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Short communication Open Access

The Potential Utility of Microsatellite Markers for Resolving the Phylogeographic Structure of the Rock Shell (*Thais clavigera*) in the Northwest Pacific

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Abstract

Rapidly evolving loci, such as microsatellites, can offer insights into the phylogeography of a species that are not revealed by more slowly-evolving genetic markers. In this short communication, the potential utility of these markers to resolve the phylogeography of the Chinese rock shell (*Thais clavigera*) is examined with three microsatellite loci for nine geographic samples. Phylogenetic and population genetic analyses of these preliminary data both support phylogeographic structure that implicates the Changjiang River and Taiwan Strait as population breakpoints and long distance dispersal as a major organizing factor of the species' geographic variation. Our phylogeographic structure, which is congruent with the findings of previous population genetic studies for this and other marine species, highlights the potential utility of microsatellite markers for the determination of rock shell phylogeography. We now call for a comprehensive microsatellite study to complement the extensive mitochondrial DNA results that already exist for the rock shell throughout its range.

Keywords: China; Marginal seas; Dispersal; Changjiang River; Taiwan Strait

Introduction

The rock shell (*Thais clavigera*, Muricidae, Gastropoda, Mollusca) is a common, predatory, sea snail of the rocky intertidal shores of Indochina, China, Korea, and Japan [1,2]. This species is of importance to humans as a bioindicator of heavy metal marine pollution and as a source of food and traditional medicines in China [3]. Recently, Guo et al. [4] conducted a detailed phylogeographic study of the rock shell in the Northwest Pacific using partial mitochondrial DNA sequences for cytochrome oxidase I (COI). In their Analysis of Molecular Variance (AMOVA) and pairwise Φ_{ST}, these authors found significant, but low, population structure that corresponded to known phylogeographic breaks at the Changjiang River and across the different marginal seas of the Northwest Pacific {i.e., South China Sea (SCS), East China Sea (ECS), and Yellow Sea (YS)} [5-7]. The freshwater outflow of the Changjiang River imposes an ecological barrier to gene flow between ECS and YS populations. When sea levels fell by >100 m during the Pleistocene glaciations (i.e., during the last glacial period ~12,000 years ago), the shallow Taiwan Strait became exposed as an emergent land barrier to gene flow between SCS and ECS/YS populations. However, Guo et al. found no phylogenetic support for their phylogeographic structure as the bootstrap scores and posterior probabilities for these divisions were all ≤ 50%. Given their mixed AMOVA, Φ_{ST}, and phylogenetic results, the authors concluded that the overall lack of phylogeographic structure in the rock shell is due to its long distance dispersal via an extended planktonic larval stage.

In this short communication, we use microsatellite DNA data to further investigate phylogeographic structure in the Chinese rock shell. Microsatellite loci evolve rapidly and are therefore valuable tools for resolving recent historical and demographic events [8,9]. Guo et al. [4] assumed an evolutionary rate of 5.2E-6 substitutions/locus/generation for their COI fragment. Conversely, although sometimes as low as 1E-6, the mutation rate for microsatellite loci usually ranges from 1E-4 to 1E-5 events/locus/generation [10,11]. This short communication provides both phylogenetic and population genetic support for the Changjiang River and Taiwan Strait as phylogeographic breakpoints and for the shallowness of this structure. These preliminary findings complement those of Guo et al., and thereby, illustrate the potential utility of microsatellite markers for the resolution of rock shell phylogeography.

Materials and Methods

Geographic samples and microsatellite data

Adult rock shells were collected from eight and one intertidal localities in mainland China and Taiwan, respectively (Figure 1). The rock shell is neither endangered nor protected, and no special permits were required for their collections. Individuals were returned alive to the laboratory, where they were sacrificed by freezing at -20°C in absolute ethanol. Total genomic DNA was isolated from the muscular feet of the thawed specimens with the Qiagen DNeasy Kit following the manufacturer's instructions (Qiagen, Hilden, Germany). The thawed carcasses were then refrozen and retained as voucher specimens and as material for future molecular work.

