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Phylogeographic Structure of Freshwater *Tor* sp. in River Basins of Sabah, Malaysia

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Abstract: We characterized the genetic diversity, phylogeography, and demography of *Tor* sp. (Cyprinidae) from Sabah, Malaysian Borneo, by examining nucleotide variation in the D-loop region of the mtDNA. Sequence analysis of 18 populations (N = 173) yielded 35 unique mtDNA haplotypes with mean haplotype and nucleotide diversity of 0.833 and 0.023, respectively. Phylogenetic reconstructions using Bayesian, neighbor-joining, and maximum parsimony methods, as well as haplotype network, revealed four well-defined clades, namely, the eastern, central, northwestern, and southwestern clades, which corresponded to evolutionarily significant units (ESUs). These ESUs were estimated to have become separated since the late Miocene to Pliocene era (between 5 and 1 million years ago), with the central highlands of Sabah Crocker Trusmadi Range (CTR) constituting the main barrier to genetic exchange between clades. Analysis of molecular variance (AMOVA) and pairwise genetic differentiation showed significant population structuring (Φ_{ct} = 0.575–1.000, $p < 0.05$). We further identified eight major groups of river systems harboring reproductively isolated *Tor* subpopulations. Neutrality statistics and Bayesian skyline plots (BSP) suggested constant population size over time for most *Tor* populations. *Tor* sp. in Sabah is comprised of four ESUs (eastern, central, northwestern, and southwestern ESUs), and that each ESU can be compartmentalized into 1–4 MUs. Due to isolation by distance, the highest number of MU occurs in the low-elevation drainages of Eastern Sabah, which is the largest in terms of land area. The evidence provided by this study supports the hypothesis that the four ESU represent genetically distinct subpopulations of *Tor* and highlight the urgent need for the in situ conservation of these subpopulations.

Keywords: phylogeography; mitochondrial DNA D-loop; *Tor* sp.; *Tor* Crocker Trusmadi Range; population genetics

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1. Introduction

Resources for the conservation of biodiversity are limited. Prioritizing allocation to conservation units (CUs) consisting of intraspecific-level groups that are ecologically, genetically, and phylogenetically distinct is an optimum approach to protecting imperiled species [1,2]. Delineating and delimiting biologically meaningful CUs in the context of freshwater biodiversity can be complicated by natural and anthropogenic fragmentation of habitat [3]. Consequently, a species inhabiting a small area may comprise highly structured subpopulations that are further divided into smaller units that occupy a continuum of genetically stratified hierarchies [4]. The two more widely recognized types of CU are the ecologically significant units (ESUs) [1] and management units (MUs) [5]. Based on definitions in the work of [5], an ESU is used herein, *sensu lato*, to refer to a conspecific group that is phylogenetically distinct (i.e., monophyletic), whereas an MU is a subset of an ESU that possesses a minor but significantly discrepant set of alleles and/or haplotypes. The latter may constitute a polyphyletic group *sensu* [6].

Anthropogenic modification of habitat from logging and agricultural activities, overharvesting of fish, and illegal fishing are the main local threats to aquatic biodiversity in Sabah [7–11], and these impacts are likely compounded by extreme changes in the global climate [12]. Delineating CUs among the freshwater biodiversity of Sabah may go a long way in strategizing efforts to protect habitats and threatened species. A previous study [13] identified two reciprocally monophyletic genodemes of *Channa striata* along the western and eastern coasts of Sabah, Malaysian Borneo, which corresponded to two ESUs comprising historically separated subpopulations with allopatrically evolving microsatellite alleles. Further, there was a lack of migration between adjacent river systems, and several drainages appeared to have formed internal clades with suitable bootstrapping support (i.e., percent bootstrap > 70%; [14]), suggesting the presence of MUs within each genome. *Channa striata* is primarily distributed along low-elevation coastal habitats [15,16], and, therefore, current knowledge on the biogeography of freshwater habitats in Sabah is limited to these areas. To fill this gap in information, we carried out a study to further identify other ESU/ESUs pertaining to the inland freshwater ecosystem of Sabah with respect to the phylogeography of *Tor* sp. We hypothesized that the central highland freshwater habitats of Sabah harbor at least one genetically isolated ESU that is distinct from the coastal ESUs in the work of [13]. Identification of MUs will also be carried out concurrently to assist in the management of freshwater biological resources in the state.

Three names have previously been applied to *Tor* specimens collected in Sabah viz. *T. douronensis*, *T. tambra*, and *T. tambroides* [11,15–17] are the most ubiquitous species names in the literature on Southeast Asian *Tor* [18,19]. The description of the three species is associated with type specimens collected from Java and Sumatra, Indonesia [18]. A taxonomic revision in 1993 reassigned *T. douronensis* and *T. tambroides* as junior synonyms of *T. tambra* [20]. However, later phylogenetic reconstructions with respect to mitochondrial DNA (mtDNA) haplotypes among *Tor* and its sister genus *Neolissochilus* as an outgroup supported the reciprocal monophyly of *T. tambra* and *T. tambroides* [21,22]. The distribution of *T. tambra* ranges from its type locality in Western Java toward mainland Southeast Asia to its north [22], while *T. tambroides* is found in its type locality in Sumatra and possibly Java [22,23] and in Peninsular Malaysia and Sarawak, Malaysian Borneo [21, 24]. Meanwhile, molecular phylogeny of specimens designated as the nominal species *T. douronensis* highlighted the presence of three deeply divergent lineages comprising Malaysian Borneo, Sumatra, and Mekong River groups [21]. A lack of Javanese topotypes in molecular systematic studies has so far precluded conclusive nomenclatural revision of specimens designated as *T. douronensis* in Sundaland. Yet, based on biogeographical distribution, the Sumatra and Mekong River lineages were suspected to be misidentified *T. tambra* specimens [18], while the Malaysian Borneo group was proposed to comprise a new species of *Tor* [18,22]. Malaysian Borneo is, therefore, home to at least two genetically distinct species of *Tor* viz. *T. tambroides* and a hitherto undescribed *Tor* sp.; both species occur in sympatry in Sarawak while only the latter is found in Sabah [21,24–26]. Kottelat [18] noted that the name *T. streeteri*, a synonymized name based on a type specimen from Sarawak, may potentially be applied to this new species, but a formal description using this name has yet to emerge. Hence, for the purpose of this study, we will refer to the Malaysian Borneo *Tor* (*sensu* [18,22]) as *Tor* sp. pending proper species designation.

In Sabah, *Tor* sp. is commonly known as *Tor*. The species has been reported to inhabit fast-flowing mountain and hill streams with rocky bottoms [15–17]. Southeast Asian *Tor* are potamodromous migratory fishes with homing behavior, capable of performing long-distance migration (50–120 km) to downstream feeding habitats and upstream spawning sites during the wetter monsoon periods [19,27]. *Tor* is a commercially important inland fish species in Sabah with market prices between 15 and 50 MYR (roughly 4–12 USD) per kilogram. From the 1970s until the late 1990s, the natural population of *Tor* sp. and other commercially harvested inland fishes in Sabah were in rapid decline; besides habitat degradation and overfishing, the absence of a legal framework to regulate inland freshwater fisheries during this period was cited as another crucial factor [9,10]. Passing of the

Natives Courts (Native Customary Laws) Rules 1995 [28] and Sabah Inland Fisheries and Aquaculture Enactment 2003 [29] were instrumental in curbing overfishing of the inland freshwater resources in the state. Provisions in Sections 58 of [28] and 35–37 in the work of [29] enabled the Sabah Department of Fisheries to implement the "tagal" system, a community-based fisheries management and conservation program that entails establishing community fisheries management zones that serve as freshwater fish refugia. The positive impacts of the tagal system and its refugia on the abundance of fish stocks (including *Tor* sp.) and livelihoods of rural communities in Sabah were apparent in the immediate years that followed [8–10]. Over a decade later, an expanding human population in Sabah brings inland fisheries into the spotlight once again due to its potentially significant role in ensuring food security and alleviating poverty [30]. Given successes in captive breeding of *Tor* spp. elsewhere [31], and its commercial appeal, *Tor* sp. is a keystone species for a state-level program in the 12th Malaysia Plan (2021–2025) aimed at developing a sustainable *Tor* aquaculture industry in Sabah. The objective of the program is to ensure an adequate supply of *Tor* sp. in the market and to concurrently restock the wild population in Sabah. In achieving these goals, information on the scale at which the Sabah *Tor* sp. is structured and its levels of genetic diversity are prerequisites for effective management and conservation of the resources.

Apart from the inclusion of small sample sizes in molecular phylogenetic studies [21,22,25], *Tor* sp. throughout Sabah had been scarcely sampled for population-level genetic analyses (but see the work of [32]) relative to its congener in Sarawak where the genetic structure of the population (even to the level of broodstock) is well-characterized [26,33]. The *Tor* sp. in Sarawak has been shown to be heterogeneous with respect to adaptive [24–26] and neutral [33,34] genetic markers, highlighting the importance of accounting for the genetic distinctiveness of *Tor* sp. broodstocks in breeding and restocking efforts.

