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Genetic diversity study on Mitochondrial Cytochrome Oxidase I gene of the Mongolian Old-World Swallowtail (*Papilio machaon Linnaeus, 1758*) Butterfly

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Abstract

Genetic diversity of the mitochondrial Cytochrome Oxidase I gene in the Mongolian Old-World Swallowtail Butterfly from the beginning of Yeru River in Mandal Sum of Selenge Province, Natural Park of Khuvsgul Province, Dadal and Numrug Sum of Khentii Province, Ikh Nart Nature Reserve of Dornogovi Province were studied. The mitochondrial COI gene sequencing results showed 22 haplotypes with 84,6% variability and 36 sites with nucleotide substitution mutation and relative high nucleotide diversity (π =0,010225). There were detected 70 transition and 37 transversion mutation in total 107 mutation. Tajima’s D of the comparison an observed nucleotide diversity against the expected nucleotide diversity was -1,9039 indicating selectively neutral diversity. Comparison of interspecies and intraspecies genetic relationships of Old-World Swallowtail Butterfly by constructing a phylogenetic tree. The Mongolian population of the Old-World Swallowtail Butterfly was divided into 5 subpopulations with the same origin, geographical location and mitochondrial COI gene nucleotide diversity.

Keyword: Mongolian Old World Swallowtail Butterfly, mitochondrial genes, cytochrome C oxidase I, COI

Introduction

Butterfly of the genus *Papilio* have been the source material for many basic studies in insect physiology, genetics, and ecology [1]. *Papilio Machaon Linneaus*, 1758 is the tape species of the genus *Papilio* of the *Papilio* nidae family and of the order Lepidoptera, which is classified as a rare species in the Mongolian Red Book.

The length of the fore wing of Old World Swallowtail Butterfly is 33-50 mm. The wing base color is black, the scales forming the spots and stripes on it are yellow, the base of the upper surface of the hind wing is yellow, and the outer edge of the wing has a yellow spot with a black border, and the inside of it has an arched stripe surrounded by a wide black line. The base color of the stripe is black, with six prominent blue spots on it, and a single red eye spot at the northern end of the inner edge of the wing. There is a long setae on the outer corner of the hind wing. From the end of V month to VIII month, the flying population breeds once a year [2]. In Mongolia they are found in the forest-steppe, river valleys of the steppe region, forest edges, and steppe meadows in the sunny slopes and mountain meadows. It mainly feeds on nectar and pollen of umbellifers (Apiaceae), sages (Lamiaceae), and composites (Asteraceae) [3].

The Old World Swallowtail Butterfly is distributed in various natural zones of Mongolia, but it spreads depending on the topology characteristics of the area, the location of permanent water sources and their remoteness from each other. It has been observed that there is a relatively large number of The Old World Swallowtail Butterfly in the areas where the mean temperature of warmest quarter ranges between 13,8C-15.4C, and have average precipitation in the driest month between -1.68 mm [4]. There are 41 subspecies in the species of *Papilio Machaon* Butterfly.

This Butterfly is found in the entire Palearctic region, from Russia to China, Japan (including the Himalayas and Taiwan), as well as Alaska, Canada, and the United States [5] [6]. Because Swallowtail Butterfly are sensitive to environmental changes, they are used as indicators of habitat quality [7], [8].

Swallowtail Butterfly are recognized as model organisms in ecology, evolutionary biology, genetics, and conservation biology but present numerous unresolved phylogenetic problems. Based on the study of mitochondrial and nuclear DNA nucleotide sequences of 51 species of the genus *Papilio*, 5 subdivisions within *Papilio* (*Heraclides, Pterourus, Chilasa, Papilio and Eleppone*) defined according to traditional taxonomy have been confirmed [9]. A study of the swallowtail Butterfly in North America using microsatellite and mtDNA polymorphisms indicates a hybrid origin of *Papilio Machaon* Butterfly. Nucleotide sequences of COI/COII and EF-1α genes and 15 microsatellites of 133 swallowtail Butterfly from 8 populations of in the *Papillio Machaon* species group were studied and the phylogenetic relationship between them was established [10].

