocus  
75 genetic datasets for non-model organisms. Because HTS using the Illumina platform involves  
76 sequencing of small fragments of DNA, this approach offers the potential to access previously  
77 unattainable genome-scale sequence data even for degraded historical samples (e.g., Prüfer et al.,  
78 2014; Palkopoulou et al., 2015). Millions of fluid-preserved specimens in museum collections  
79 predate the development of allozyme and DNA sequencing technologies, and thus lack specially  
80 preserved tissue samples for genetic analysis. Formalin-fixed fluid specimens usually having  
81 highly fragmented and cross-linked DNA, are often refractory to sequencing efforts using  
82 traditional Sanger sequencing. However, recent studies have shown that it is possible to obtain  
83 genomic DNA sequences from some of these fluid-preserved museum specimens. Hykin, Bi &  
84 McGuire (2015) demonstrated that low-coverage genomic sequences could be recovered from a  
85 30-year old formalin-fixed museum specimen, though they were unsuccessful with a ~100-year  
86 old specimen. Ruane and Austin (2016) sequenced Ultra-Conserved Elements (UCEs) from both  
87 formalin-fixed (n=11) and ethanol-fixed (n=10) museum specimens, including one sample that  
88 was collected between 1878 and 1911. Both had mixed success, with the quantity of DNA  
89 recovered in the extraction stage likely playing the largest role in the performance of their  
90 sequencing efforts. Notably, the samples that failed in Ruane and Austin's (2016) experiment  
91 included subsets of both their formalin- (7 of 16) and alcohol-fixed (4 of 5) samples, indicating  
92 that old alcohol-preserved museum specimens are not necessarily less problematic than those  
93 initially fixed with formalin. This is surprising given that contemporary tissue samples  
94 earmarked for genetic analysis are routinely stored in 95% ethanol.

95 Our objective in this study was to address an otherwise intractable problem in flying  
96 lizard taxonomy using Illumina HTS and ancient DNA methods for a 145-year old fluid-  
97 preserved holotype specimen. The nettlesome taxonomic issue involves a small clade of poorly  
98 known flying lizards (genus *Draco*, Agamidae) that, for reasons outlined below, was unlikely to  
99 be resolved without obtaining genetic data from the holotype specimen of one of the constituent  
100 species, *Draco cristatellus*. Determining species limits within this small clade (the *Draco*  
101 *fimbriatus* group) has proven challenging for taxonomists, and we first describe the convoluted  
102 taxonomic history of the clade as a justification for our ultimate solution to this question  
103 involving HTS. The *D. fimbriatus* group currently includes four recognized species: *D.*  
104 *abbreviatus*, *D. cristatellus*, *D. fimbriatus*, and *D. maculatus*. This taxonomic framework is  
105 based on Manthey (2008) and was followed by the widely utilized Reptile Database (Uetz, 2006).  
106 For reasons that we will describe in a subsequent paper, we instead utilize an alternative  
107 taxonomy that includes *D. cristatellus*, as well as *D. fimbriatus* (= *D. abbreviatus* above), *D.*  
108 *hennigi*, *D. punctatus* (= *D. fimbriatus* above), and *D. maculatus*. We note that our taxonomy  
109 differs from that of Manthey (2008) primarily as a consequence of having information that  
110 indicates that the type locality of *D. fimbriatus* is the Malay Peninsula rather than Java. We  
111 further note that our recognition of both *D. cristatellus* and *D. punctatus* is tentative, as a primary  
112 objective of this paper is to resolve whether these are in fact distinct species.

113 The *Draco fimbriatus* group is taxonomically challenging. Although *Draco maculatus* is  
114 abundant, easily sampled in the field, and easily distinguished from other members of the group  
115 based on external phenotype, the remaining members of this clade are only rarely encountered,  
116 with relatively few specimens represented in museum collections. These species are canopy  
117 specialists (McGuire, 2003), making them more difficult to detect and more challenging to  
118 collect than other *Draco* taxa. Furthermore, the species comprising the *D. fimbriatus* group, as  
119 well as several other *Draco* clades are primarily distinguished on the basis of differences in  
120 coloration of their display structures (dewlap and patagia for most *Draco* taxa, just the dewlap  
121 among the relevant members of the *D. fimbriatus* group). For example, two major clades — the  
122 ‘Philippines *volans* group’ (McGuire and Alcala, 1999) and the ‘*Draco lineatus* group’  
123 (McGuire et al., 2007) — are each composed of multiple species that are primarily distinguished  
124 on the basis of coloration. Because coloration fades in preservative, recognizing species-specific  
125 coloration characteristics generally requires experience with the species in the field and/or access  
126 to color imagery of the specimen in life. Thus, as museum specimens, the members of these  
127 clades are functionally cryptic sympatric species. In summary, for the *D. fimbriatus* group, the  
128 paucity of museum specimens, and the rarity with which specimens are observed in the field  
129 from throughout their collective ranges by single observers, has greatly impeded taxonomic  
130 progress.

131 Within the *Draco fimbriatus* group, a particularly challenging issue relates to the  
132 taxonomic standings of *D. cristatellus* Günther 1872 and *D. punctatus* Boulenger 1900. *Draco*  
133 *cristatellus* was described based on a single specimen collected by Mr. Alfred Hart Everett in  
134 Matang, Sarawak between 1869 and March of 1872 (when Günther’s manuscript describing the  
135 species was submitted for publication). Although Everett collected the type specimen, the  
136 Trustees of the British Museum purchased it from Mr. W. Cutter, thereby making it available to  
137 Günther for description (see Günther, 1872). Because Günther presumably did not see the living  
138 specimen, he did not evaluate the coloration of the dewlap in life, which is essential for species  
139 identification within this group. Günther described the dewlap as ‘golden-yellow, with a brown  
140 anterior edge’, presumably from its preserved state. Subsequently, Boulenger (1900) described *D.*