Management of genetically distinct populations as a single entity could otherwise result in overexploitation of populations, leading to reduced genetic diversity and perhaps even extirpation [35,36]. Here, we employed a partial 5′ region of the displacement loop (D-loop) of the mtDNA in characterizing the population genetic structure and demographic history of *Tor* sp. The mtDNA D-loop has been successfully used in fisheries management and conservation [37,38] despite some debates on its usage as a marker for molecular diversity [39,40]. The mtDNA is useful in reconstructing the phylogeny of metazoa as it provides an unbroken genealogy of the maternal lines of ancestry. Lineage sorting is also expected to occur more rapidly with mtDNA haplotypes due to its four-fold smaller effective population size relative to nuclear loci [38].

2. Materials and Methods

2.1. Collection of Samples

Collection of samples was carried out at 18 localities consisting tributaries along 10 major rivers (or Sungai in Malay) throughout Sabah: Sorinsim (abbr. KSO), Tangkol (KTA), Sunsui (KSU), and Marak Parak (KMP) representing Sungai Kanarom; Luanti (SLU) and Poring (SPO) representing Sungai Sugut; Kinarasan (SKI) and Paus (SPA) representing Sungai Labuk; Pinipi (SPI) and Kironggu (SKIR) representing Sungai Kinabatangan; Menserulong (IME) and Rugading (IRU) representing Sungai Pagalan; Tokulung (WCTO) and Lingkubang (WCLI) representing Sungai Kadamaian; Gontung (WCGO) representing Sungai Tuaran; Babagon (WCBA) representing Sungai Moyog; Doingin representing Sungai Kimanis; and Telantang (WCTE) representing Sungai Bongawan (Figure 1). Sungai Kanarom, Kadamaian, Tuaran, Moyog, Kimanis, Bongawan, and Pagalan flow into the South China Sea while Sungai Sugut, Labuk, and Kinabatangan flow into the Sulu Sea. At each sampling locality, *Tor* sp. individuals were caught using either a seine net or cast net depending on the depth and width of the tributary. Species identity of individuals caught was confirmed by referring to the work of [16,19,41], following which a clipping from the caudal fin was extracted and preserved in 95% ethanol. At the laboratory, the samples were kept at −80 °C for long-term storage.

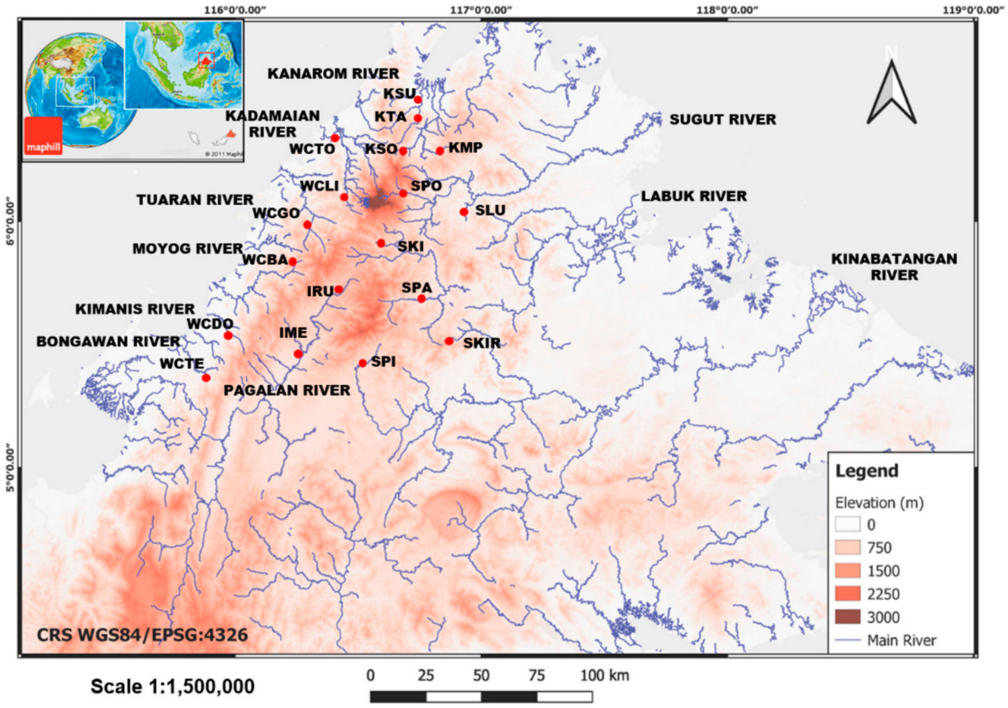

Figure 1. Sample codes and the location of *Tor* populations. The inset shows the areas of collection in relation to the Malaysian Peninsular and Malaysian Borneo, Sarawak.

2.2. Laboratory Analysis

Genomic DNA was isolated using the Wizard Genomic DNA Extraction Kit (Promega, Madison, WI, USA) with validated modifications to the protocol. An mtDNA segment spanning the 3′ end of the transfer RNA gene for proline (tRNAPro) and the partial 5′ end of the D-loop region was amplified using forward primer TD_TRNAPRO_F3 (5′-AGC CAG AAT TCT AAA CTA AAC TAT-3′) and reverse primer TD_CSBD_R3 (5′-TTG GCA TGG GTA AT-3′). These primers were designed using PrimerSelect module [42] in DNAStar v 5.05 [43] by using multiple sequence alignments of *Tor* sp. D-loop regions available from Genbank as reference (AP011326 *Tor putitora*, JX444718 *Tor tambroides*, KC914620 *Tor putitora*, KF305826 *Tor sinensis*, KP795444 *Tor tor*, KR868704 *Tor tor*, KR868706 *Tor khudree*, KU870466 *Tor mosal mahanadicus*). The expected molecular-weight size of the amplicon was between 480 and 490 base pairs (bp).

PCR reactions contained 1X Qiagen TopTaq master mix buffer (Qiagen, Valencia, CA, USA), 0.2 µM of each primer, approximately 50–100 ng of template DNA and adjusted to a final volume of 50 µL with sterile double-distilled water. DNA was initially denatured at 94 °C for 3 min, then 35 cycles of 94 °C denaturating for 30 s, 58 °C annealing for 30 s, and 72 °C extension for 45 s, followed by a final extension period of 10 min at 72 °C. The amplified products were visualized on 1.3% agarose gels containing SYBR Safe (Invitrogen, Waltham, MA, USA) cyanine dye, run for approximately 55 min at 75 V and photographed under blue light. A 100 bp DNA ladder (Fermentas, Vilnius, Lithuania) was used as a standard size marker. The PCR products were further purified using QIAquick Gel Purification Kit (Qiagen, Germantown, MD, USA). All purified PCR products were sent to Aitbiotech Pte Ltd. (Singapore) for sequencing using the forward and reverse primers (TD_TRNAPRO_F3 and TD_CSBD_R3), enabling the identification of ambiguities.

2.3. Data Analysis

The sequence chromatograms were visually inspected on Chromas v 2.6.6 (Technelysium, South Brisbane, QLD, Australia) (http://technelysium.com.au, accessed on 1 January 2021), and the paired-end reads were concatenated in MEGA X v 10.2.3 [44]. No consistently ambiguous bases in the chromatograms were detected and hence assumed lack of mtDNA

sequence heteroplasmy with respect to the sequenced region. Trimming of the flanking primer sequences and the remaining five bases at the 3′ end of the tRNAPro region yielded 438–443 nucleotide positions. Sequences were validated for taxonomic identity using the Basic Local Alignment Search Tool Program v 2.2.6 available online at the NCBI website (http://www.ncbi.nlm.nih.gov/index.html, accessed on 1 January 2021). The sequences obtained herein showed high similarity with D-loop sequences from other congeneric taxa, with E-values approaching zero. No amplification or co-amplification of putative paralogs previously reported in other *Tor* spp. were detected where a different combination of primers was used [45]. Multiple sequence alignments for the edited sequences were performed using the CLUSTALW program of MEGA X v 10.2.3 [44].

2.3.1. Genetic Diversity

The genetic variation within and among the populations was reported as haplotype (h) and nucleotide (π) diversity calculated using DnaSP v 6.12.03 [46]. The selection of the best model to describe the nucleotide substitution was based on the lowest Bayesian information criterion calculated using the "Model Selection (ML)" feature in MEGA X v 10.2.3 [44]. Out of the 24 candidate models, the Tamura three-parameter plus gamma rate model (TN92 + G) [47] nucleotide substitution model assuming a mixture of gamma distributed (+G) was the best-fit model.