Nine microsatellite loci were selected from a set of 15 loci recently reported for the rock shell by Li et al. [12]. These nine loci (A4, TCA26, TCA31, TCA33, TCA47, TCA137, TCA150, TCA160, and 50) were PCR amplified with the primers and procedures of these authors. Microsatellite genotypes were scored on an ABI 3130xl automated sequencer (Applied Biosystems, Waltham, MA) with GENEMARKER (Softgenetics, State College, PA).

Reliable genotyping proved difficult for all loci, except for A4, TCA31, and TCA160. At each of the six loci other than A4, TCA31,

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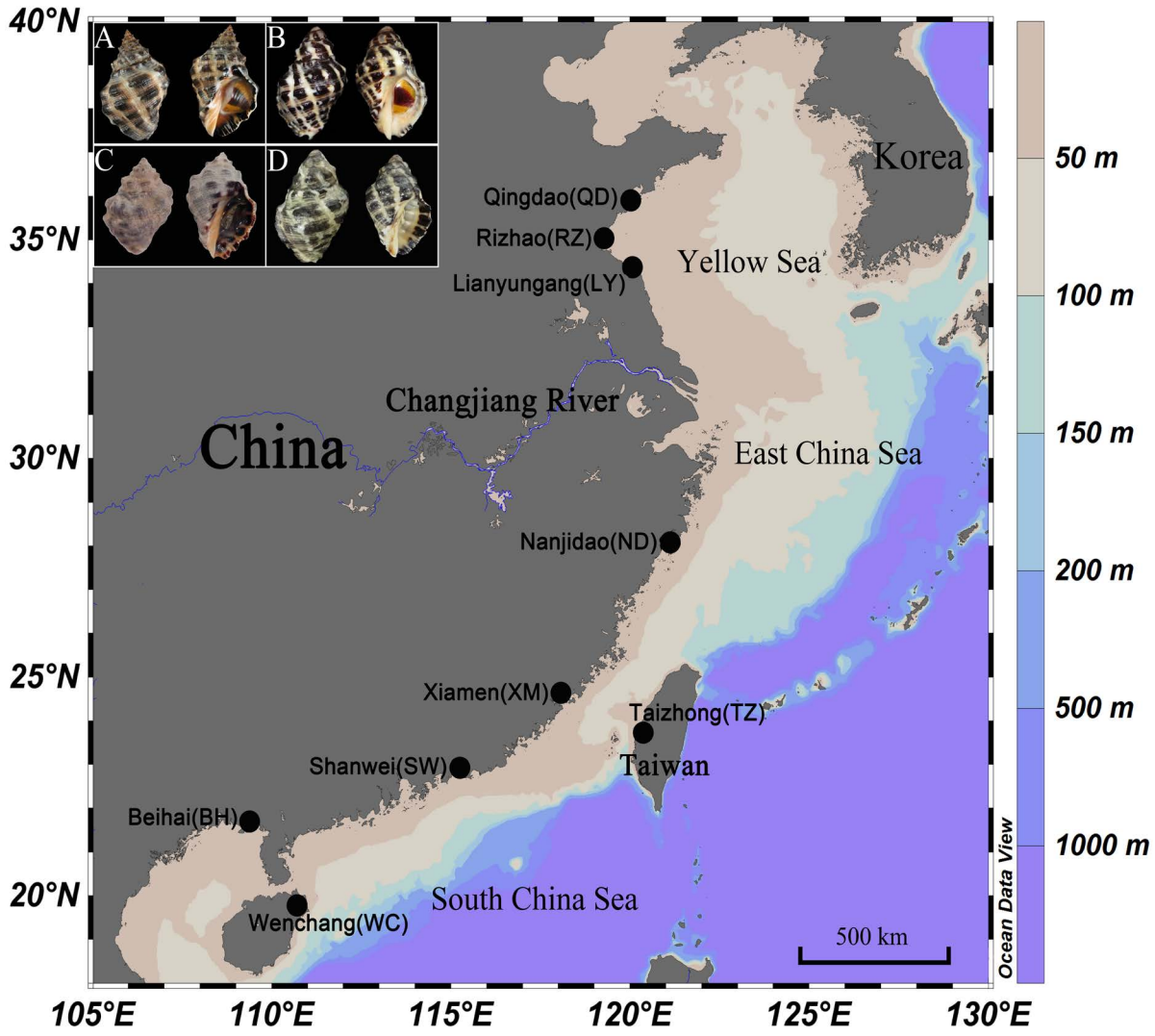