141 *punctatus* from Bukit Larut on the Malay Peninsula, noting that the dewlap was lemon yellow in  
142 coloration. Although he did not attempt to diagnose *D. punctatus* from *D. cristatellus*, Boulenger  
143 (1900) was clearly aware of the latter species and explicitly considered his *D. punctatus* holotype  
144 to be taxonomically distinct. Indeed, Boulenger (1900) noted that he had examined a second  
145 specimen of *D. punctatus* from Sarawak that was also collected by Everett, remarking that he  
146 had previously referred that second specimen to *D. cristatellus*. Boulenger (1900) might be the  
147 last author to have had a clear idea about the taxonomic distinctiveness of *D. cristatellus* and *D.*  
148 *punctatus*, and it is a pity that he did not identify the character differences that he used to render  
149 his taxonomic decision. Although de Rooij (1915) recognized *D. fimbriatus*, *D. cristatellus* and  
150 *D. punctatus* as distinct species, subsequent authors synonymized one or more members of the  
151 group. Hennig (1936) synonymized *D. cristatellus* with *D. fimbriatus*, while continuing to  
152 recognize *D. punctatus*. In his monographic *Draco* taxonomic study, Musters (1983) opted to  
153 synonymize both *D. cristatellus* and *D. punctatus* with *D. fimbriatus*. In his competing  
154 taxonomic treatment, Inger (1983) recognized two species, *D. cristatellus* and *D. fimbriatus*, as  
155 valid species, but placed *D. punctatus* in the synonymy of *D. cristatellus*. Inger's (1983)  
156 recognition of two species was based in part on Grandison's (1972) report on two sympatric *D.*  
157 *fimbriatus* group species with distinct dewlap colorations on Gunung (Mt.) Benom on the Malay  
158 Peninsula. Whereas Grandison (1972) identified the two sympatric species as *D. fimbriatus* and  
159 *D. punctatus* (without commenting on the status of the Bornean *D. cristatellus*), Inger (1983)  
160 instead opted to treat *D. punctatus* as a synonym of *D. cristatellus*. This sensible decision was  
161 presumably made on the basis of the similar dewlap colorations of the *D. cristatellus* and *D.*  
162 *punctatus* holotypes ('golden-yellow, with a brown anterior edge' vs. 'lemon yellow'). Inger  
163 (1983) furthermore attempted to differentiate his conceptions of *D. fimbriatus* and *D. cristatellus*  
164 using a statistical analysis of eight linear measurements and scale counts. Although he  
165 successfully sorted his sample into two groups on the basis of overlapping but significantly  
166 distinct character state differences, his *a priori* placement of *D. punctatus* in the synonymy of *D.*  
167 *cristatellus* effectively precluded the possibility that three species — *D. cristatellus*, *D.*  
168 *fimbriatus*, and *D. punctatus* — might all co-occur on the Greater Sunda Shelf (and particularly  
169 on Borneo). Here we address this open question taking advantage of two critical developments:  
170 (1) the acquisition and analysis of a key specimen (TNHC 56763) obtained by JAM from  
171 Santubong, Sarawak, Malaysian Borneo in 1996, and (2) an analysis of the 145-year old *D.*  
172 *cristatellus* holotype using ancient DNA extraction methods and HTS on the Illumina platform.

173

## 174 MATERIALS AND METHODS

175

### 176 (a) DNA extraction and sequencing from the *Draco cristatellus* holotype

177 We obtained from the Natural History Museum in London liver tissue from the holotype of  
178 *Draco cristatellus* (specimen BMNH 1872.2.19.4). This specimen was originally collected and  
179 prepared prior to March 1872, well before the advent of formalin-fixation.

180 At that time, the standard practice for fluid preservation of reptiles, amphibians, and fishes was  
181 direct preservation in "pure spirits of wine" (Günther, 1880). Thus, the holotype was most likely  
182 initially fixed in 90-100% ethanol (= Günther's "pure spirits of wine") and never exposed to  
183 formalin. Nevertheless, we opted to perform our initial DNA extraction using the methodology  
184 described in Hykin, Bi & McGuire (2015) for formalin-fixed tissues, with the goal being to  
185 perform an exome-capture experiment with this sample. The Hykin, Bi & McGuire (2015)  
186 procedure involves a series of initial ethanol washes followed by treatment in a heated alkali

187 buffer solution to break cross-linkages before standard phenol-chloroform extraction. When this  
188 extraction returned a very low (potentially zero) yield, we performed a second pair of phenol-  
189 chloroform extractions involving phase-lock gel tubes followed by SPRI bead clean-up. This  
190 second round of extractions was performed with and without exposure to heated alkali solution.  
191 These extractions also failed to return sufficient DNA to move forward with library preparation.  
192 Despite minimal DNA yield, we made an attempt to PCR-amplify and sequence a short fragment  
193 of the mitochondrial ND2 gene from both DNA extracts. These experiments resulted in the  
194 amplification and sequencing of human ND2 in two separate experiments. Both of our low-yield  
195 DNA extractions were then sent to MYcroarray Inc. in Ann Arbor, MI where they were  
196 subjected to an extra silica purification designed for low-concentration fragment retention, and  
197 prepared as libraries. However, the library preparation retrieved only artifact and we did not  
198 proceed to targeted enrichment of selected exons or sequencing.