Patterns of nucleotide divergence in COI-COII within species and between species pairs of Lepidoptera were studied and 600 bp location of the COI-COII gene was found to be the most optimally informative [11]. It was established that the mitochondrial cytochrome c oxidase I (COI) gene can serve as the core of a global bio identification system for animals and 648 nucleotides of the COI gene were obtained as barcodes. A COI identification system will provide a reliable, cost-effective and accessible solution to the current problem of species identification. Its variability will also generate important new insights into the diversification of life and the rules of molecular evolution [12]. Using DNA barcode for exploring boundaries among taxa, understanding the geographical distribution of cryptic diversity and evaluating the status of purportedly endemic taxa by Swiss Butterfly were found the species biological characteristics in relation to geographic distribution and ecology and the usefulness for determining the status of endemic taxa and development conservation strategies [13].

Assessing the effects of geographic and ecological isolation on the genetic diversity of Swallowtail Butterfly , the species composition of arctic Butterfly may change due to climate change in the next century [14].

We conducted the genetic diversity study of the Mongolian Old World Swallowtail Butterfly population analyzing sequence data set of BOLD (The Barcode of Life Data System) fragments of the mitochondrial cytochrome C oxidase I enzyme (COI) gene.

Material and methods

We collected 50 samples of Old World Swallowtail Butterfly from valleys of the Yero River in Mandal Sum of Selenge Province, in the Natural Park of Khuvsgul Province, Dadal and Nymrug Sums of Khentii Province, and in Ikh Nart Nature Reserve of Dornogovi Province. Butterfly samples were frozen or stored in 96% ethanol.

DNA was isolated from the Butterfly’s body and head using the EnzyMix ExtraGene DNA Prep kit (IsoGene, Moscow) following the supplier’s instructions.

The PCR reaction for the amplification of the BOLD fragment of the mtDNA CO1 gene was carried out with the GenePak PCR Core kit using primers LCO1490: 5'-ggtcaacaaatcataaagatattgg-3', HC02198: 5'-taaacttcagggtgaccaaaaaatca-3'. The PCR program comprised an initial denaturation phase at 95˚C for 3 min, followed by 35 cycles at 95˚C for 1 min, at 45˚C for 1 min and at 72˚C for 1 min 30 s, with a final elongation step at 72˚C for 4 min. PCR product purity was checked by 2% agarose gel electrophoresis.

Positive PCR products were sequenced on an ABI 3031 automated sequencer (Applied Biosystems) by Zanaa Specs LLC. Population genetic diversity analyses were performed using BioEdit V7.2.5 [16], nucleotide sequence date edited by C lustalW [17] and phylogenetic parameter analysis were performed using Mega V11 [18] programs.

Results

A molecular genetic study of the Mongolian Old World Swallowtail Butterfly population from various geographical areas was conducted analyzing nucleotide diversity of the BOLD fragment of the mitochondrial cytochrome C oxidase I enzyme (COI) gene.

PCR of the mitochondrial cytochrome C oxidase I enzyme gene (COI) of 40 DNA samples from Swallowtail Butterfly carried out and got PCR products and proved their DNA purity and size using 2% agarose gel electrophoresis. Figure 1 shows the PCR products by 2% agarose gel electrophoresis.