2.3.2. Haplotype Relationship, Divergence Time

The level of divergence and relationships among haplotypes of *Tor* was inferred via neighbor-joining (NJ) [48] and maximum-likelihood (ML) techniques implemented in the program MEGA X v 10.2.3 [44] and Bayesian method using the program BEAST v 1.10.4 [49]. The freshwater *Neosochillus strateyi* (GenBank accession no.: NC 031555) was used as outgroup taxa. The Tamura three-parameter plus gamma rate model (TN92+G) [47] was selected by MEGA X v 10.2.3 [44] as the best-fitting substitution model for NJ and ML methods based on the Bayesian information criterion. Bayesian information criterion was demonstrated to be the most appropriate model-selection criteria because of their high accuracy and precision [50]. The confidence level at each node was assessed by 1000 bootstrap replication [51]. BEAST v 1.10.4 [49] was used to jointly estimate the mitochondrial phylogeny and divergence timings using a Bayesian approach. Because no fossils are known for this lineage, it was not possible to calibrate the molecular clock using fossil-based minimum ages [21]. Thus, to calibrate the mitochondrial tree, a normal prior on the D-loop substitution rate with mean 0.017 mutations/site/Myr and standard deviation 0.0025 mutations/site/Myr were applied. This prior provided upper and lower 95% confidence intervals of 0.022 and 0.012 mutations/site/Myr, respectively, which encompasses published D-loop substitution rates of various families of fishes [52]. Appropriate substitution models were chosen for D-loop using MEGA Xv 10.2.3 [44] under the Bayesian information criterion. Preliminary analyses of different combinations of molecular clock model (relax, strict) and population growth (exponential, constant) resulted in approximately the same results with ESS values of more than 200 indicating strong support. Thus, a coalescent tree prior with exponential population growth rate and a lognormal relaxed clock model was specified in the analysis. The analysis consisted of 10^7 generations run, sampling the Markov chain Monte Carlo (MCMC) chain every 1000 generations. The first 10% of the run was discarded as burn-in. The output of runs was imported into Tracer v 1.7.1 [53] to assess whether the number of MCMC steps was sufficient to bring effective sample sizes (ESS) above the minimum threshold of 200, as recommended in the BEAST v 1.10.4 [49] documentation. All ESS values greatly exceeded 200. A maximum clade credibility (MCC) time-calibrated tree was then selected from the posterior sample of trees using Treeannotator [49] and annotated with posterior clade probabilities and 95% confidence intervals (CI) for node ages. Tree diagram with divergence time estimates was visualized in FigTree v 1.4.4 [54]. The trees were compared against the haplotype network,

which was created using TCS network methods [55] in PopART [56]. TCS is a statistical parsimony approach to determine connections between haplotypes [55].

2.3.3. Genetic Differentiation and Population Structure

Genetic differentiation among populations was estimated by computation Φ_{st} (using genetic distances with Tamura) as implemented in Arlequin v 3.5.2.2 [57]. Significance levels of pairwise Φ_{st} values, under the null hypothesis of no differentiation, were computed by permutation tests from 10,000 random permutations between populations and, when appropriate, populations between groups. Population structures were analyzed in several ways. First, genetic differentiation due to linear geographic distance for the 18 populations was analyzed. This isolation-by-distance analysis regressed estimates of pairwise population genetic distance against the linear distance separating pairs of populations. This regression was calculated using a Mantel test in GenAlEx v 6.5 [58], with 1000 permutations to assess statistical significance. Second, linear distance is not always the best predictor of genetic differentiation, as different geographic and historical forces may contribute to large genetic differentiation even over very small spatial scales. To ascertain a potentially better phylogeographic predictor of genetic variance, two hypotheses using AMOVAs [59], performed with 10,000 permutations across and within the sampled loci in Arlequin v 3.5.2.2 [57] was addressed. Five models were constructed that reflect putatively different genetic structures across the landscape: (1) clustering of populations into groups based on current river basins/watersheds, and (2) clustering of populations into groups based on location relative to the CTR separation. To test these models, five grouping schemes were employed. For the first and second models, populations were grouped into river systems according to biogeographical division and phylogeographic results, yielding 10 groups and 8 groups, respectively. Sabah river basins/watershed data were obtained from the Department of Irrigation and Drainage (https://www.water.gov.my, accessed on 1 February 2021). The third to five models were constructed to assess population structure separated by the CTR, which sorted the sampled locations into two, three, and four groups. For each model, pairwise phi-statistics were calculated and with 10,000 permutations to assess statistical significance. Lastly, all 173 individuals were concluded with adequate genotype information from all 18 populations to explore population structure with Bayesian Analysis of Population Structure v 6.0 (BAPS) software [60]. BAPS was run with 10 replicates for every level of k (1–18) without origin information ("clustering of individuals"), and the results were averaged according to the resultant likelihood scores.

2.3.4. Demography

Three neutrality tests were calculated to examine whether the haplotype data deviated from the expected values obtained under the neutral model and the assumption of demographic equilibrium. Fu and Li's D* [61], Fu's Fs [62] and R2 [63] tests were powerful tests to detect sudden population expansion, sudden contraction and bottleneck events [63,64]. The tests were applied by population and major drainage using the program DnaSP v 6.12.03 [46] with 10,000 replications. Any departure from the assumption of neutrality indicates the occurrence of non-neutral processes, such as gene flow, changes in population size, or selection. A significant negative value is indicative of a recent population expansion, whereas a significant positive value signifies a recent demographic decline or a strong population structure [62]. Since departures from neutrality are often due to changes in effective population size, Bayesian skyline plots (BSP) [65] were also applied as implemented in BEAST v 1.10.4 [66]. BSP [65] plots were generated by population and major drainage. No BSP [65] analyses were inferred from major clades because of the confounding effect of population structure on BSP [67] inferences on demographic history. An HKY + G model of mutation, the closest model available in BEAST v 1.10.4 [66] to the model T92 + G that was suggested by the best-fit DNA substitution model by MEGA X v 10.2.3 [44], was used. MCMC runs of 100 million iterations, sampling every 10 thousand steps, were performed assuming a strict molecular clock. Bayes factor for Bayesian skyline

plot and constant model were determined to test the fit of the empirical data between demographic models and to ensure that biological conclusions are driven by the data and not by prior model selection. The first 10% of iterations were discarded as burn-in. Tracer v 1.7.1 [53] was used to check convergence by measuring effective sample sizes (ESS) of all parameters (ESS > 200) and to calculate the mean value, the upper and lower bounds of the 95% highest posterior density interval of effective population sizes, and to draw skyline plots. Estimation of time since expansion event was inferred from converting mutations units in estimates of years using a D-loop mutation rate of 0.017 mutations/site/Myr [52].

3. Results

3.1. Genetic Diversity

The partial D-loop sequence was successfully sequenced across 173 *Tor* individuals resulted in a final alignment of 447 nucleotide positions in the final data set after multiple pairwise alignments. The sequenced region was A-T-rich, containing, on average, 37.4% A, 33.3% T, 15.5% C, and 13.7% G. A total of 332 sites (74.3%) were conserved while the 115 segregating sites included 11 positions with insertion/deletion (InDel) mutations. The latter consisted of 1- and 4-bp gaps with an average length of 1.31 bp. All the gaps in the aligned sequences were included in the subsequent analyses because each one was present in multiple haplotypes. The sequences generated were deposited in Genbank (accession nos.: MH686405–MH686439).

A total of 35 unique mtDNA haplotypes were identified (Table 1), where 27 were private haplotypes that occurred in only one locality. The subpopulation sampled from SPA (n = 10) had the highest number of private haplotypes with eight. Seven haplotypes were shared among at least two subpopulations of *Tor* sp. although they were still within the same river systems, namely, Hap1 (shared among KMP, KTA, KSO, KSU in Sungai Kanarom); Hap3 (WCTO and WCLI in Sungai Kadamaian); Hap6 and Hap11 (IRU and IME in Sungai Pagalan); Hap17 (SKI and SPA in Sungai Labuk); Hap18 (SPI and SKIR in Sungai Kinabatangan); and Hap19 (SLU and SPO in Sungai Sugut). Hap2 was present in WCGO in Sungai Tuaran and WCBA in Sungai Moyog, making it the only mtDNA type common between subpopulations from different river systems. The mean overall haplotype (h) and nucleotide diversity were 0.833 and 0.023, respectively, with nine subpopulations exhibiting a lack of genetic variation.

Table 1. Summary of genetic diversity of *Tor* sp. subpopulation in each locality.

Locality	n	h	Haplotype Designation (Individual Number)	S	π	H_d
Kanarom River						
KMP	10	1	Hap1 (10)	0	0.000	0.000
KSU	10	2	Hap1 (9), Hap26 (1)	1	0.000	0.200
KTA	8	2	Hap1 (7), Hap27 (1)	1	0.001	0.250
KSO	10	1	Hap1 (10)	0	0.000	0.000
Sugut River						
SLU	10	1	Hap19 (10)	0	0.000	0.000
SPO	10	1	Hap19 (10)	0	0.000	0.000
Labuk River						
SKI	10	6	Hap12 (1), Hap13 (1), Hap14 (3), Hap15 (3), Hap16 (1), Hap17 (1)	21	0.021	0.800
SPA	10	7	Hap17 (3), Hap20 (1), Hap21 (1), Hap22 (2), Hap23 (1), Hap24 (1), Hap25 (1)	26	0.023	0.911
Kinabatangan River						
SKIR	10	1	Hap18 (10)	0	0.000	0.000
SPI	10	1	Hap18 (10)	0	0.000	0.000
Pagalan River						
IRU	10	4	Hap6 (6), Hap11 (1), Hap34 (1), Hap35 (2)	5	0.003	0.644
IME	9	6	Hap6 (4), Hap7 (1), Hap8 (1), Hap9 (1), Hap10 (1), Hap11 (1)	13	0.008	0.833
Kadamaian River						
WCTO	9	1	Hap3 (9)	0	0.000	0.000

Table 1. *Cont.*

Locality	n	h	Haplotype Designation (Individual Number)	S	π	H_d
WCLI Tuaran River	7	4	Hap3 (4), Hap31 (1), Hap32 (1), Hap33 (1)	13	0.009	0.714
WCGO Moyog River	10	1	Hap2 (10)	0	0.000	0.000
WCBA Kimanis River	10	1	Hap2 (10)	0	0.000	0.000
WCDO Bongawan River	10	3	Hap28 (4), Hap29 (5), Hap30 (1)	4	0.004	0.644
WCTE	10	2	Hap4 (9), Hap5 (1)	3	0.001	0.200

n: number of individuals, h: number of haplotypes, S: number of polymorphic sites, $H_{d:}$ haplotype diversity, π: nucleotide diversity.