199 At this stage, we engaged with a lab specializing in genetic analysis of ancient DNA  
200 samples, with extraction and sequencing performed in a facility specifically designed for work  
201 with ancient samples (the Shapiro Lab at UC Santa Cruz). No reptile work had previously been  
202 done in this facility and all work followed lab standards for working with historical samples  
203 (Fulton, 2011). The DNA extraction protocol was based on Dabney et al. (2013), Tin, Economo  
204 & Mikheyev (2014), and D. D. Cotoras et al. (2017). An initial subsample of 40 mg of tissue was  
205 subdivided into ~1mm pieces and suspended in lysis buffer. The composition of the 100 mL lysis  
206 buffer aliquot was: 5.3 mL 1 M Tris-HCl (pH 8.0), 5.3 mL 0.2 M EDTA, 10.6 mL 20% Sarkosyl,  
207 1 mL 2-mercaptoethanol, and 77.8 mL distilled water. The tissue was digested with a total of 1  
208 mL of lysis buffer with 1 mg/mL proteinase K, initially incubated overnight at 56°C, and then  
209 raised to 72°C for 1 hr. The 72°C incubation step was undertaken in case the sample had been  
210 exposed to formalin in order to reverse any potential crosslinks. Silica-based purification  
211 followed the centrifugation-based protocol described by Dabney et al. (2013). Briefly, 0.5 mL of  
212 3M sodium acetate was added to the lysate and then transferred to a tube with 13 mL of binding  
213 buffer. The binding buffer is prepared in a 50 mL tube by first adding 23.88 g of guanidine  
214 hydrochloride and then adding water to bring the volume to 30 mL. A key element of this  
215 purification protocol is the high salt concentration of this binding buffer, which enhances  
216 recovery of short DNA fragments. After complete dissolution of the guanidine hydrochloride, 25  
217 µL of Tween-20 and sufficient isopropanol to bring the total volume to 50 mL were added. The  
218 mixture of sample, binding buffer, and sodium acetate was transferred into a Zymo extension  
219 reservoir attached to a MiniElute spin column. The spin column was then centrifuged for 10  
220 minutes at 1,000 rpm, after which the spin column was transferred to a 1.5 mL Eppendorf tube.  
221 We performed a dry spin for 1 minute at 13,000 rpm, followed by 2 washes with 750 µL of PE  
222 buffer (1 minute spin at 6,000 rpm). To ensure the entire PE buffer was removed, we did a dry  
223 spin for 1 minute at maximum speed. We eluted the purified extract in two volumes of 25 µL of  
224 TET. Each sample was centrifuged for 30 seconds at 13,200 RPM after 3-5 minutes of  
225 incubation. Because the elution displayed pigmentation, 25 µL of the extract was purified on a  
226 column filled with cross-linked polyvinylpyrrolidone (PVPP) (Arbeli and Fuentes, 2007). We  
227 also produced an extraction control consisting of lysis buffer that was subjected to the same set  
228 of procedures.

229 For genomic sequencing, we prepared two barcoded Illumina sequencing libraries (one  
230 for the holotype sample and one for the control) using the Meyer and Kircher (2010) protocol,  
231 starting with 5 µL of the PVPP purified DNA extraction. The same volume was used for the  
232 extraction control. The libraries were sequenced on an Illumina MiSeq machine using 150-cycle



233 v3 chemistry (2x75). Following sequencing, adaptors were removed from reads and sequences  
234 were merged using SeqPrep2 (<https://github.com/jeizenga/SeqPrep2>). Default parameters were  
235 used with the exception of the following: -q 20 -L 30 -A AGATCGGAAGAGCACACGTC -B  
236 AGATCGGAAGAGCGTCGTGT. FastQC ([https://www.bioinformatics.babraham.ac.uk/  
237 projects/fastqc/](https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)) confirmed that the sequence quality was good, with the normal base quality drop  
238 in the final five bases.

239

#### 240 **(b) Sanger sequence data for *Draco fimbriatus* group specimens**

241 Our team has generated a large number of complete ND2 sequences for *Draco* specimens,  
242 including for 65 exemplars representing the *D. fimbriatus* group. These sequences were available  
243 for comparison with ND2 sequence fragments obtained from the *D. cristatellus* holotype. PCR-  
244 amplification was undertaken using the primers Metf1 and ALAr2, with cycle sequencing  
245 involving these external primers plus the internal primers Metf5 and ND2r6 (see McGuire and  
246 Kiew 2001 for details).

247

#### 248 **(c) Exome-capture and screening of mitochondrial DNA**

249 For another project (McGuire et al. in prep), we generated an exome-capture data set using the  
250 MyBaits in-solution capture system for a set of 350 samples spanning all of *Draco*. This sample  
251 set included 14 *D. fimbriatus* group samples. The target loci for the exome capture include 1400  
252 exons and flanking sequences derived from transcriptome sequences (jointly representing 709  
253 loci), which were supplemented with an additional 540 lizard-specific UCE loci. Libraries  
254 enriched for our target loci were barcoded and sequenced on an Illumina Hi Seq 4000. Although  
255 our experiment was specifically designed to avoid capturing mitochondrial genes, mitochondrial  
256 sequences are so abundant in genomic DNA extractions that some mitochondrial molecules  
257 inevitably find their way into the off-target by-catch (non-target DNA sequences that are  
258 obtained during an exome-capture experiment). We took advantage of this imperfect filter to  
259 obtain mitochondrial sequences for comparison with the *D. cristatellus* holotype. For TNHC  
260 56763, we used Geneious version 8.1.7 (Kearse et al. 2012) to obtain a mostly complete  
261 representation of mitochondrial coding genes by mapping our raw exome capture data (including  
262 off-target sequences) to the complete mitochondrial genome of *Acanthasaura armata* available  
263 on GenBank (AB266452.1). A preliminary assessment of the identity of the sequences was  
264 performed with a BLAST search after collapsing duplicate sequences with fastx\_collapser  
265 ([http://hannonlab.cshl.edu/fastx\\_toolkit/commandline.html#fastx\\_collapser\\_usage](http://hannonlab.cshl.edu/fastx_toolkit/commandline.html#fastx_collapser_usage)). The result  
266 of the BLAST search was visualized with the program MEGAN (Huson et al., 2007). Processed  
267 reads were mapped with BWA mem (Li and Durbin, 2009) against the reference partial  
268 mitochondrial genome of TNHC 56763. Duplicates were removed with samtools rmdup (Li et al.,  
269 2009). A total of 17 unique reads mapped against the reference after duplicate removal. The  
270 represent a total of 777 bp of the 8114 bp reference. Most of the mapped regions had 1x  
271 coverage and portions of four contigs had 2x coverage. The average length of the mapped reads  
272 was 53 bp. Finally, for each of the 14 *D. fimbriatus* group samples included in our exome-  
273 capture experiment, we used Geneious to map our raw sequencing reads to 10 mitochondrial  
274 contigs obtained for the *D. cristatellus* holotype. The raw sequence data is available on the SRA  
275 database.

