No reliable evidence supports the presence of the Javan tiger: data issues related to the DNA analysis of a recent hair sample

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Abstract A recent study published in *Oryx* proposed that the extinct Javan tiger *Panthera tigris sondaica* may still survive on the Island of Java, Indonesia, based on mitochondrial DNA analysis of a single hair sample collected from a location where a tiger was reportedly encountered. However, upon reanalysing the genetic data presented in that study, we conclude that there is little support for this claim. The sequences of the putative tiger hair and Javan tiger museum specimens generated are not from tiger cytoplasmic mitochondrial DNA but more likely the nuclear pseudogene copies of mitochondrial DNA. In addition, the number of mismatches between the two Javan tiger sequences is unusually high for homologous sequences that are both from tigers, suggesting potential issues with data reliability. The paper provides insufficient details on quality control measures, making it impossible to rule out the possibility that errors were introduced during the analysis. Consequently, it is inappropriate to use the sequences presented in that study to infer the existence of the Javan tiger.

Keywords Extinct species, genetic analysis, Indonesia, Java, Javan tiger, mitochondrial DNA, Numt, *Panthera tigris sondaica*

There has been no confirmed sighting of the Javan tiger *Panthera tigris sondaica* since the 1970s and the subspecies has been declared extinct (Seidensticker, 1987; Jackson & Nowell, 1996, 2008). In 2019 a putative encounter with a tiger was reported near a community plantation in West Java, and DNA from a single hair sample collected from a fence nearby was subsequently analysed (Wirdateti et al., 2024). Our re-examination of the genetic data from the paper raises concerns regarding the credibility of the data and hence the reliability of the conclusion.

Wirdateti et al. amplified and sequenced a 1,043 bp cytochrome b mitochondrial DNA (mtDNA) segment from the hair collected and compared it to those of leopards and tiger subspecies of known origin, including a Javan tiger museum specimen collected in 1930. Phylogenetic trees showed that the hair sample aligned most closely with the Javan tiger museum specimen, forming a clade distinct from other tiger subspecies and the Javan leopard. Based on the results the authors concluded that the hair belongs to the Javan tiger, implying that this tiger subspecies is not extinct. However, after reanalysing the data presented by Wirdateti et al., we conclude there is no support for the authors' conclusions, for the following three reasons: (1) the sequences that the authors obtained are not genuine tiger mtDNA, (2) the sequences are probably nuclear pseudogene copies of mtDNA (Numt), and (3) the sequences generated from the putative and the control Javan tiger specimens are more divergent from one another than the mean difference between other tiger sequences, yet readers cannot evaluate the reliability of the original data because few details concerning quality control were provided in the paper.

Firstly, the sequences that the authors obtained are not tiger mtDNA segments. In the paper, the genetic clade including the hair sample in question (NCBI Accession OQ601561.1) and the Javan tiger museum specimen from 1930 (OQ601562.1) is an outgroup to the tiger mtDNA clade and is phylogenetically equidistant from both tigers and leopards, which is a pattern that was not observed in previous studies involving the Javan tiger (Xue et al., 2015; Sun et al., 2023). To investigate this issue, we conducted a phylogenetic analysis of the two putative Javan tiger sequences produced by Wirdateti et al., along with published mtDNA sequences from *Panthera* species (28 *Panthera tigris* including one *Panthera tigris sondaica* museum specimen, three *Panthera pardus*, three *Panthera leo*, three *Panthera onca* and three *Panthera uncia*; Table 1). We used *MUSCLE 5.1* (Edgar et al., 2022) for multi-sequence alignment of the 42 sequences. We manually trimmed 72 bp from both sides of the alignment, resulting in a 971 bp nucleotide DNA sequence matrix without missing data.

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Table 1 *Panthera* species mitogenome DNA sequences assessed in this study.