3.2. Molecular Phylogeny and Divergence Times

Phylogenetic trees of *Tor* sp. reconstructed by using Bayesian, neighbor-joining (NJ), and maximum-likelihood (ML) methods were broadly congruent in their topologies with only minor differences in the level of bootstrap support (Figure 2). The phylogeny inferred using the Bayesian method treated Labuk as a single lineage while the NJ and ML method split the Labuk subpopulation into two lineages. The haplotype network indicated more clearly the structuring into clades and substructuring within each clade (Figure 3). The result showed a phylogeographic structure with high bootstrap support (99–100%) and high posterior probabilities (0.91–1.0) in which two distinct haplogroups corresponded well to two independent evolutionary clades: (1) the western clade representing populations from Kadamaian River, Tuaran River, Moyog River, Kimanis River, and Bongawan River; (2) the eastern clade representing populations from Kanarom River, Sugut River, Labuk River, Kinabatangan River, and Pagalan River. However, lower bootstrap support (44–61%) and posterior probabilities (0.36–0.42) were showed within the eastern clade populations, which might indicate insufficient time for lineage sorting to complete. Clade 1 from the western region was further subdivided to the north (Kadamaian River, Tuaran River, Moyog River,) and south (Kimanis River and Bongawan River) clades, whereas clade 2 from the eastern region were subdivided to central (Pagalan River) and eastern (Kanarom River, Sugut River, Labuk River, Kinabatangan River) clades. Figure 4 presents the geographical distribution of the four mtDNA D-loop clades. No haplotypes were shared between clades. Each clade was further sub-structured to correspond with each river system except for Tuaran and Moyog River, which were designated as one cluster. Within clades, four haplotypes (Hap5, Hap14, Hap16, and Hap22) were shared among different river systems. Haplotype Hap16, Hap22, and Hap24 (found in four fish, one from SKI and three from the SPA population of the Labuk River system) were found to be more closely related to Kinabatangan River haplotypes, whereas haplotype Hap5 (found in one fish from WCDO) was more closely related to Kimanis River haplotypes.

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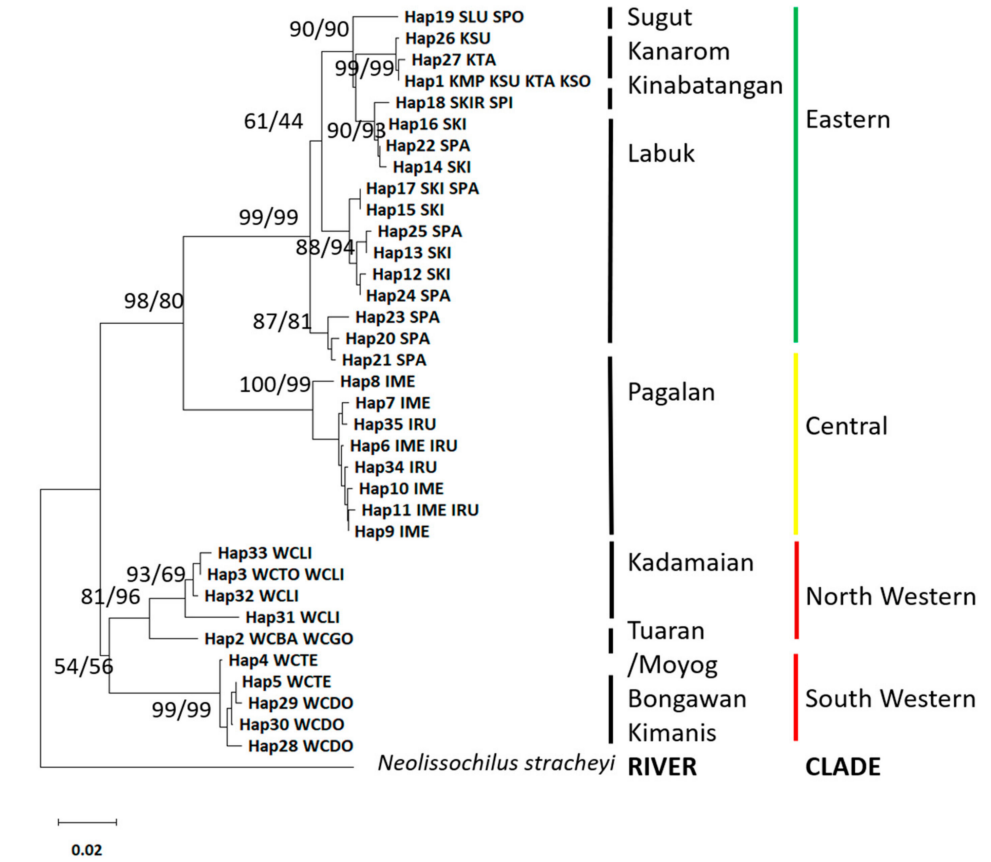
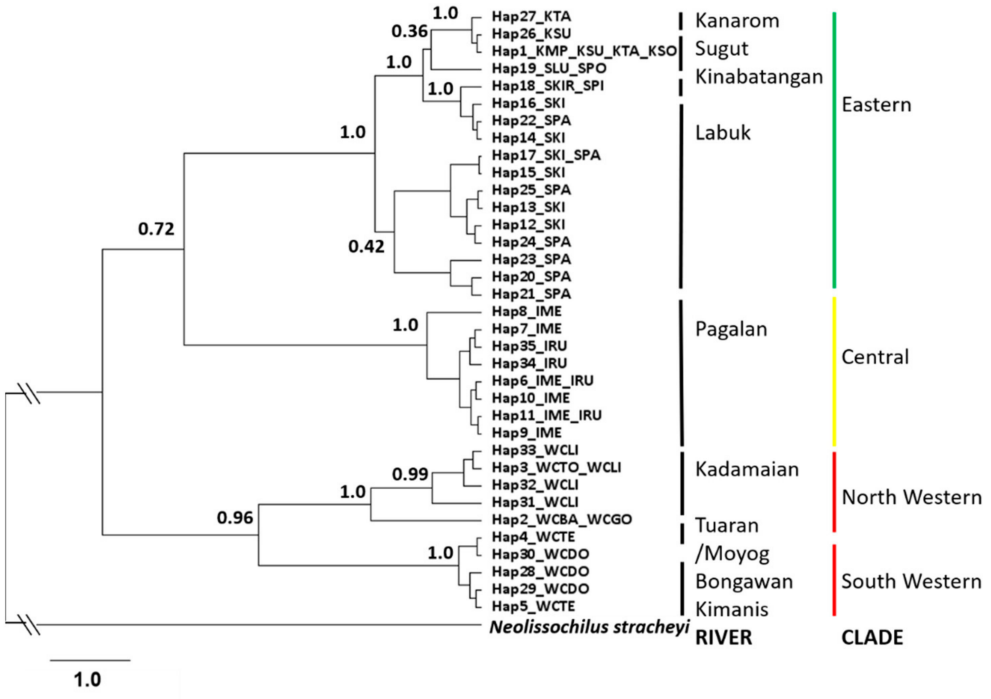


(**a**)



(**b**)

Figure 2. Juxtaposition of phylogenetic trees reconstructed using (**a**) neighbor-joining (NJ) and maximum-likelihood (ML) methods and (**b**) Bayesian method showing the evolutionary relationship of *Tor* sp. among different sampling localities and river systems in Sabah with respect to their mtDNA haplotypes. Percentage bootstrap values for the NJ/ML tree posterior probabilities Bayesian-inferred tree are shown above the branch.

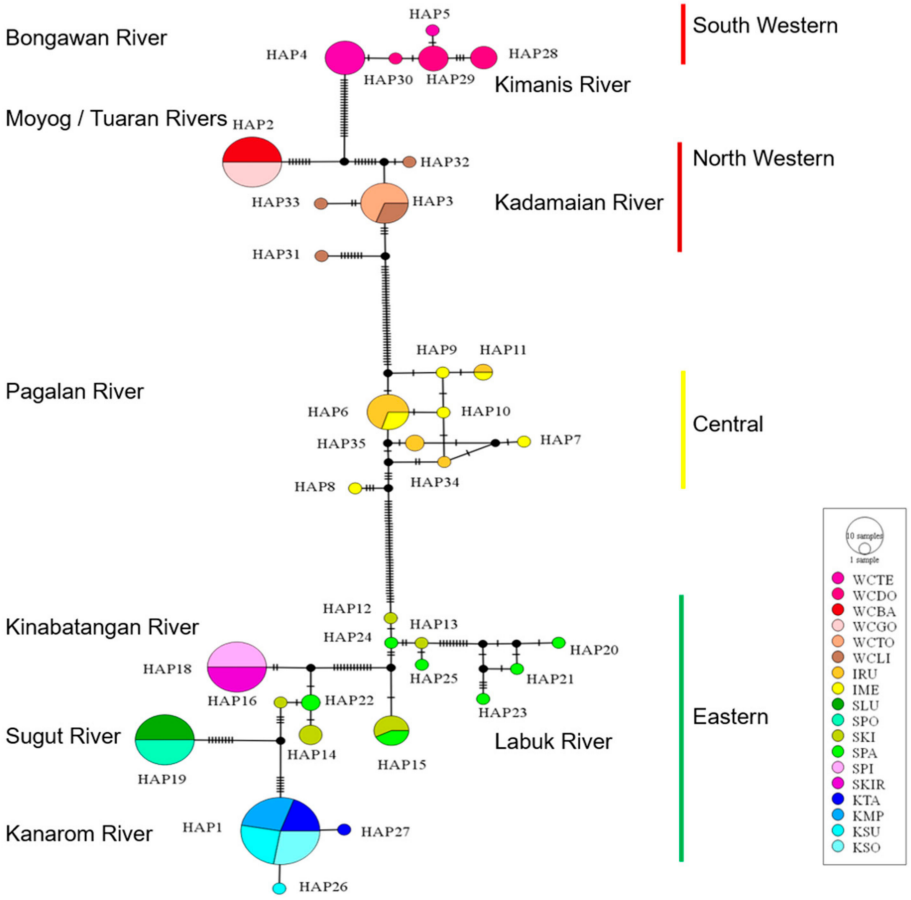


Figure 3. TCS haplotype network of mtDNA D-loop for *Tor*. Lines represent mutational steps that separate haplotypes. Circle size of the haplotypes indicates relative haplotype frequency. Colors represent the sampling locations.