276

#### 277 **(d) Analysis of DNA sequence variation**

278 Our analysis of DNA sequence variation included alignment of homologous DNA sequences and  
279 a simple count of nucleotide base substitutions between the *Draco cristatellus* holotype, sample  
280 TNHC 56763 from Santubong, Sarawak, and our selection of *D. fimbriatus* and *D. punctatus*  
281 samples from the Malay Peninsula, Sumatra, the Mentawai Islands, Java, and Borneo. Specimens  
282 examined are listed in Table 1. We also performed a heuristic parsimony analysis to obtain a  
283 phylogram for the *D. fimbriatus* group and performed a non-parametric bootstrap analysis with  
284 1000 replicates to assess branch support. We did not perform a more rigorous maximum  
285 likelihood or Bayesian analysis because our primary objective was to assess uncorrected relative  
286 branch lengths. Phylogenetic analyses were performed in PAUP version 4 (Swofford 2002).

287

#### 288 (e) Data availability and Permits

289 Sanger sequence data are available on GenBank (will be submitted if accepted) and a matrix for  
290 the mitochondrial ND2 gene for the *D. fimbriatus* group is included as supplemental materials.  
291 This research was undertaken in accordance with UC Berkeley Animal Use Protocol Number  
292 AUP-2014-12-6954. Fieldwork was undertaken with research permits issued by the Economic  
293 Planning Unit of Malaysia (UPE:40/200/19 SJ.363) and the Indonesia Institute of Sciences  
294 (LIPI: No. 2411/FRP/SM/X?2008 and No. 0115/FRP/SM/VI/2009).

295

## 296 RESULTS

297

298 The MiSeq run generated a total of 1,086,926 paired-end reads after combining the data from 2  
299 different libraries (prepared identically) from the same extract. Raw sequences were merged if  
300 possible and duplicates collapsed, producing a total of 538,995 reads, which were BLAST  
301 searched (Altschul et al. 1990) against the NCBI database. 115,266 reads were successfully  
302 assigned, and of these, 47,400 hits corresponded to bacteria, 40,751 were assigned to mammals  
303 (of which 29,720 were specifically assigned to human), and 48 were assigned to *Anolis*  
304 *carolinensis*. Only three reads were assigned to *Draco*, each of which involved the mitochondrial  
305 ND2 sequence posted on GenBank for *Draco cristatellus* sample TNHC 56763 (see below). The  
306 paucity of *Draco* hits is not surprising given that there are no *Draco* reference genomes to map  
307 to. The extraction control was sequenced producing a total of 104,417 PE reads. After processing  
308 (adaptor removal, merged if possible, and duplicate removal) a total of 15,480 reads were  
309 assigned by the BLAST search. Bacteria were represented by 6,805 reads, mammals by 4,711  
310 reads (of those, 3103 were assigned to human, and reptiles were assigned no reads (one read was  
311 assigned to chicken (*Gallus gallus*)).

312 Of the three holotype ND2 reads, two were broadly overlapping, and the joint ND2 data  
313 obtained from the holotype totaled only 125 bp. To search for additional mitochondrial contigs in  
314 the holotype MiSeq data, we first generated a partial mitochondrial genome for TNHC 56763  
315 from exome-capture off-target by-catch, which returned 8114 bp of protein-coding gene  
316 sequence data. Mapping the holotype data to the TNHC 56763 mitochondrial assembly resulted  
317 in the recovery of an additional 10 reads totaling 596 bp of mitochondrial sequence data  
318 representing six genes (COI, COXIII, ATPase8, ND4, ND4L, ND5). Thus, a total of 13 contigs  
319 were assembled with an average length of 61 bp. Most of the mapped regions had 1x coverage  
320 and parts of four contigs had 2x coverage. No reads were recovered when mapping the extraction  
321 blank against the same reference. In comparing the holotype sequence data with TNHC 56763  
322 across the 721 bp of homologous sequence data, we found that the two samples were only  
323 weakly divergent from one another, sharing the same base calls at 710 of 721 positions for a raw

324 sequence divergence of 1.5%. We then mapped the raw reads from the exome captures for the  
325 remaining 13 *D. fimbriatus* group samples to the 10 *D. cristatellus* holotype contigs, which  
326 returned as few as two and as many as six homologous sequences per sample.

327 Comparison of the mitochondrial data obtained from the *Draco cristatellus* holotype with  
328 homologous data obtained for *D. fimbriatus* group samples found that the sample TNHC 56763  
329 from Santubong, Sarawak, Malaysian Borneo was much more similar to the holotype than were  
330 any other *D. fimbriatus* group samples. When limiting our comparison to the six gene fragments  
331 for which we had between six and 13 corresponding sequences for comparison to the holotype,  
332 we found that TNHC 56763 was 0.9% divergent from the holotype, whereas all other samples  
333 ranged between 11.9% and 15.1% divergent. TNHC 56763 differed from the holotype at just five  
334 of 547 base positions, whereas the other samples differed from the holotype at from 36 of 303 bp  
335 to 70 of 465 base positions. The ND2 comparisons were most comprehensive because we had  
336 access to complete ND2 sequences for 65 *D. fimbriatus* group samples. Whereas TNHC 56763  
337 differed from the holotype at three of 183 base positions, all other samples differed by at least 24  
338 base positions. Notably, the two *D. punctatus* samples from Sarawak (the type locality for *D.*  
339 *cristatellus*) differed from the holotype at 28 and 30 of 183 base positions (15.3% and 16.4%,  
340 respectively).