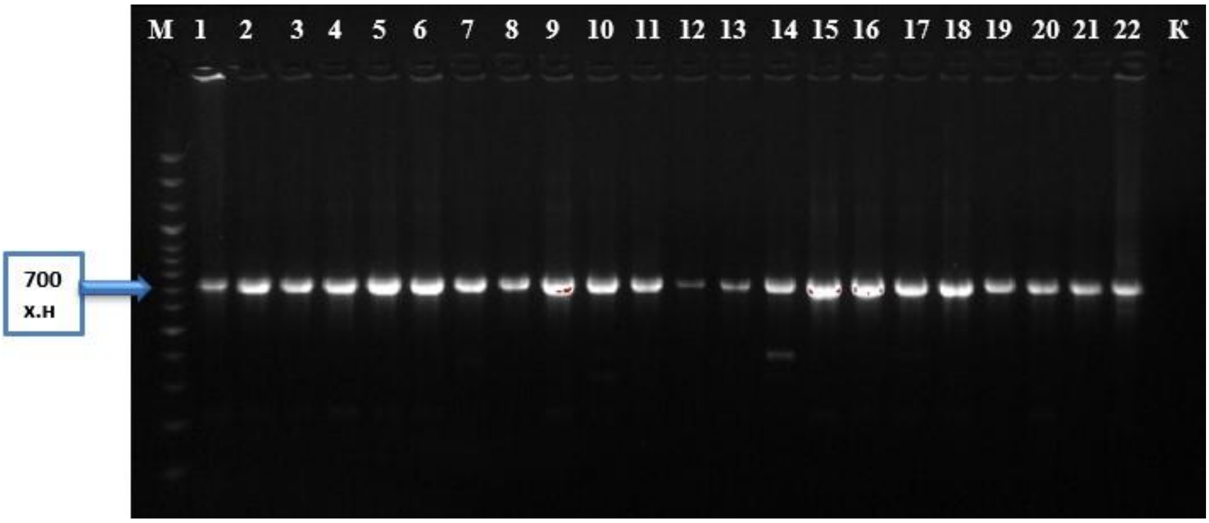

Figure 1. Gel electrophorogram of COI-PCR products (Marker - GenPak DNA marker 100)

PCR products reviewed at the Figure 1, it can be seen that the PCR product is 700 nucleotide base pairs (bp).

In Tables 1-3 and Figure 2 are shown the sequence results of the region of the mitochondrial cytochrome C oxidase enzyme COI gene of the Mongolian Swallowtail Butterfly.

The length of the sequence product was 640 bp, and it was 455 bp after processing and alignment. Nucleotide substitutions or mutations were determined by comparison with the reference sequence (NC_018047.1) of the Chinese Swallowtail Butterfly from the World Gene Bank. Mitochondrial BOLD part of COI gene sequences was carried out for 26 Butterfly specimens and the results are represented in Table 1. Twenty-two haplotypes were detected in the mitochondrial COI gene BOLD region. in the Mongolian population of the Swallowtail Butterfly. Mitochondrial BOLD part of COI gene sequences was carried out for 26 Butterfly specimens and it resulted mutations detected through alignment to reference sequence HM243594.1 generated from GenBank. 70 transition mutations and 37 transversion mutations and 4 nucleotide locations with unique mutations were observed (Table 2).

Table 2 shows the mutation characteristics of the nucleotide sequence of 1458-2116 bp of the mitochondrial COI gene in Swallowtail Butterfly, or the BOLD region of the gene subunit I (COI) cytochrome C oxidase I enzyme.

The evolutionary factors influence analyses on the nucleotide diversity of the mitochondrial COI gene region in the Mongolian Swallowtail Butterfly population were presented at Table 3. A total of 36 mutational sites or nucleotide locations were found in the Mongolian Swallowtail Butterfly population, and the nucleotide diversity (π=0.010225) was high. It was observed that the population size has a positive effect on the diversity of nucleotides, while there is no effect of selection.

Table 1.

Mitochondrial COI gene haplotypes of the Mongolian Swallowtail Butterfly

N	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Transitions	3	3	7	5	4	2	2	2	2	2	3	2	3
Transversions	-	2	8	4	4	1	1	-	1	-	2	-	5
N	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
Transitions	6	4	3	3	2	1	2	2	2	2	3	1	2
Transversions	5	-	-	-	2	-	-	1	1	-	-	-	-

Table 2.

Number of 4-nucleotide mutations or substitutions calculated by maximum composite likelihood

	A	T	C	G
A	-	*6.83*	*2.77*	**11.57**
T	*5.62*	-	**7.71**	*2.46*
C	*5.62*	**18.99**	-	*2.46*
G	**26.36**	*6.83*	*2.77*	-

The number of nucleotide transitions or mutations is indicated in **bold**, and the number of transversal mutations is indicated in *italics*.

Table 3.

Results of genetic diversity of the COI gene

	m	S	p_s	Θ	π	D
Ikh nart	8	15	0.032967	0.012715	0.010204	-1.010270
Khentii	4	3	0.006623	0.003612	0.003679	0.167656
Khonin nuga	5	23	0.050885	0.024425	0.014159	-3.120736
Ikh nart -II	3	1	0.002208	0.001472	0.001472	n/c
Khuvsgul	6	7	0.015453	0.006768	0.006917	0.128407
Total	**26**	**36**	**0.079121**	**0.020734**	**0.010225**	**-1.903906**

m = total number of sequences, S = number of mutated sites, ps = S/n mutation frequency, Θ = ps/a1 distribution probability, π = nucleotide diversity, D - Tajima test statistic

The phylogenetic relationships of the Mongolian Swallowtail Butterfly in a population and the interspecies diversity were determined by constructing a NJ (Neighbor Joining) phylogenetic tree (Figure 2).