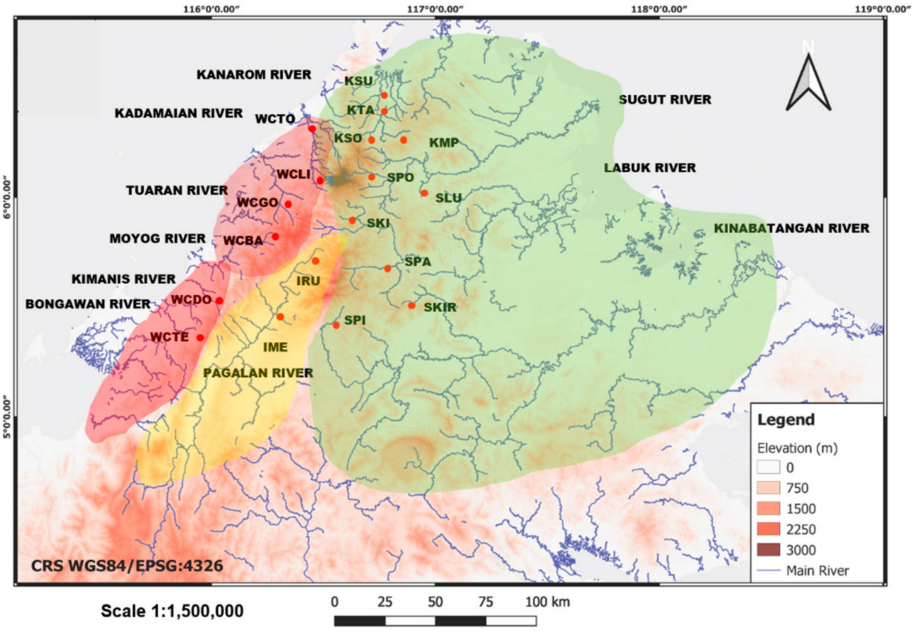


Figure 4. Map of Sabah showing geographical distributions of four mtDNA D-loop clades shown by different colors; southwestern clade (red), northwestern clade (brown), central clade (yellow), and eastern clade (green).

Through the Bayesian tree, the estimates of eastern and western clades divergence occurred in the 4.81 mya (7.32–2.97 mya) (Figure 5). The eastern clade was further subdivided into central inland and eastern coastal subclades in the 3.77 mya (5.81–2.14 mya), whereas the western clade was subdivided into northern and southern subclades in the 2.88 mya (4.64–1.58 mya). Subsequent major river divergence within subclades occurred in the 1.4–0.29 mya (2.39–0.09 mya).

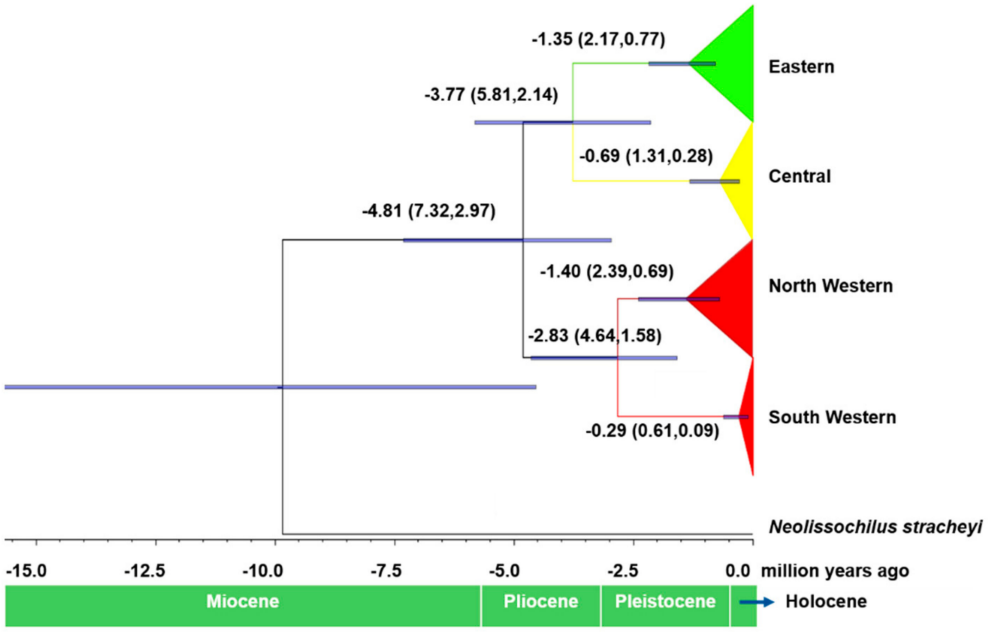


Figure 5. A time-calibrated mtDNA D-loop tree of *Tor*. Node bars represented the age 95% HPD intervals and age of each cluster labeled on the branches. Branches within each clade are collapsed.

3.3. Genetic Differentiation and Population Structure

AMOVAs were performed to estimate the amount of variance explained by each model (Model 1 and 2; populations grouped by current river distributions, and Model 3 to 5; populations grouped by current river distributions relative to the Crocker Trusmadi Range separation). Rivers grouped as 10 accounted for slightly higher variations (95.63%, Φ_{ct} = 0.956, p = 0.000 ± 0.000) than group of 8 (95.29%, Φ_{ct} = 0.956, p = 0.000 ± 0.000) whereas four groups of clades accounted for most variations (81.19%, Φ_{ct} = 0.812, p = 0.000 ± 0.000) rather than two (58.34%, Φ_{ct} = 0.583, p = 0.000 ± 0.000) and three clades (73.77%, Φ_{ct} = 0.738, p = 0.000 ± 0.000). AMOVA results indicate that there is a substantial amount of genetic structuring at all hierarchical levels except for populations within the same river (Table 2).

The findings of pairwise Φ_{ct} revealed high genetic differentiation among all populations (Φ_{ct} = 0.575–1.000, $p < 0.05$) except for populations within the same river systems (Figure 6). The highest and most significant values of pairwise comparisons of genetic differentiation (Φ_{ct}) were detected between all the four clades averaging more than 0.960 (Table 3). The values of Φ_{st} within clades were high and significant, averaging more than 0.603 except in the central subclades. Geographic and genetic distances exhibited a weak correlation (R^2 = 0.206, Figure 7).

Table 2. Summary of results of the hierarchical analysis of molecular variance (AMOVA).

Source of Variation	% of Variation	Φ Statistics
Grouped by 8 rivers (Kanarom/Sugut/Labuk/Kinabatangan/Pagalan/Kadamaian/Tuaran + Moyog/Kimanis + Bongawan)		
Among 8 groups	95.29	Φct = 0.956
Among 18 populations within 8 groups	0.41	**Φsc = 0.001**
Within 18 populations	4.30	Φst = 0.956
Grouped by 10 rivers (Kanarom/Sugut/Labuk/Kinabatangan/Pagalan/Kadamaian/Tuaran/Moyog/Kimanis/Bongawan)		
Among 10 groups	95.63	Φct = 0.956
Among 18 populations within 10 groups	0.01	**Φsc = 0.001**
Within 18 populations	4.37	Φst = 0.956
Grouped by 2 clades (western/central + eastern)		
Among 2 groups	58.34	Φct = 0.583
Among 18 populations within 2 groups	38.44	Φsc = 0.923
Within 18 populations	3.21	Φst = 0.968
Grouped by 3 clades (western/central/eastern)		
Among 3 groups	73.77	Φct = 0.953
Among 18 populations within 3 groups	22.94	Φsc = 0.875
Within 18 populations	3.29	Φst = 0.957
Grouped by 4 clades (northwestern/southwestern/central/eastern)		
Among 4 groups	81.19	Φct = 0.812
Among 18 populations within 4 groups	15.48	Φsc = 0.823
Within 18 populations	3.33	Φst = 0.967

All Φst, Φct and Φsc values are significant with $p < 0.001$. Non-significant p-values are indicated in bold. Western (WCTO, WCLI, WCBA, WCGO, WCDO, WCTE), Northwestern (WCTO, WCLI, WCGO, WCBA), Southwestern (WCDO, WCTE), Eastern (KMP, KSU, KTA, KSO, SLU, SPO, SKI, SPA, SPI, SKIR), central (IME, IRU).