341 A parsimony phylogenetic analysis of the ND2 gene including the *D. cristatellus*  
342 holotype strongly supports the monophyly of the holotype together with TNHC 56763 to the  
343 exclusion of all other *D. fimbriatus* group samples with 100% bootstrap support (Figure 1).  
344 Further, the 0.9% mitochondrial sequence divergence between TNHC 56763 and the holotype is  
345 within the scope of ND2 sequence variation that we observe between *D. punctatus* samples from  
346 the same locality. This does not consider the possibility that one or more of the five documented  
347 base substitutions could be sequencing errors resulting either from damage to the holotype DNA  
348 or random errors in our low coverage data, as we did not apply informatics pipelines developed  
349 to identify post-mortem damage of ancient DNA to our data (e.g. Mateiu and Rannala 2008,  
350 Molak and Ho 2011). Notably, one of the three inferred ND2 substitutions is a first position C-  
351 >T change that would result in a proline to serine amino acid replacement, suggesting that this  
352 might be the result of post-mortem deamination of the template molecular or sequencing error.

## 353 **DISCUSSION**

354  
355 In the present study, we applied HTS to a 145-year old fluid-preserved holotype specimen in an  
356 effort to disentangle an otherwise intractable taxonomic question. The problem stems from the  
357 fact that one of the species in this group, *Draco cristatellus*, was described using limited color  
358 information, and because fluid-preserved specimens representing multiple sympatric *D.*  
359 *fimbriatus* group species are often indistinguishable from one another without color information.  
360 Indeed, sympatric species in this complex are effectively cryptic once they have been prepared as  
361 museum specimens. This combination of circumstances rendered it virtually impossible to  
362 resolve the species status of *D. cristatellus* relative to *D. punctatus* and *D. fimbriatus*, two  
363 widespread species on the Greater Sunda Shelf. Importantly, a sample (TNHC 56763) collected  
364 in 1996 by JAM provides phylogenetic evidence for a third *D. fimbriatus* group species on  
365 Borneo, with the natural question being whether this sample is conspecific with the name-  
366 bearing holotype specimen of *D. cristatellus* housed in the British Museum of Natural History.  
367 Several species composition outcomes were possible, all of which were considered in a  
368 hypothesis-testing framework. In Hypothesis 1, *D. cristatellus* and *D. punctatus* are synonyms,  
369

370 together representing a single species distinct from *D. fimbriatus* and TNHC 56763. In  
371 Hypothesis 2, *D. cristatellus* and *D. fimbriatus* are synonyms. In Hypothesis 3, *D. cristatellus* is  
372 a species distinct from *D. punctatus* and *D. fimbriatus* but conspecific with TNHC 56763.  
373 Finally, in Hypothesis 4, *D. cristatellus*, *D. fimbriatus*, *D. punctatus*, and TNHC 56763 all  
374 represent distinct species, with TNHC 56763 representing a fourth sympatric species on Borneo.  
375 The only way to conclusively test these alternative hypotheses was to obtain informative genetic  
376 data from the holotype specimen of *D. cristatellus* for comparison with TNHC 56763, and  
377 representative specimens of *D. fimbriatus* and *D. punctatus*.

378 We initially believed that genetic data would easily be retrieved from the *Draco*  
379 *cristatellus* holotype. The holotype was prepared before the advent of formalin-fixation, and we  
380 consequently had reason to believe that the specimen was originally fixed with ethanol and had  
381 never been exposed to formalin. Because tissue samples collected for genetic analysis are  
382 routinely stored in ethanol, we were confident that the holotype would still hold high molecular-  
383 weight DNA suitable for genomic sequencing. Our hope was to perform exome-capture with this  
384 sample and include it in a larger *Draco* phylogenomic data set. However, our initial attempts at  
385 extracting DNA from the sample using methods appropriate for historical and formalin-fixed  
386 tissues failed, forcing us to adjust both our approach and our expectations. Fortunately, our  
387 alternative hypotheses proved testable without comprehensive genomic data from the holotype.  
388 Indeed, analysis of the initial 125 bp of mitochondrial ND2 data identified when the holotype  
389 sequence data was subjected to GenBank BLAST allowed us to reject hypotheses 1, 2, and 4 in  
390 favor of hypothesis 3. The additional 422 bp of mitochondrial data obtained via mapping of  
391 holotype contigs to the reconstructed mitochondrial genome for TNHC 56763 simply provided  
392 additional confirmation that *D. cristatellus* and *D. punctatus* are each valid species, and that our  
393 specimen TNHC 56763 from Santubong, Sarawak is indeed a true *D. cristatellus* exemplar. This  
394 finding was a best-case scenario because all future specimens for which tissue samples are  
395 obtained can now be compared with known *D. cristatellus*, *D. punctatus*, and *D. fimbriatus*  
396 samples for genetic identification.

397 What lessons can be learned from our attempt to obtain genetic data from the *Draco*  
398 *cristatellus* holotype? First, even when initial attempts at extraction and quantification of DNA  
399 suggest that none is present, small numbers of DNA molecules may survive in the sample. For  
400 questions of simple species identification involving old and highly degraded samples, it may  
401 only be necessary to obtain limited data — even a few hundred base pairs of mitochondrial data  
402 may be sufficient to address the question. Our study shows that this is indeed possible even when  
403 initial assessments suggest that DNA in a tissue sample has been highly degraded. Obtaining  
404 data in these instances will likely require highly specialized extraction procedures such as the  
405 silica-column based extraction methodology utilized here, followed by short-fragment  
406 sequencing. Finally, we believe that the difficulty we confronted with this ethanol-fixed sample  
407 — which is consistent with the problems experienced by Ruane and Austin (2016) with their  
408 presumed alcohol-fixed samples (4 of 5 of which failed to sequence) — suggests that hydrolyzed  
409 ethanol-fixed tissues might be more problematic than formalin-fixed samples for genomic  
410 sequencing efforts (see Handt et al. 1994 for a description of DNA hydrolysis). This has  
411 important implications for curatorial practices. Not only is potential hydrolytic damage cause for  
412 concern with whole fluid specimens stored in ethanol, but it could be particularly problematic for  
413 tissue samples stored in ethanol that are not maintained in sub-zero degree conditions.  
414 Evaporation of ethanol from tissue vials might render even modern tissue samples virtually  
415 unusable for genetic analysis.