Figure 1: Sampling localities for the Chinese rock shell, which are hereafter referred to by their two-letter abbreviations in parentheses. This map is rendered with ODV v4.7.3 [26]. Bathymetric depths are indicated by the offshore shading. (Inset) Four adult rock shells from XM (A), TZ (B), QD (C), and BH (D), illustrating the diversity of shell patterns.

and TCA160, significant genotyping errors were detected by MICROCHECKER v2.2.2 [13] in four to all nine of the geographic samples (e.g., at TCA26/TCA33 and 50, eight and nine collections were plagued by such misidentifications, respectively). Furthermore, 36% of the genotypes for these six loci were missing among the total 398 individuals. Conversely, for A4, TCA31, and TCA160, significant genotyping errors were found at only TZ TCA31 and 26% of the genotypes for these three loci were missing among the total 398 specimens (Table S1). Thus, our initial dataset was reduced to a final matrix with only A4, TCA31, and TCA160 and special procedures were adopted for the handling of TZ TCA31 in our microsatellite data analyses (as described below).

Within-sample data analyses

Estimates of within-sample genetic diversity (n_a, n_e, H_o, and H_e for observed and effective numbers of alleles and observed and expected heterozygosities, respectively) were calculated with POPGENE v1.3.2 [14]. Wright's F_{IS} was estimated for each locus of a sample with FSTAT v2.9.3 [15]. Each locus of a sample was tested against its Hardy-Weinberg expectations with the G-test for goodness-of-fit in POPGENE. Each pair of loci for a sample was then tested for its genotypic linkage disequilibrium with the χ^2 test in POPGENE. To correct for multiple comparisons, a standard Bonferroni correction (that is more conservative than a sequential adjustment) was applied in the G- and χ^2 tests on a locus- and paired-locus-wide basis, respectively [16].

Between-sample data analyses

F_{ST} distances were calculated for all pairs of geographic samples with the Weir and Cockerham [17] method in GENEPOP v4.4.3 [18]. An unrooted neighbor-joining (NJ) tree was then inferred from these pairwise F_{ST} with APE v3.4 [19]. The reliability of the groups in the NJ tree was estimated with expected bootstrap scores that were determined exactly rather than approximately with *n* replications (Appendix). Briefly, these expected scores were exactly determined by inferring the NJ trees for all ten of the possible bootstrap datasets for the three loci and then quantifying the frequencies at which the groups of the original NJ tree were recovered by these ten solutions.

The genotype frequencies for each pair of samples were next compared on a locus-by-locus basis with the G-test of heterogeneity in DEDUCER v0.7-7 [20]. To correct for multiple testing, a standard Bonferroni correction was applied on a locus-wide basis.

Following these two analyses, the phylogeographic relationships of only TZ remained ambiguous (as described below). To assess whether the ambiguous affinities of TZ were related to admixture, we performed an additional analysis with STRUCTURAMA v2.0 [21]. This analysis was designed to infer the number of source populations *(K)* for TZ and to assign its 48 individuals to one or more of these sources. The Markov chain Monte Carlo sampler was run for 100,000,000 cycles with samples taken every 5000 generations after a 10% burn-in. The prior mean and variance for *K* were set to ~1.5 and ~0.5 by fixing the shape and scale of the hyperprior gamma distribution for the alpha parameter of the Dirichlet process prior to 0.4 and 3.0, respectively. The STRUCTURAMA analysis was repeated three independent times to ensure the convergence of its results.

In the end, three phylogeographic groups were supported (see below). Pairwise regional F_{ST} (θ) and their associated genetic distances (*d*, events/locus) were estimated for the three phylogeographic groups, with the latter calculated with the equation: $d = -\ln(1-\theta)$ [22]. A range of divergence times was then estimated from the *d* for each phylogeographic pair by assuming a usual spread of 1E-4 to 1E-5 events/locus/generation for the microsatellite mutation rate [10,11] and a generation time for the rock shell of one year [4].