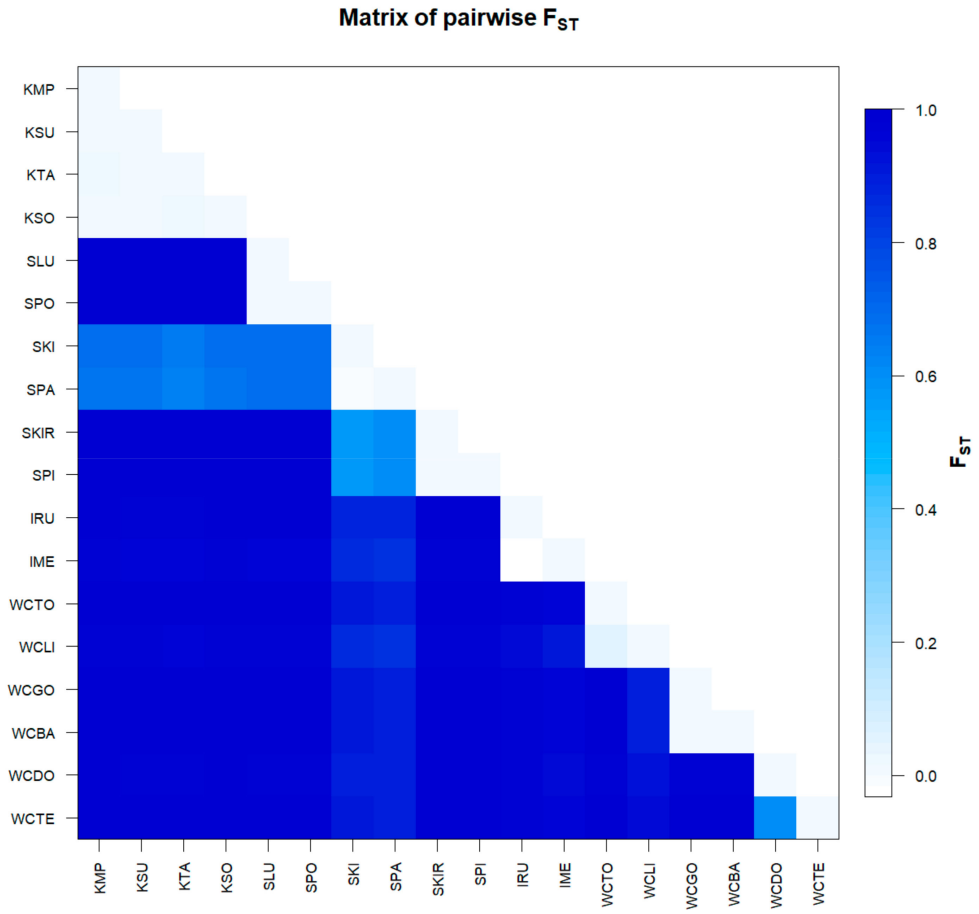


Figure 6. Heat map of pairwise Φ_{st} estimates for each locality, using mtDNA D-loop; dark blue squares represent high Φ_{st} values, and light blue squares represent low Φ_{st} values between localities.

Table 3. Summary of average pairwise Φst, Φct, and Φsc values for *Tor* within and among clades.

Population Comparisons	No. of Populations	No. of Groups	Φ_{st}	Φ_{ct}	Φ_{sc}
Within Eastern	10	1	0.81451		
Within Central	2	1	−0.03271		
Within Northwestern	4	1	0.93037		
Within Southwestern	2	1	0.60349		
Between Eastern and Central	12	2	0.95987	0.80554	
Between Eastern and Northwestern	14	2	0.96731	0.62898	0.91188
Between Eastern and Southwestern	12	2	0.96648	0.64202	0.90636
Between Central and Northwestern	6	2	0.97096	**0.81383**	0.84399
Between Central and Southwestern	4	2	0.96448	**0.94703**	0.32938
Between Northwestern and Southwestern	4	2	0.97169	**0.73008**	0.89511
Among Eastern, Central, Northwestern, Southwestern	18	4	0.96666	0.81187	0.82279

All Φ_{st}, Φ_{ct}, and Φ_{sc} values are significant with $p < 0.001$. Non-significant *p*-values are indicated in bold.

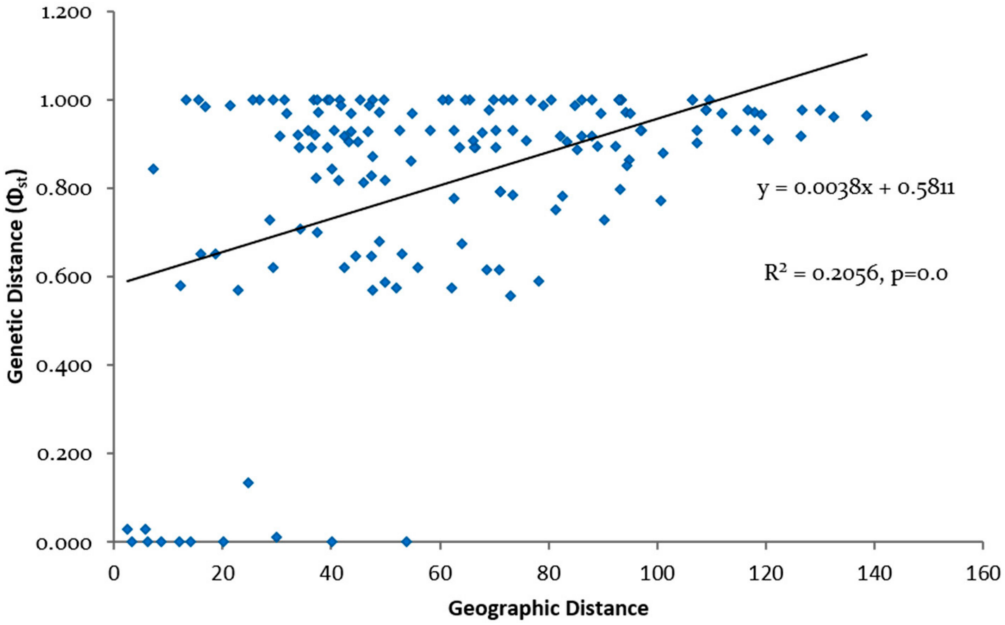


Figure 7. Scatter plots illustrating the pairwise relationship between genetic distance (Φ_{st}) and geographic distances (km) between 18 populations. Lines of best fit and R^2 values are shown.

The Bayesian Analysis of Population Structure (BAPS) analysis at the individual level mixture resulted in eight genetic clusters (Figure 8) with log marginal likelihoods of −2178.02. The clusters were distributed according to major river systems. In addition, populations from different river systems in the present time were clustered in the same genetic cluster, such as WCGO and WCBA from Tuaran and Moyog River systems and WCDO and WCTE from Bongawan and Kimanis river systems. SPA and SKI populations from Labuk River systems showed populations mixture with SPI and SKIR from the Kinabatangan River system.

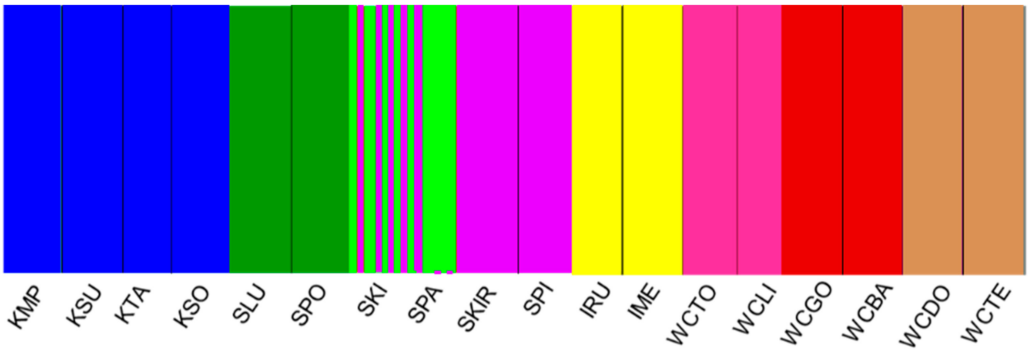
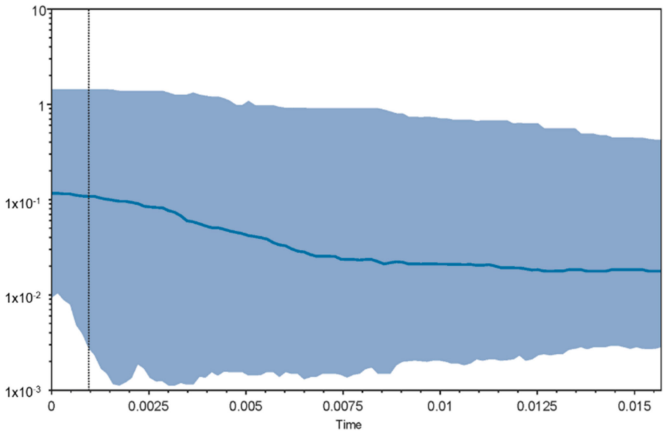


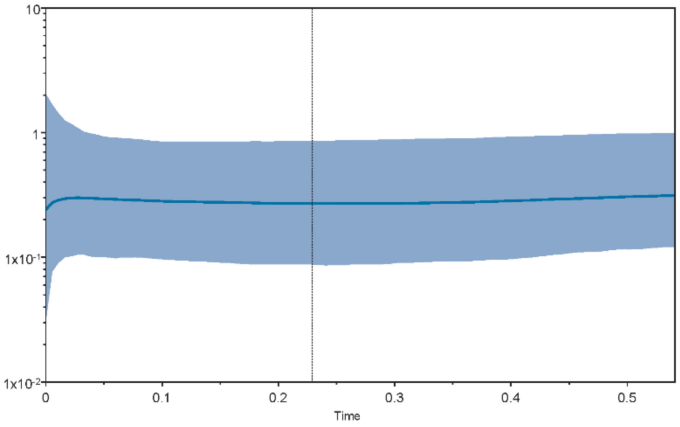
Figure 8. Proportional membership of each individual of *Tor* in the cluster identified by BAPS (K = 8). The locality of origin for each individual is indicated on the *x*-axis.