416

417 **CONCLUSIONS**

418

419 The development of HTS has revolutionized biological research by making genome-scale data  
420 readily available at a reasonable cost, even for non-model organisms.

421 Systematists have fully embraced these advances in data acquisition for freshly sampled  
422 specimens, but are just beginning to harness HTS for the millions of fluid-preserved historical  
423 samples housed in natural history collections around the world. As we have shown here,  
424 acquiring genetic data from old museum specimens will sometimes present special challenges,  
425 but the information that can be gleaned from such specimens may be the only way to  
426 conclusively resolve previously intractable evolutionary and taxonomic questions.

427

428 **ACKNOWLEDGEMENTS**

429

430 The authors would like to thank Patrick Campbell and Donney Nicholson of The Natural History  
431 Museum, London for assistance processing the loan of liver tissue from the *D. cristatellus*  
432 holotype. Colin McCarthy and Oliver Crimmen kindly provided suggestions for locating  
433 information on A.H. Everett's Bornean collections and the history of fluid preservation at  
434 BMNH, respectively. We acknowledge Alison Devault and Jacob Enk at MYcroarray and  
435 MYreads both for advice and for generating the library preparation confirming the low yield of  
436 our initial DNA extraction. David Wake, Rauri Bowie, and members of the McGuire and Bowie  
437 labs provided valuable suggestions that improved the manuscript. JAM thanks the Economic  
438 Planning Unit of Malaysia and the Indonesian Institute of Sciences (LIPI) for providing scientific  
439 collecting permits.

440

441

442 **REFERENCES**

443

- 444 Altschul, S. F., W. Gish, W. Miller, E. W. Myers, & D. J. Lipman. 1990. Basic local alignment  
445 search tool. *Journal of Molecular Biology* 215:403-410.
- 446 Arbeli, Z. & C. L. Fuentes, 2007. Improved purification and PCR amplification of DNA from  
447 environmental samples. *FEMS Microbiology Letters* 272:269–275.
- 448 Boulenger, G. A. 1900. Descriptions of new batrachians and reptiles from the Larut Hills, Perak.  
449 *Annals and Magazine of Natural History Series 7*, Volume 6:186-193.
- 450 Cotoras, D. D., G. G. R. Murray, J. Kapp, R. G. Gillespie, C. Griswold, W. B. Simison, R. E.  
451 Green, & B. Shapiro. 2017. Ancient DNA resolves the history of *Tetragnatha* (Araneae,  
452 Tetragnathidae) spiders on Rapa Nui. *Genes* 8:403.
- 453 Dabney, J., M. Knapp, I. Glocke, M.-T. Gansauge, A. Weihmann, B. Nickel, C. Valdiosera, N.  
454 Garcia, S. Paabo, J.-L. Arsuaga, & M. Meyer. 2013. Complete mitochondrial genome  
455 sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments.  
456 *Proceedings of the National Academy of Sciences*, 110:15758–15763.
- 457 Fulton, T. L. 2011. Setting Up an Ancient DNA Laboratory. *Ancient DNA Methods and*  
458 *Protocols* (ed. by M. Shapiro, B.; Hofreiter), pp. 1–11. Humana Press, New York.
- 459 Grandison, A. G. C. 1972. The Gunong Benom Expedition 1967. 5. Reptiles and amphibians of  
460 Gunong Benom with a description of a new species of *Macrocalamus*. *Bulletin of the British*  
461 *Museum (Natural History)* 23:45-101.