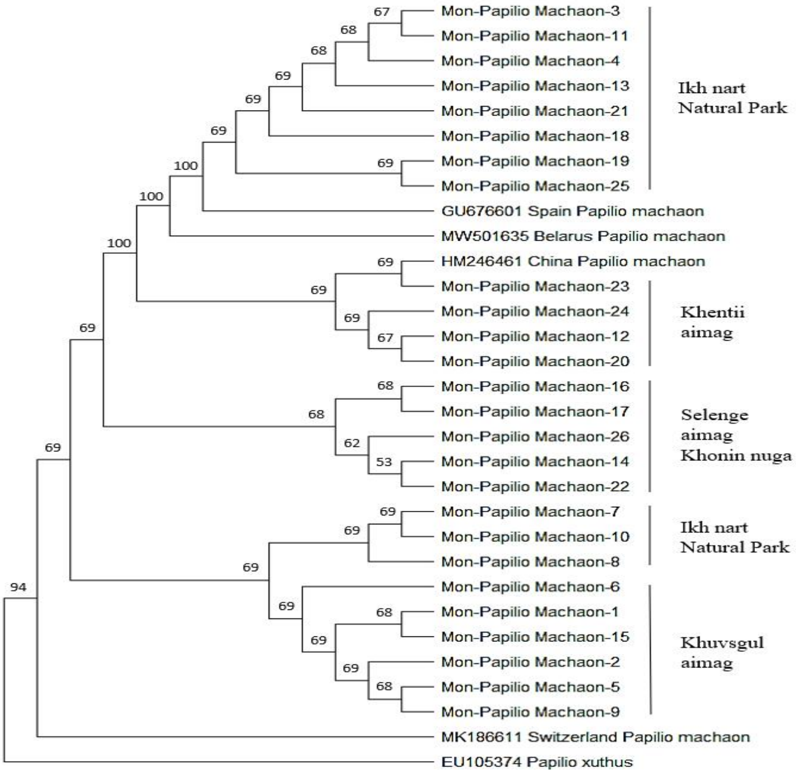

Figure 2. NJ (Neighbor Joining) phylogenetic tree of the Mongolian Old World Swallowtail Butterfly

A phylogenetic tree was constructed using Mega v11 software to explore the Haplotype and nucleotide diversity of the mitochondrial COI gene of the Mongolian Old World Swallowtail Butterfly distributed in different geographic locations (Figure 2). The COI BOLD sequence data of *Papilio Xuthus* as an out group and of Swallowtail P from Spain, Belarus, Switzerland and China as interspecies controls were obtained from GenBank. The phylogenetic tree showed Mongolian Old-World Swallowtail Butterfly are divided into 5 subpopulations. Butterfly from Ikh nart were divided into phylogenetically distinct two large and small subpopulations and Butterfly from Khentii, Khonin Nuga, and Khuvsgul formed each another 3 phylogenetic subpopulations of the same origin.The genetic diversity of Ikh nart Butterfly of 2 groups differed considerable (Table 3), and the group with a relatively larger number of Butterfly with a higher variability was phylogenetically closer to the Spanish and Belorussian Butterfly. Close to the subpopulation of Khentii's Butterfly is the Chinese Swallowtail Butterfly. It has been observed that Swiss Swallowtail Butterfly is relative different from Mongolian *Papilio Machaon* Butterfly.

Discussion

We first conducted the molecular genetic study of the Mongolian Swallowtail Butterfly from various geographical location, although they are distributed in nearly all geographical areas in our country, but it is rarely available to collect them, there was a loss in DNA sample due to separation difficulties from the Butterfly, and it was necessary to select a PCR-DNA samples for the sequencing. Despite the reduced number of identified samples, a sufficient number of Butterfly were included in the population study of the Mongolian Swallowtail Butterfly in Mongolia.