Handling of TZ TCA31

To correct for its significant genotyping errors, MICROCHECKER recommended the incorporation of an unobserved null allele with a frequency of 0.12 at TZ TCA31. Thus, the observed allele frequencies at TZ TCA31 were proportionally decreased to incorporate this null allele and its 0.12 frequency. This correction allowed for the inclusion of TCA31 in the various analyses of TZ, which were conducted at the level of the allele (n_a, n_e, H_e, F_{ST}, the NJ tree, and linkage disequilibrium) rather than the genotype (H_o, F_{IS}, the tests of Hardy-Weinberg equilibrium and between-sample genotype frequencies, and the STRUCTURAMA analysis).

Results and Discussion

Within-sample genetic diversities

Considerable genetic variation exists within the nine geographic samples as indicated by their mean observed and effective numbers of alleles of 7.7-10.7 and 2.8-5.8 and their average observed and expected heterozygosities of 0.63-0.91, and 0.62-0.74, respectively (Table 1). None of the three pairs of loci in any sample is in significant genotypic linkage disequilibrium after Bonferroni correction. Conversely, in five cases of A4 in BH, ND, QD, TZ, and WC, genotype frequencies deviate significantly from their Hardy-Weinberg expectations after Bonferroni correction, because of a heterozygote excess as indicated by their negative F_{IS}. Indeed, although not significant in their Hardy-Weinberg tests, F_{IS} for all but three of the remaining 21 sample/locus pairs (A4 in LY and TCA31 in BH and XM) are also negative for a heterozygote excess. Heterozygote excess is uncommon among marine invertebrates [23] and may be related in the rock shell to its long distance dispersal that can lead to outbreeding (see [24] for a discussion of this and other potential factors).

Phylogeographic structure in the Chinese rock shell

With one exception, the branching pattern of the unrooted NJ tree for the pairwise F_{ST} parallels the marginal sea distributions of the nine Chinese samples (Figure 1 and Figure 2A). Specifically, SW, WC, and XM from the SCS form a well-supported group at a bootstrap score of 96%. The southernmost BH then joins to these three SCS samples at a borderline bootstrap score of 59%. In turn, LY, QD, and RZ from the YS form a reasonably well-defined group at a bootstrap score of 70%. The one exception involves TZ, which does not join with the four other SCS samples. Rather, ND from the ECS joins first to BH, SW, WC, and XM at a borderline bootstrap score of 59%.

This branching pattern of the NJ tree is corroborated by the per-locus between-sample G-tests of the genotype frequencies (Figure 2B). Specifically, at each locus, none of the six SCS tests between BH, SW, WC, and XM is significant after Bonferroni correction and the same is true of the three YS comparisons between LY, QD, and RZ. Conversely, at A4, these four SCS and three YS samples differ significantly in ten of their 12 tests. Similarly, at A4 and TCA160, ND from the ECS and the former four SCS samples vary significantly twice each, whereas this sample and the three from YS do so two and three times, respectively. Conversely, TZ from the SCS varies significantly with another sample only in its A4 test with ND. Thus, unlike the four other SCS samples, no significant differences exist between TZ and the three YS collections.

In the STRUCTURAMA analysis of TZ, K=1, 2, 3, and ≥ 4 receive 65.9%, 27.4%, 5.8%, and 0.9% of the total posterior probability, respectively. Thus, a single population is preferred for TZ. Furthermore, only one of its 48 individuals is assigned to a separate group. Still, the possibility of multiple source populations for TZ remains as $K \geq 2$ is supported by 34.1% of the total posterior probability.

As in many other intertidal species of the Northwest Pacific [5,7], the Chinese rock shell includes three phylogeographic groups from the SCS, ECS, and YS (Figure 1 and Figure 2). This congruence among intertidal species further implicates the freshwater outflow of the Changjiang River and the Taiwan Strait as major phylogeographic breakpoints along the coastline of China. Regional F_{ST} and divergence times for these three phylogeographic groups range from 0.029-0.062 and 290-6400 years ago, respectively (Table 2). F_{ST} of 0.029-0.062 are largely indicative of little genetic differentiation according to Wright [25], whereas divergence times of 290-6400 years ago broadly overlap with the last half of the Holocene Epoch. Thus, the phylogeographic structure of the rock shell qualifies as both "low" and "shallow" according to its regional F_{ST} and divergence times.