- 462 Günther, A. 1872. On the reptiles and amphibians of Borneo. *Proceedings of the Zoological*  
463 *Society of London* 1872:586-600.
- 464 Günther, A. 1880. An introduction to the study of fishes. Adam and Charles Black. Edinburgh.  
465 743 pp.
- 466 Handt, O., M. Höss, M. Krings, & S. Pääbo. 1994. Ancient DNA: methodological challenges.  
467 *Experientia* 50:524-529.
- 468 Huson, D. H., A. F. Auch, J. Qi, & S. C. Schuster. 2007. MEGAN analysis of metagenomic data.  
469 *Genome Research* 17:377-386.
- 470 Hykin, S. M., K. Bi, & J. A. McGuire. 2015. Fixing Formalin: A Method to Recover Genomic-  
471 Scale DNA Sequence Data from Formalin-Fixed Museum Specimens Using High-  
472 Throughput Sequencing. *PLoS ONE* 10(10): e0141579. doi:10.1371/journal.pone.0141579
- 473 Inger, R. F. 1983. Morphological and ecological variation in the flying lizards (genus *Draco*).  
474 *Fieldiana Zoology, New Series* 18:vi+35 pp.
- 475 Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A.  
476 Cooper, S. Markowitz, C. Duran, T. Thierer, B. Ashton, P. L. Meintjes, & A. J. Drummond.  
477 2012. Geneious Basic: An integrated and extendable desktop software platform for the  
478 organization and analysis of sequence data. *Bioinformatics* 28:1647-1649.
- 479 Li, H. & R. Durbin. 2009. Fast and accurate short read alignment with Burrows-Wheeler  
480 Transform. *Bioinformatics*, 25:1754-60. [PMID: 19451168]
- 481 Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R.  
482 Durbin, & 1000 Genome Project Data Processing Subgroup. 2009. The Sequence  
483 alignment/map (SAM) format and SAMtools. *Bioinformatics* 25, 2078-9. [PMID: 19505943]
- 484 Manthey, U. 2008. Agamid Lizards of Southern Asia. Draconinae 1. Edition Chimaira, Frankfurt  
485 am Main, Germany.
- 486 Mateiu, L., & B. Rannala. 2008. Bayesian inference of errors in ancient DNA caused by  
487 postmortem degradation. *Molecular Biology and Evolution* 25:1503-1511.
- 488 McGuire, J. A. 2003. Allometric prediction of locomotor performance: An example from  
489 Southeast Asian flying lizards. *American Naturalist* 161:347-369.
- 490 McGuire, J. A., & A. C. Alcalá. 2000. A taxonomic revision of the flying lizards of the  
491 Philippine Islands (Iguania: Agamidae: *Draco*), with a description of a new species.  
492 *Herpetological Monographs* 14:81-138.
- 493 McGuire, J. A., & B. H. Kiew. 2001. Phylogenetic systematics of Southeast Asian flying lizards  
494 (Iguania: Agamidae: *Draco*) as inferred from mitochondrial DNA sequence data. *Biological*  
495 *Journal of the Linnean Society* 72:203-229.
- 496 McGuire, J. A., R. M. Brown, Mumpuni, A. Riyanto, & N. Andayani. 2007. The flying lizards of  
497 the *Draco lineatus* group (Squamata: Iguania: Agamidae): A taxonomic revision with  
498 descriptions of two new species. *Herpetological Monographs* 21:180-213.
- 499 Molak, M., & S. Y. W. Ho. 2011. Evaluating the impact of post-mortem damage in ancient  
500 DNA: A theoretical approach. *Journal of Molecular Evolution* 73:244-255.
- 501 Meyer, M. & M. Kircher. 2010. Illumina sequencing library preparation for highly multiplexed  
502 target capture and sequencing. *Cold Spring Harbor Protocols*, 5, 1–11.
- 503 Musters, C. J. M. 1983. Taxonomy of the genus *Draco* L. (Agamidae, Lacertilia, Reptilia).  
504 *Zoologische Verhandelingen* 199:1-120 + 4 plates.
- 505 de Rooij, N. 1915. The Reptiles of the Indo-Australian Archipelago. I. Lacertilia, Chelonia,  
506 Emydosauria. E. J. Brill Ltd., Leiden.

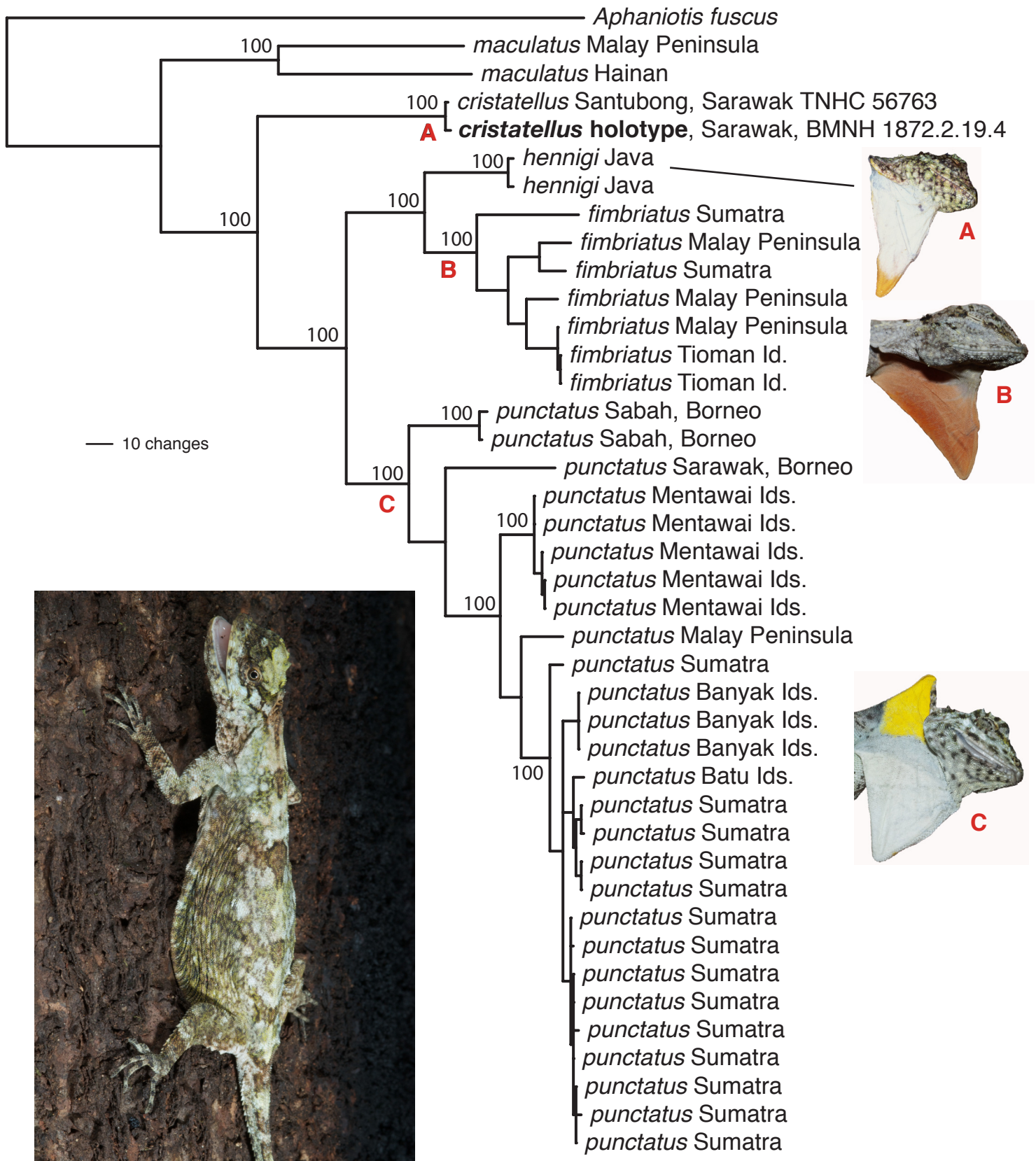
- 507 Palkopoulou, E., S. Mallick, P. Skoglund, J. Enk, N. Rohland, H. Li, A. Omrak, S. Vartanyan, H.  
508 Poinar, A. Götherström, D. Reich, & L. Dalén. 2015. Complete genomes reveal signatures of  
509 demographic and genetic declines in the woolly mammoth. *Current Biology* 25:1395-1400.
- 510 Prüfer, K., F. Racimo, N. Patterson, F. Jay, S. Sankararaman, S. Sawyer, A. Heinze, G. Renaud,  
511 P. H. Sudmant, C. de Filippo, H. Li, S. Mallick, M. Dannemann, Q. Fu1, M. Kircher, M.  
512 Kuhlwilm, M. Lachmann, M. Meyer, M. Ongyerth, M. Siebauer, C. Theunert, A. Tandon, P.  
513 Moorjani, J. Pickrell, J. C. Mullikin, S. H. Vohr, R. E. Green, I. Hellmann, P. L. F. Johnson,  
514 H. Blanche, H. Cann, J. O. Kitzman, J. Shendure, E. E. Eichler, E. S. Lein, T. E. Bakken, L.  
515 V. Golovanova, V. B. Doronichev, M. V. Shunkov, A. P. Derevianko, B. Viola, M. Slatkin,  
516 D. Reich, Janet Kelso, & S. Pääbo. 2014. The complete genome sequence of a Neandertal  
517 from the Altai Mountains. *Nature* 505(7481):43-49.
- 518 Ruane, S., & C. C. Austin. 2016. Phylogenomics using formalin-fixed and 100+ year-old  
519 intractable natural history specimens. *Molecular Ecology Resources* doi:10.1111/1755-  
520 0998.12655.
- 521 Swofford, D. L. 2002. PAUP\*. Phylogenetic Analysis Using Parsimony (\*and Other Methods).  
522 Version 4. Sinauer Associates, Sunderland, Massachusetts.
- 523 Tin M. M.-Y., E. P. Economo, & A. S. Mikheyev. 2014. Sequencing Degraded DNA from Non-  
524 Destructively Sampled Museum Specimens for RAD-Tagging and Low-Coverage Shotgun  
525 Phylogenetics. *PLoS ONE* 9(5): e96793. doi:10.1371/journal.pone.0096793.
- 526 Uetz, P. 2006. The EMBL Reptile Database CD-ROM edition, March 2006.