3.4. Demographic History

Site-specific and drainage tests for deviation from mutation-drift and gene-flow drift equilibrium did not generally support the hypothesis for population expansion except for Kadamaian River (WCLI), which shows significant Fu and Li's D values (WCLI = −1.741, WCLI + WCTO = −3.1202, $p < 0.01$) supporting demographic-spatial expansion. However, this is not supported by the Bayesian skyline plot (BSP) analysis (Figure 9). To test the fit of the empirical data between demographic models and to ensure that biological conclusions are driven by the data and not by prior model selection, Bayes factor was performed to each major river. Comparison of Bayes factor between BSP model to constant demographic model shows higher support for the constant model to all populations except for Labuk River (SKI + SPA) and Kimanis River (WCDO). Bayesian skyline plot (with ESS scores > 1000) revealed population growth for Labuk River (SKI + SPA) and Kimanis River (WCDO) populations. When BSP was run within SKI and SPA populations separately, Bayes factor support was higher for the constant population model. Haplotype network structure and phylogeography dendrogram supported the constant demography model for Tor.

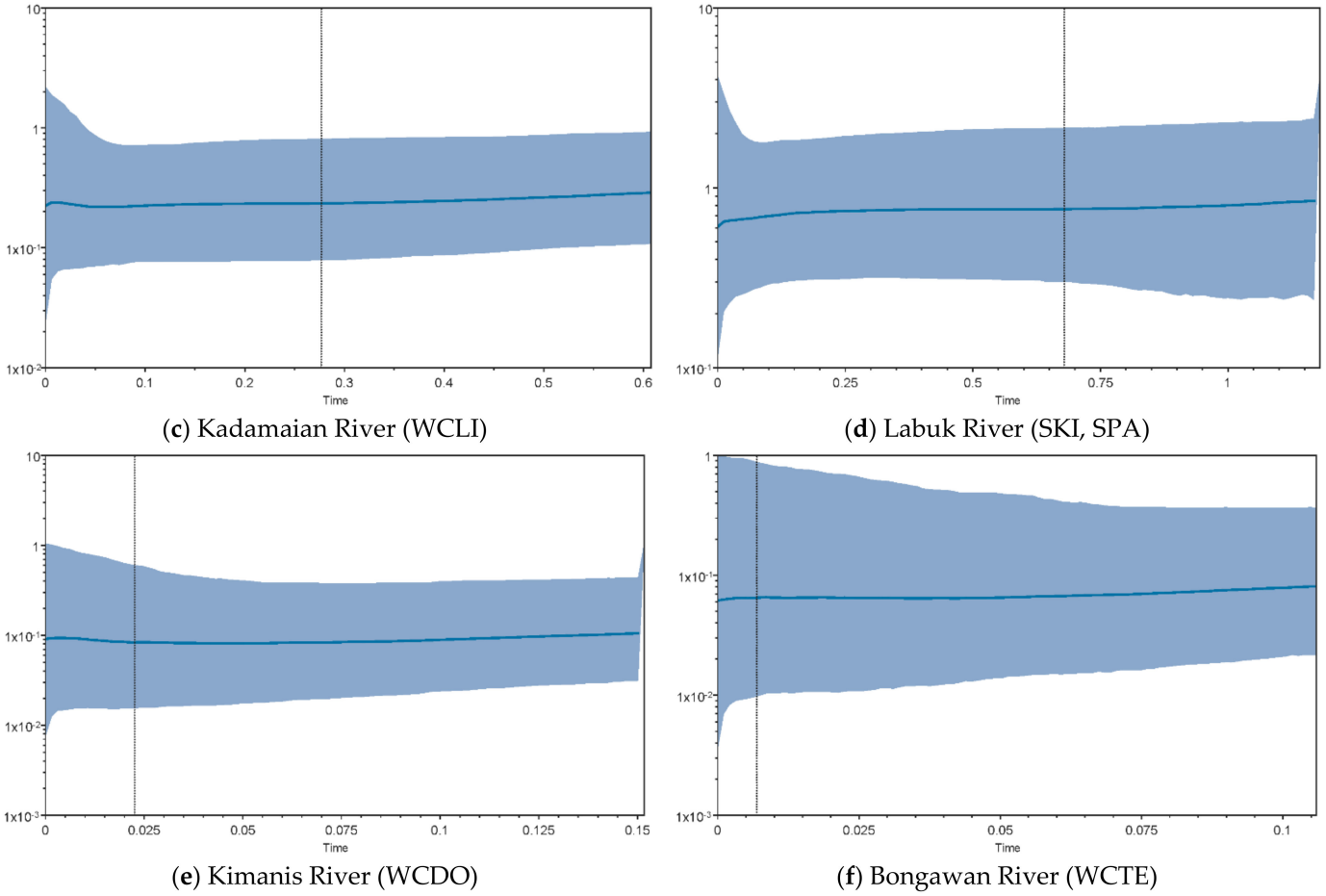


(**a**) Kanarom River (KSU, KTA)



(**b**) Pagalan River (IRU, IME)

Figure 9. *Cont*.



(**c**) Kadamaian River (WCLI) (**d**) Labuk River (SKI, SPA)

(**e**) Kimanis River (WCDO) (**f**) Bongawan River (WCTE)

Figure 9. BSPs to evaluate the shape of each population growth of *Tor* over historical time. (**a**) BSP of Kanarom River (**b**) BSP of Pagalan River (**c**) BSP of Kadamaian River (**d**) BSP of Labuk River (**e**) BSP of Kimanis River (**f**) BSP of Bongawan River populations. The x-axis represents time in units of million years. The y-axis represents effective population size as Neτ on a log scale. The black line depicts the median population size, and the shaded areas represent the 95% highest posterior density intervals.

4. Discussion

4.1. Genetic Diversity and Demography

The genetic diversity of *Tor* mtDNA D-loop region (H_d = 0.000–0.911, π = 0.001–0.021) was within a broader range compared to those of other *Tor* spp. (H_d = 0.000–0.777, π = 0.000–0.008) [26,68], but this was probably of the faster mutation rate in the non-coding D-loop region. The neutrality test did not reveal any major concern for studied populations. Neutrality statistics and BSPs show constant population size over time for most *Tor* populations. Despite the decline in abundance reported in previous decades [9,10], we did not find any evidence to suggest that there was a bottleneck event in the past that influenced the genetic diversity and population structure of the present *Tor* sp. in Sabah. The current finding seems to support the notion that *Tor* sp. is resilient against anthropogenic threats [11]. However, this proposition will need to be further tested with a larger sample size and with other molecular markers.

Nine populations showed an absence of genetic variation, which we believe was independent of sample size. For instance, the seven individuals sampled from IME had among the highest haplotype diversity (H_d = 0.714), whereas subpopulations with monomorphic mtDNA types were all represented by 10 individuals. Being an important fish could lead to a small effective population size because of one or more likely, several factors such

as overexploitation, habitat fragmentation, or habitat loss due to human perturbation, including human activities resulting in genetic bottlenecks that may have led to inbreeding.

Rivers located in the central region of Sabah, i.e., Kadamaian, Pagalan, and Labuk rivers, harbored *Tor* populations with higher genetic diversity relative to other rivers in other locations. A high species diversity and a high degree of endemism in Sabah are well known for many plant and animal taxa, particularly for the central mountain ranges, that is, the Crocker Range, Mount Kinabalu, and the Trusmadi Range [69,70]. This finding supports the studies of other taxa that proposed the mountain ranges in Sabah play a role in the maintenance of ancient lineages and species diversity [69,70]. In this study, Labuk River appeared to have potentially retained large proportions of ancestral genotypes as shown by its location in the basal position of the phylogeographic tree, the same results obtained for *Channa striata* [13]. This shows Sandakan is the origin of colonization before the expansion of populations to eastern clades.

4.2. Phylogeography and Population Structure

Through phylogeographic tree, haplotype network, and BAPS clustering, there are two major clades (i.e., eastern and western) with each clade sub-structured to two subclades (northwestern, southwestern, eastern, central), followed by clustering of each subclade to major rivers totaling up to eight clusters, i.e., four subclusters for the western clades and four subclusters for the eastern clades. The clustering coincides with the separation of Sabah in the center by CTR and in accordance with major river systems. The results of this study were also obtained by the authors of [13], where *Channa striata* is subdivided into two genodeme, namely Genodeme 1 and Genodeme 2, which were known in this study as western and eastern clusters, respectively.