Sequence ID	Taxonomy	Source
Seven sequences of *Panthera tigris* from Wirdateti et al. (2024)		
OQ601561.1	*P. t. sondaica* (putative)	NCBI Accession: OQ601561.1
OQ601562.1	*P. t. sondaica* (putative)	NCBI Accession: OQ601562.1
OQ629467.1	*P. t. sumatrae*	NCBI Accession: OQ629467.1
OQ629468.1	*P. t. sumatrae*	NCBI Accession: OQ629468.1
OQ629469.1	*P. t. sumatrae*	NCBI Accession: OQ629469.1
OQ629470.1	*P. t. sumatrae*	NCBI Accession: OQ629470.1
OQ629471.1	*P. t. sumatrae*	NCBI Accession: OQ629471.1
24 sequences of *Panthera tigris* from other studies		
NC_010642.1	*P. tigris*	NCBI Accession: NC_010642.1
pti183	*P. t. sumatrae*	Sun et al. (2023)
pti184	*P. t. sumatrae*	Sun et al. (2023)
pti096	*P. t. sumatrae*	Sun et al. (2023)
pti105	*P. t. tigris*	Sun et al. (2023)
pti103	*P. t. tigris*	Sun et al. (2023)
pti331	*P. t. tigris*	Sun et al. (2023)
PTV02	*P. t. virgata*	Sun et al. (2023)
PTV17	*P. t. virgata*	Sun et al. (2023)
pti305	*P. t. corbetti*	Sun et al. (2023)
pti306	*P. t. corbetti*	Sun et al. (2023)
pti307	*P. t. corbetti*	Sun et al. (2023)
pti247	*P. t. jacksoni*	Sun et al. (2023)
pti269	*P. t. jacksoni*	Sun et al. (2023)
pti272	*P. t. jacksoni*	Sun et al. (2023)
RUSA06_cap	*P. tigris*	Sun et al. (2023)
RUSA23_cap	*P. tigris*	Sun et al. (2023)
RFET0002	*P. t. altaica*	Sun et al. (2023)
RFET0007	*P. t. altaica*	Sun et al. (2023)
pti220	*P. t. amoyensis*	Sun et al. (2023)
HPS	*P. t. amoyensis*	Sun et al. (2023)
M2	*P. t. amoyensis*	Sun et al. (2023)
Maza0008	*P. t. sondaica*	Sun et al. (2023)
Nobb0004	*P. t. balica*	Sun et al. (2023)
12 sequences of other *Panthera* species		
JF720183.1	*P. pardus*	NCBI Accession: JF720183.1
MH588626.1	*P. pardus*	NCBI Accession: MH588626.1
NC_010641.1	*P. pardus*	NCBI Accession: NC_010641.1
NC_028302.1	*P. leo*	NCBI Accession: NC_028302.1
KP001504.1	*P. leo*	NCBI Accession: KP001504.1
KP001505.1	*P. leo*	NCBI Accession: KP001505.1
NC_022842.1	*P. onca*	NCBI Accession: NC_022842.1
KM236783.1	*P. onca*	NCBI Accession: KM236783.1
KF483864.1	*P. onca*	NCBI Accession: KF483864.1
NC_010638.1	*P. uncia*	NCBI Accession: NC_010638.1
MT423723.1	*P. uncia*	NCBI Accession: MT423723.1
MT423722.1	*P. uncia*	NCBI Accession: MT423722.1

We constructed a maximum likelihood phylogenetic tree using *IQ-TREE 2.3.0* (Nguyen et al., 2015), with the HKY+G model selected by *jModelTest 2.1.10* (Darriba et al., 2012), and we evaluated statistical support based on 10,000 bootstraps. Prima facie, our results (Fig. 1) appear to recapitulate the pattern documented by Wirdateti et al. (2024), in which the clade including OQ601561.1 and OQ601562.1 is an outgroup of the tiger mtDNA clade. The clade exhibits an unusually elongated branch length in comparison to those of all other tiger subspecies. This pattern was not observed in previous studies based on partial (Xue et al., 2015) or full (Sun et al., 2023) mtDNA sequences from Javan tiger specimens of known origin, and therefore suggests that the two sequences generated by the authors do not originate from Javan tiger mtDNA.

We further evaluated the pairwise genetic distances (p-distances) amongst the sequences using *Biopython 1.83* (Cock et al., 2009). The mean distance amongst the 28 published tiger mtDNA sequences is $5.645 \times 10^{-3} \pm$ SD 2.733×10^{-3} (378 pairwise distance calculations), whereas the mean distance between the putative Javan tiger sequences generated by the authors and the published tiger mtDNA sequences is $0.07353 \pm$ SD 2.872×10^{-3} (56 pairwise distance calculations), which is 13 times greater than the mean between-tiger genetic distance. For comparison, the mean mtDNA genetic distance between a non-tiger *Panthera* species and a tiger is $0.1049 \pm$ SD 4.854×10^{-3} (336 pairwise distance calculations), which is only slightly greater than the level of genetic distance between the putative Javan tiger and published tiger subspecies.