**Figure 1**(on next page)

Phylogenetic tree for the *Draco fimbriatus* group including the *D. cristatellus* holotype.

Figure 1. Phylogenetic tree for the *Draco fimbriatus* group based on a parsimony analysis of the complete mitochondrial ND2 gene (1032 bp). The *D. cristatellus* holotype includes 183 bp of sequence data. Only two of 28 available *D. maculatus* samples were included to simplify the image. Non-parametric bootstrap values (1000 replicates) are superimposed on the single most parsimonious phylogram for select clades. The photo in the bottom left is *Draco punctatus*.





**Table 1** (on next page)

Numbers of base substitutions and sequence divergence values between the *Draco cristatellus* holotype and 14 exemplars representing the *D. fimbriatus* group.

Base pair differences between the *Draco cristatellus* holotype and each of 14 *D. fimbriatus* group samples for six mitochondrial genes. For ND2, the data used for comparisons were generated using standard Sanger sequencing. For all other genes, the data were derived from exome-capture off-target sequences. Mean sequence divergence values relative to the holotype are provided for each species.

1

	<b>COX III contig3a</b>	<b>COI contig12a</b>	<b>COI contig12b</b>	<b>ND4L contig13</b>	<b>ND5 contig15</b>	<b>ND2 Sanger</b>	<b>Total</b>	<b>%</b>
<i>crstatellus</i> Borneo TNHC 56763	0/43	1/79	1/81	0/79	0/64	3/183	5/547	0.9%
<i>punctatus</i> Borneo TNHC 56766		7/79	7/69			30/183	44/331	13.3%
<i>punctatus</i> Borneo TNHC 56764		6/79	8/81	10/69		28/183	52/412	12.6%
<i>punctatus</i> Malay Pen. LSUHC 5617	5/43			10/79		32/183	47/305	15.4%
<i>punctatus</i> Mentawai MVZ 270632	4/43	10/79	7/81	12/79		37/183	70/465	15.1%
<i>punctatus</i> Batu Ids MVZ 270636	4/43	10/79	7/81	12/79		35/183	68/465	14.6%
<i>punctatus</i> Sumatra MVZ 270835	4/43	9/79	7/81	11/79		35/183	66/465	14.2%
<i>punctatus</i> Banyak Ids MVZ 270829	4/43	10/79	7/81	9/59		37/183	67/465	14.4%
<i>fimbriatus</i> Mal Pen TNHC 57954	4/43	7/71	7/81	9/79	12/64	29/183	68/521	13.1%
<i>fimbriatus</i> Mal Pen TNHC 58565	4/43	10/79	7/80	9/79	12/64		42/345	12.2%
<i>fimbriatus</i> Sumatra MZB Lace.14276		9/79	5/56	8/79		32/183	54/397	13.6%
<i>fimbriatus</i> Sumatra MVZ 239473		9/79	8/81	8/79	11/64		36/303	11.9%
<i>hennigi</i> LSUMZ 81446		11/79		8/79	12/64	23/183	54/405	13.3%
<i>hennigi</i> LSUMZ 81447		11/79		8/79	13/64	23/183	55/405	13.6%
						<i>crstatellus</i>	5/547	0.9%
							414/290	
						<i>punctatus</i>	8	14.2%
							200/156	
						<i>fimbriatus</i>	6	12.8%
						<i>hennigi</i>	111/810	13.7%

2

3

4

5

6

7

8