Phylogeographical distribution of freshwater fish species is strongly influenced by present and historical connections among rivers and became the basis of the interpretation of the observed genetic relationships among populations [71–75]. In Sabah, Crocker and Trusmadi Range (CTR) became the major geological barrier that separates the western and eastern riverine and floodplains habitats. Crocker Range (CR) is the highest range in the mountainous western part of Sabah with an average altitude of 2000 m, more than 40 km wide, stretches about 200 km along, and mountains up to 4000 m in height [76]. Situated adjacent to the CR is the 80 km Trusmadi Range (TR), which houses the second-highest mountain peak in Malaysia. CTR and its mountains became the headwater source of rivers discharging to the South China Sea in the east, central and north coast of Sabah. Extending toward the western coasts, southern plains, and the interior or central part of Sabah are lower mountain ranges and plains with occasional hills disconnecting major rivers such as Kinabatangan River, Labuk River, and Sugut River, which drained into the Sulu Sea. Generally, the length of the western and northern rivers is shorter (less than 100 km) than the eastern and central rivers, which range from 100 to 560 km long, with Kinabatangan River being the longest and widest floodplain.

The subdivision of eastern, central, northwestern, and southwestern clades occurred in the late Miocene and Pliocene (5–1 mya range), which coincides with Borneo mountain ranges uplift and oscillated tropical storm [77]. The overall effect of these alternating wetter tropical storm and mountain uplifts isolates and join rivers but also shift the position, size, and types of habitats covering CTR and its surrounding areas, creating various scenarios of population subdivision and colonization and, thus, diversification that can be seen today. Further subdivision of major river systems occurred in the Pleistocene era (2.58–0.02 mya). Climate changes during the Pleistocene could have shaped contemporary species distribution patterns through dispersal and subsequent population differentiation due to geographical isolation. Alternatively, the low sea level in the Pleistocene allows dispersal in the outlets of rivers and subsequent isolation during the rises of sea levels.

Following the stream hierarchy model (SHM) [78], obligate freshwater fish populations are often genetically structured and differentiated (freshwater fish, mean Fst = 0.222) [79] at both among and within river basin scales as the results of gene-flow restriction and

dispersal limitation among populations [80–84]. Isolation by distance (IBD) [85], isolation by barriers (IBB) (e.g., dams, culvert, waterfalls, rapids) [86], and isolation by resistance (IBR) (e.g., temperature, stream gradient, number of confluences, drainage basin, seasonal precipitation, seasonal water flow, and high flow events) [87,88] are reported to influence genetic variation in riverine freshwater fishes [89].

The significant high genetic differentiation between clades and river systems shows historical isolation by CTR and contemporary geographical isolation of the rivers following the SHM, respectively, which are responsible for genetic variations in *Tor* populations. In addition, significant Mantel test analysis indicated that the genetic structure of *Tor* populations was also affected by geographical distance following the IBD model. The pattern of genetic differentiation according to mountain ranges and river systems has been reported previously in *Tor* spp. in Malaysian Borneo [21,32].

In Sabah, most rivers are separated from each other by mountain ridges due to rugged landscape; thus, neighboring rivers are not connected to each other, and genetic differentiation would be expected between populations. However, some populations from contemporarily separated but geographically closed river basins are found to be highly homogeneous (e.g., between WCBA (Moyog River) and WCGO (Tuaran River), WCDO (Kimanis River) and WCTE (Bongawan River), SKIR (Kinabatangan River) and SKI, SPA (Labuk River)), which imply gene flows of individuals. The high similarity of kinship between Moyog and Tuaran populations was also reported in *Channa striata* using microsatellites [13]. The lack of genetic patterning could be explained by dispersal through a geomorphological phenomenon such as river capture, stream piracy, floods events, the historical connection between river systems [90], and anthropogenic activities such as restocking programs and translocation [91].

In Sabah, tectonic movements have been recorded from 1911 to 2009 [92]. The west coast of Sabah, located on the edge of the Sunda Plate, is seismically active, as illustrated by the recent occurrence of magnitude 6.0 earthquake in the Sabah region on 5 June 2015. The tectonic movements could have resulted in river capture events where horizontal migration of the water divide occurred, resulting in dynamic reorganization of the drainage network. Stream piracy events also have been reported in Sabah [15,16]. These events could have caused the changes in freshwater fish connection and dispersal. River capture, in particular, has been implicated in the diversification of many freshwater faunas worldwide [93,94]. Besides river capture, dispersal across drainage divides could also be facilitated by flood events that allow connectivity across a divide or dispersal through the outlets of a lake or swamp that drain into catchments on both sides of the divide [90,91,95]. Alternatively, the other explanation is human-mediated translocation or stocking due to the public awareness of *Tor* high commercial value.

4.3. Management Implications

In the short-term fisheries management context, genetic stocks are comparable to management units (MU) [4] with the objective of maintaining productive fisheries resources and sustaining local abundance by avoiding overexploitation, whereas, in the long-term fisheries conservation context, genetic stocks refers to evolutionary significant units (ESUs), which represent important components of adaptive diversity and long-term evolutionary potentials with the objective of preserving sustainable local populations [4,5]. MU is used to describe individuals that require separate management with respect to exploitation, and the latter is used to identify groups of individuals of special interest with respect to conservation. ESUs may constitute one or more subpopulations (MUs), and each ESU represents a significant proportion of the total genetic diversity present within a species across its natural geographical range. Each ESU and MU delineation identified by this study can be used to guide existing management measures such as stocking programs, assigning quotas, modeling alternative harvesting scenarios, and designing monitoring programs [79,96].

The current study showed *Tor* populations appear to be significantly structured into geographically discrete subpopulations across drainages. Given that all clades are historically isolated, monophyletic, and having a high level of genetic differentiation [79], fulfilling the criteria of an ESU [5,97,98]; thus, this study suggests that all clades were treated as an ESU. Because of insignificant genetic differentiation values, River Bongawan and River Kimanis, as well as River Moyog and River Tuaran, can be combined as a unit. Thus, eight rivers namely River Kanarom, River Sugut, River Kinabatangan, River Labuk, River Pagalan, River Kadamaian, the combined River Bongawan Kimanis and River Moyog Tuaran were treated as MU.

This study showed a high degree of genetic differentiation among the clades and major drainage. Thus, in order to minimize the negative genetic impacts of stocking programs, stocking should not be carried out between drainage basins where different genetic stocks have been identified [79,99]. Each identified ESU/MU be managed separately, no inter-clade stocking should be performed, and only releasing fish into the same ESU/MU from which their dams and sires were collected in order to avoid introducing different genetic stocks that may hybridize with the local population. By taking these precautions, the potential negative genetic impacts of stocking/translocation, e.g., outbreeding depression, loss of genetic diversity, and loss of between-population variation [100,101], can be minimized.

Each MU appear to constitute members of a stock distinct to each other with limited dispersal ability, which implies that potential for recruitment to the local area from other river basins is limited, and hence local population declines due to overfishing are unlikely to be compensated for in the short term at least, by recolonization from other parts of the river basin. The overall pattern of isolation by distance among all sites suggests that dispersal is likely to be low at this spatial scale. Thus, it is recommended that monitoring programs be implemented in order to prevent overfishing based on the delineation of MUs.

From a longer-term evolutionary perspective, local genetic diversity in a population is essentially unique, and if lost, cannot be regained by colonization of individuals from other parts of the drainage. Genetic diversity potentially allows individuals to use a wider array of environments, protects against short-term environmental fluctuations, and provides the building blocks for surviving future environmental changes [102–104]. Populations from Labuk, Pagalan, Kadamaian, and Kimanis rivers showed relatively high genetic diversity, which could serve as founder for hatchery stock for commercial aquaculture purposes to achieve substantial genetic variations in the successive populations, whereas populations that showed reduced genetic diversity could be targeted in stocking programs to increase the effective population size and genetic variation [105].

The results of this study demonstrate how mtDNA can contribute in a positive way to help inform management plans for species of management and conservation concerns. However, mtDNA is a single locus; thus, its ability to resolve population structure is relatively limited. mtDNA is not functionally neutral; thus, mtDNA diversity may be influenced by selective sweeps or/and background selection, rather than accurately reflecting population history and demography [39,106]. Microsatellites have become a widely used genetic marker to infer population genetic structure in fisheries management and conservation [107,108] due to their biparental inheritance in a co-dominant Mendelian manner and highly polymorphic. Therefore, the incorporation of both mtDNA and microsatellite markers would greatly improve the understanding of the historical and present events that shape the population structure of a species as well as greatly improving the overall assessment of genetic diversity.

5. Conclusions

Based on mtDNA D-loop phylogeographic and population genetic analysis of *Tor* populations across Sabah, this study identified four major groups that can be considered as ESU and eight groups as MU. The pattern of phylogeographical structuring and significant high genetic differentiation between clades and river systems shows CTR and river isolation are responsible for genetic variations in the *Tor* population influencing population genetic

structure. It was also found that most of the *Tor* populations have low genetic diversity, highlighting the need for immediate attention for conservation. However, populations from Labuk, Pagalan, Kadamaian, and Kimanis showed relatively high genetic diversity that can serve as founders for hatchery stock to achieve substantial genetic variations in the successive populations. However, the incorporation of both mtDNA and microsatellite markers would greatly improve the understanding of the historical and present events that shape the population structure and genetic diversity of *Tor* in Sabah.

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Conflicts of Interest: The authors declare no conflict of interest.

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