From the perspectives of both phylogenetic pattern and genetic distance, the two putative Javan tiger sequences generated by the authors exhibit significant disparities from the mtDNA sequences of all tiger subspecies, including the published Javan tiger mtDNA haplotype (Mazaoo08; Sun et al., 2023). Such differences cast doubt on the genuine mtDNA origins of the two Javan tiger sequences. It is improbable for these two sequences to have originated from tiger

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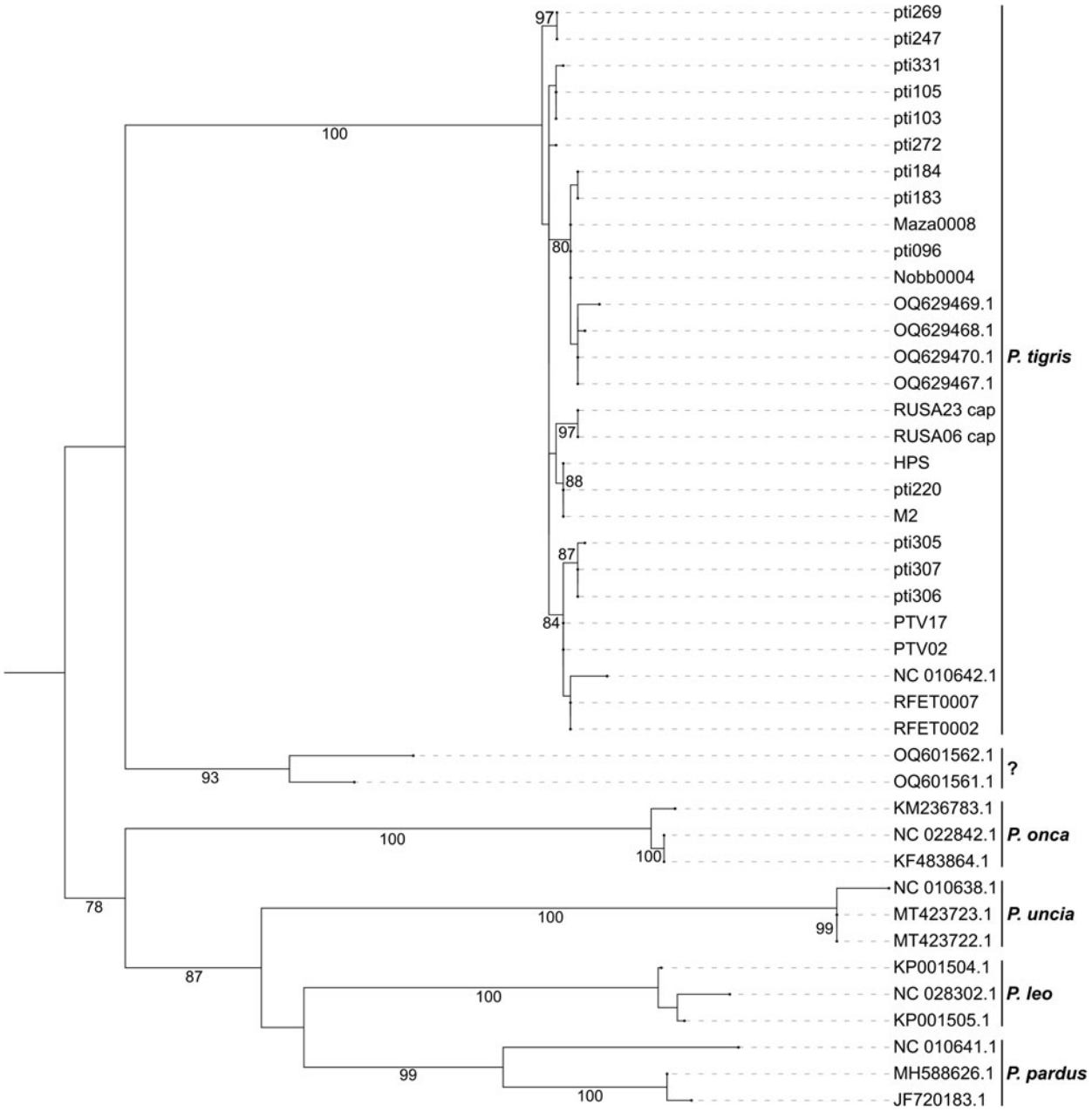

FIG. 1 A maximum likelihood phylogenetic tree inferred from the 971-bp mtDNA cytochrome b sequence assessed in this study. Haplotypes are labeled by their sample IDs. The sequences in question (OQ601561.1 and OQ601562.1) are marked with a question mark. Nodes with bootstrap values larger than 70% based on 10,000 bootstrap replicates are labelled. The reference *Panthera tigris* sequences included all nine subspecies (Table 1).

mtDNA (let alone Javan tiger mtDNA). This would explain why the two sequences do not cluster with other tigers.