The genetic diversity of mitochondrial mtDNA of the Swallowtail Butterfly has allowed us to more precisely define the differences between species and interspecies variability and asses the processes of evolution factors and adaptation characteristics within species. It was observed that the nucleotide diversity of the mitochondrial COI gene of the Mongolian Swallowtail Butterfly is higher than that of the Swallowtail Butterfly of other countries such as France, Czechoslovakia and Japan [14].

The nucleotide diversity of the mitochondrial COI gene of the Mongolian Swallowtail Butterfly in different geographical locations is considerable different, which indicates the highly variable population genetic structure.

The Tajima test (D) is calculated by comparing the sum of the differences between pairs of individuals in the population to the number of mutations, and shows the influence of evolutionary factors. D=-1.9039 is negative for the Mongolian Swallowtail Butterfly, which indicates that the population is in a natural balance.

A study based on the nucleotide sequence data of the mtDNA gene of the Butterfly *P. machaon* found that it is mostly compatible with the traditional taxonomy based on allozymes and morphological characters, and that it is possible to define the differences between species and subspecies within the Butterfly in detail. Mt DNA studies of *P. Machaon* And *P. Hospiton, P. Polyxenes, P. Zelicaon, P. Indra,* and *P. Alexanor* Butterfly from different geographic locations revealed that *P. Machaon* Butterfly are closest phylogenetically to *P. Polyxenes And P. Zelicaon* Butterfly. At the same time, the variability of *P. Machaon* Butterfly is high, and the presence of interspecies hybrid individuals in the population has been revealed [14].

Our phylogenetic study results showed the diversification of the Mongolian Old-World Swallowtail Butterfly population and revealed 5 phylogenetically different subpopulations with the same origin, geographical location and mitochondrial COI gene nucleotide diversity. The comparison of the Mongolian Old-World Swallowtail Butterfly to the Swallowtail Butterfly from other countries indicated the close phylogenetic relationship between the Swallowtail Butterfly from Khentii and the *P. Machaon* from China, relatively close relationship of the Ikh Nart Old World Swallowtail Butterfly to the Swallowtail Butterfly from Europe, and phylogenetically distinct relation of the Mongolian Old-World Swallowtail Butterfly to the Swallowtail Butterfly from Switzerland.

The Mongolian Old-World Swallowtail Butterfly population has a highly variable genetic structure and genetic diversification of the unique population structure depending on the natural condition and ecology. The expanding molecular genetic study of the Mongolian Old-World Swallowtail Butterfly geophylogenetically in depth can be used to evaluate ecosystem changes and develop conservation strategies.

Conclusion

In the Mongolian Old-World Swallowtail Butterfly population were detected 22 haplotypes of the mitochondrial COI gene with 84.6% variability. It revealed nucleotide substitution mutations at 36 locations of 455 bp in the BOLD region of the mitochondrial COI gene with frequency of 0.079.

The higher nucleotide diversity (π=0.010225) of the mitochondrial COI gene of the Mongolian Swallowtail Butterfly in comparison to Swallowtail Butterfly from other foreign countries was observed.

The Tajima's test statistic (D=-1.9039) calculated by comparing the number of nucleotide locations with mutations in the BOLD part of the mitochondrial COI gene to the nucleotide diversity is negative, indicating that the Mongolian Swallowtail Butterfly population is in a natural equilibrium.

The phylogenetic study of the Mongolian Swallowtail Butterfly population revealed 5 interspecific subpopulations differed in their geographic location and mitochondrial COI gene nucleotide diversity.

Authors' Contribution

A.Ts. designed and performed the study and analyzed data; Ts. Ts. supervised the experiment and written; Ts. B. reviewed and edited this work.

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References

[1] M.S.Caterino and F.A.H. Sperling, "Papilio Phylogeny Based on Mitochondrial Cytochrome Oxidase I and II Genes," Mol. Phylogenet. Evol., vol. 11, no. 1, pp. 122-137, Feb. 1999, https://doi.org/10.1006/mpev.1998.0549

[2] Ч.Гантигмаа, Б.Анударь, and Л.Жаргалсайхан, Монгол орны эрвээхэй. Боть I. Улаанбаатар, 2021.