Secondly, the sequences that the authors obtained are probably nuclear mtDNA segments. Nuclear mtDNA pseudogene segments (Numts) result from the transfer of cytoplasmic mtDNA (Cymt) copies into the nuclear DNA, a scenario that is often found in the tiger and *Panthera* genomes (Luo et al., 2004; Kim et al., 2006). Given their common origin, there is a possibility that both Cymt and Numt segments can be amplified. This issue is particularly common in the *Panthera* genus as Numt co-amplifications frequently appear in Cymt-targeting PCR experiments in these species (Zhang et al., 2006; Creecy et al., 2024). In GenBank, some pseudogene tiger Numt sequences are mistakenly labelled as tiger Cymt sequences (Morgan et al., 2021).

A *BLASTn 2.14.1* (Camacho et al., 2009) search against the latest tiger genome assembly ptil_mat1.1 (NCBI Accession GCF_018350195.1) indicates Numts are the most probable sources of OQ601561.1 and OQ601562.1. The best

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Table 2 *BLASTn* results of the mtDNA sequences assessed in this study.

Query[1]	Subject[2]	% identity[3]	Alignment length (bp)[4]	Mismatches (bp)	Gaps	Start in query	End in query	Start in subject	End in subject	*E*-value[5]	Bit score[6]
OQ601561.1	NC_056676.1	97.528	971	24	0	1	971	5,568,675	5,569,645	0	1,661
OQ601561.1	NC_056676.1	97.425	971	25	0	1	971	5,585,283	5,586,253	0	1,655
OQ601561.1	NC_010642.1	92.482	971	73	0	1	971	15,188	16,158	0	1,389
OQ601561.1	NC_056668.1	87.449	972	120	2	1	971	24,377,331	24,378,301	0	1,118
OQ601561.1	NC_056677.1	82.851	968	159	4	1	968	68,227,587	68,228,547	0	861
OQ601561.1	NC_056663.1	85.845	763	89	4	1	763	107,153,940	107,154,683	0	793
OQ601561.1	NC_056660.1	79.915	946	166	18	33	971	897,423	898,351	0	673
OQ601561.1	NC_056666.1	79.239	973	190	7	1	971	58,091,713	58,092,675	0	667
OQ601562.1	NC_056676.1	97.528	971	24	0	1	971	5,568,675	5,569,645	0	1,661
OQ601562.1	NC_056676.1	97.425	971	25	0	1	971	5,585,283	5,586,253	0	1,655
OQ601562.1	NC_010642.1	92.181	972	74	2	1	971	15,188	16,158	0	1,373
OQ601562.1	NC_056668.1	87.879	957	113	3	1	955	24,377,331	24,378,286	0	1,122
OQ601562.1	NC_056660.1	80.064	938	167	15	39	971	897,429	898,351	0	678
OQ601562.1	NC_056673.1	79.158	974	190	9	1	971	24,734,451	24,735,414	0	662
OQ601562.1	NC_056666.1	79.137	973	191	7	1	971	58,091,713	58,092,675	0	662
Maza0008	NC_010642.1	98.867	971	11	0	1	971	15,188	16,158	0	1,733
Maza0008	NC_056676.1	90.628	971	91	0	1	971	5,568,675	5,569,645	0	1,290
Maza0008	NC_056676.1	90.525	971	92	0	1	971	5,585,283	5,586,253	0	1,284
Maza0008	NC_056666.1	79.725	947	184	4	1	947	58,091,713	58,092,651	0	678
OQ629467.1	NC_010642.1	98.866	970	11	0	1	970	15,188	16,157	0	1,731
OQ629467.1	NC_056676.1	90.619	970	91	0	1	970	5,568,675	5,569,644	0	1,288
OQ629467.1	NC_056676.1	90.515	970	92	0	1	970	5,585,283	5,586,252	0	1,282
OQ629467.1	NC_056666.1	79.725	947	184	4	1	947	58,091,713	58,092,651	0	678
OQ629468.1	NC_010642.1	98.763	970	12	0	1	970	15,188	16,157	0	1,725
OQ629468.1	NC_056676.1	90.619	970	91	0	1	970	5,568,675	5,569,644	0	1,288
OQ629468.1	NC_056676.1	90.515	970	92	0	1	970	5,585,283	5,586,252	0	1,282
OQ629468.1	NC_056666.1	79.725	947	184	4	1	947	58,091,713	58,092,651	0	678

[1]The queries include the putative mtDNA sequences of Wirdateti et al. (2024) and one previously published Javan tiger mtDNA sequence (Sun et al., 2023).
[2]The tiger reference genome.
[3]The per cent of identity across the aligned sequence between Query and Subject.
[4]The sequence length of the aligned sequence between Query and Subject.
[5]The statistical significance of the sequence alignment between Query and Subject.
[6]Evaluation of the alignment quality.

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(NC_056676.1:5,568,675-5,569,645) and second best (NC_056676.1:5,585,283-5,586,253) matched regions of OQ601561.1 and OQ601562.1 are both located on an autosomal scaffold corresponding to tiger chromosome F2. The nucleotide sequence identities of these matches are all $>$ 97.4% across the 971 bp trimmed sequences, whereas the similarity to the tiger mtDNA (NC_010642.1) is $\leq$ 92.5%. In contrast, mtDNA segments from a previously published Javan tiger sequence (Mazaoo08; Sun et al., 2023) and the Sumatran tigers *Panthera tigris sumatrae* acquired by the authors (OQ629467.1 and OQ629468.1) matched the tiger mtDNA, with $>$ 98.75% sequence similarity (Fig. 1, Table 2). These results suggest that the two supposed Javan tiger sequences generated by the authors are not completely derived from Cymt but more likely from Numt or a mixture of the two.

Thirdly, the high variant rate in the putative Javan tiger sequences prompted concerns related to data accuracy and quality control. There are 24 mismatches in the 971 bp sequence between the hair and the museum Javan tiger specimen, corresponding to a genetic distance of 2.473×10^{-2}. The sequences are twice as divergent from one another as from the mean pairwise difference between other tiger mtDNA haplotypes, or 10 times more divergent than amongst Sunda tigers. In population genomic analyses including all tiger subspecies, only 196 variants were found across the mtDNA (15.5 kb in length with the control region removed), which is c. 12.6 variants per 1,000 bp (Liu et al., 2018). The genetic difference amongst the Javan, Bali *Panthera tigris balica* and Sumatran tigers from Sundaland is even lower, with 44 variants across the 15.5 kb mtDNA sequence, corresponding to c. 2.84 variants per 1,000 bp (Sun et al., 2023). For the tiger nuclear DNA, the single-nucleotide variant rate in different subspecies varies, ranging between 0.026% and 0.072%, which equates to 0.26–0.72 variants per 1,000 bp (Liu et al., 2018). Regardless of their mitochondrial or nuclear DNA origins, the presence of such a large number of variant sites between the putative Javan tiger sequences generated by the authors is unusual for two homologous sequences that are both from tigers, and this is indicative of data unreliability.

There are various potential reasons for these errors, but they cannot be identified based on the information provided by Wirdateti et al. (2024). Processing DNA from single hair or museum specimens requires stringent precautions, including contamination prevention, elimination of potential inhibitors, multiple replications to exclude non-specific stochastic amplifications from trace amounts of the DNA template, and data quality measurements, amongst others. For instance, residual hair keratin could inhibit PCR and Sanger sequencing reactions, hence reducing the efficiency and quality of DNA sequence analysis (Schrader et al., 2012). However, we are not able to determine from the article whether the DNA extraction and downstream experiments were handled with the precautions that are required for working with degraded genetic material, nor how such precautions might have been implemented. As few details with regard to quality control are provided, it is inappropriate to use these sequences to draw conclusions regarding the existence of the Javan tiger.

The report of the rediscovery of the Javan tiger by Wirdateti et al. (2024) garnered widespread attention from the general public as well as amongst scientists and conservationists. We would all be thrilled to learn that the Javan tiger is not extinct and we agree with the authors that '[w]hether the Javan tiger actually still occurs in the wild needs to be confirmed with further genetic and field studies' (Wirdateti et al., 2024, p. 472). However, the authors' initial conclusions based on DNA analysis of one putative tiger hair sample are more likely to be erroneous than to reflect the survival of the Javan tiger, because of the flawed experimental design employed and the lack of scientific stringency. Clear and reliable visual, physical or genetic evidence will be required to conclude that the Javan tiger still survives in Java nearly half a century since the last confirmed sighting.

Author contributions Study design: S-JL; data analysis: Z-YS, S-JL; writing: all authors.

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Conflicts of interest None.

Ethical standards This research abided by the *Oryx* guidelines on ethical standards.

Data availability All the data are published, available in GenBank and on Github at github.com/xinsun1/Xin_etal_2023_NEE_TigerPopGen.

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