[3] Ч.Гантигмаа, Б.Анударь, and Л. Жаргалсайхан, Монгол орны эрвээхэй. Боть II. Улаанбаатар, 2022.

[4] B.Anudari, C.Gantigmaa, and D.Bayarmaa, "Distribution characteristics of Old-World Swallowtail (Papilio Machaon Linnaeus, 1758) Butterfly in Mongolia.," Proceedings of the Institute of Biology, vol. 37, pp. 47-51, 2021.

[5] N.Mark Collins and G.Michael Morris, Threatened Swallowtail Butterfly of the World: The IUCN Red Data Book., Gland&Cambridge: International Union for Conservation of Nature. Switzerland and Cambridge U.K, 1985.

[6] M.A.Salmon, P.Marren, and B. Harley, The Aurelian legacy: British Butterfly and their collectors. Berkeley: University of California Press, 2000. https://doi.org/10.1163/9789004475472

[7] M.S.Warren et al., "Rapid responses of British Butterfly to opposing forces of climate and habitat change," Nature, vol. 414, no. 6859, pp. 65-69, Nov. 2001, https://doi.org/10.1038/35102054

[8] J. C. Habel, A. Segerer, W. Ulrich, O. Torchyk, W. W. Weisser, and T. Schmitt, "Butterfly community shifts over two centuries: Shifts in Butterfly Communities," Conserv. Biol., vol. 30, no. 4, pp. 754-762, Aug. 2016, https://doi.org/10.1111/cobi.12656

[9] E. V. Zakharov, M. S. Caterino, and F. A. H. Sperling, "Molecular Phylogeny, Historical Biogeography, and Divergence Time Estimates for Swallowtail Butterfly of the Genus Papilio (Lepidoptera: Papilio nidae)," Syst. Biol., vol. 53, no. 2, pp. 193-215, Apr. 2004, https://doi.org/10.1080/10635150490423403

[10] J.R.Dupuis and F.A.H. Sperling, "Repeated Reticulate Evolution in North American Papilio Machaon Group Swallowtail Butterfly," PLOS ONE, vol. 10, no. 10, p. e0141882, Oct. 2015, https://doi.org/10.1371/journal.pone.0141882

[11] A. D. Roe and F. A. H. Sperling, "Patterns of evolution of mitochondrial cytochrome c oxidase I and II DNA and implications for DNA barcoding," Mol. Phylogenet. Evol., vol. 44, no. 1, pp. 325-345, Jul. 2007, https://doi.org/10.1016/j.ympev.2006.12.005

[12] P. D. N. Hebert, A. Cywinska, S. L. Ball, and J. R. deWaard, "Biological identifications through DNA barcodes," Proc. R. Soc. Lond. B Biol. Sci., vJ. Litman et al., "A DNA barcode reference library for Swiss Butterfly and forester moths as a tool for species identification, systematics and conservation," PLOS ONE, vol. 13, no. 12, p. e0208639, Dec. 2018, https://doi.org/10.1371/journal.pone.0208639

[13] Z. G. MacDonald, J. R. Dupuis, C. S. Davis, J. H. Acorn, S. E. Nielsen, and F. A. H. Sperling, "Gene flow and climate-associated genetic variation in a vagile habitat specialist," Mol. Ecol., vol. 29, no. 20, pp. 3889-3906, Oct. 2020, https://doi.org/10.1111/mec.15604

[14] F. A. H. Sperling and R. G. Harrison, "Mitochondrial DNA Variation Within and Between Species of The Papillio Machaon Group of Swallowtail Butterfly," Evolution, vol. 48, no. 2, pp. 408-422, Apr. 1994, https://doi.org/10.1111/j.1558-5646.1994.tb01320.x

[15] T. A. Hall, "BIOEDIT: A user friendly biological sequence alignment editor and analysis program for windows 95/98/NT.," pp. 95-98, 1999.

[16] J. D. Thompson, D. G. Higgins, and T. J. Gibson, "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice," Nucleic Acids Res., vol. 22, no. 22, pp. 4673-4680, 1994,

[17] K. Tamura, G. Stecher, D. Peterson, A. Filipski, and S. Kumar, "MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0," Mol. Biol. Evol., vol. 30, no. 12, pp. 2725-2729, Dec. 2013, https://doi.org/10.1093/molbev/mst197