Comparison to the COI study

Our current microsatellite investigation and the previous COI study [4] both support the existence of separate phylogeographic groups among the marginal seas of the Northwest Pacific. However, one difference between the two is that our phylogeographic structure is corroborated by phylogenetic evidence (Figure 2A), whereas that of the COI study is not. This phylogenetic corroboration offers support for the potential utility of microsatellite markers for the phylogeographic resolution of the rock shell throughout its range.

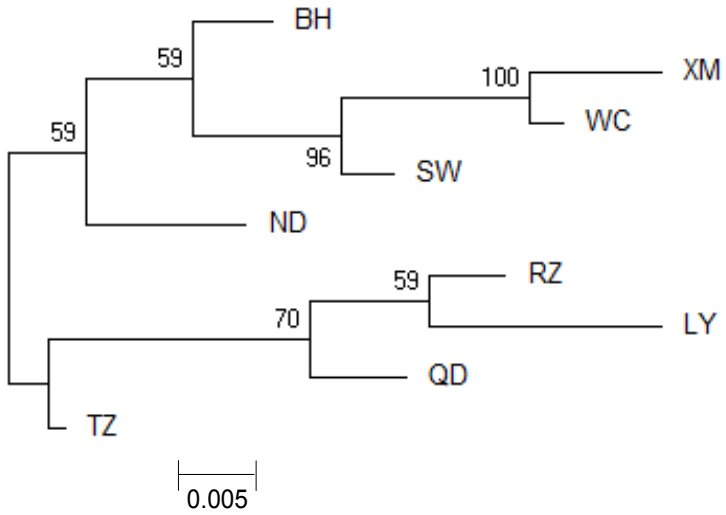
Otherwise, our findings and those of the COI study are comparable. In particular, one similarity between the two is that both support a "low and shallow" level of phylogeographic structure (Table 2). As emphasized by Guo et al. [4], this low and shallow level of divergence can be tied to the long distance dispersal of the rock shell during its extended planktonic larval stage. Thus, the surprisingly recent divergence times for our phylogeographic groups can be connected to this long distance dispersal and its homogenizing effect, rather than the historical events that underlie their earlier separations (i.e., the emergence of the shallow Taiwan Strait as a land barrier to gene flow

Geographic sample	n	n_a	n_e	H_o	H_e	F_{IS}
LY	31.7(25-40)	7.7 (6-9)	2.8 (2.6-3.1)	0.71 (0.63-0.80)	0.66 (0.62-0.69)	-0.11 (-0.30 to 0.01)
RZ	38.7 (20-49)	9.7 (7-11)	3.0 (2.6-3.2)	0.81 (0.80-0.83)	0.67 (0.62-0.71)	-0.23 (-0.30 to -0.15)
QD	31.6 (26-35)	10.0 (4-14)	2.9 (1.7-3.6)	0.72 (0.47-0.97)	0.62 (0.42-0.74)	-0.18 (-0.38 to -0.01)
TZ	33.3 (23-40)	9.0 (7-11)	4.0 (2.7-5.2)	0.72 (0.57-0.81)	0.74 (0.63-0.83)	-0.16 (-0.03 to -0.30)
ND	41.0(25-51)	9.3 (9-14)	3.5 (2.8-4.6)	0.91 (0.80-0.98)	0.71 (0.65-0.80)	-0.32 (-0.53 to -0.02)
BH	34.3 (30-41)	10.0 (8-13)	3.4 (1.7-4.9)	0.63 (0.44-0.77)	0.66 (0.42-0.81)	0.01 (-0.05 to 0.14)
SW	25.3 (18-31)	9.0 (7-12)	4.2 (2.4-7.0)	0.89 (0.77-1.00)	0.72 (0.60-0.88)	-0.27 (-0.35 to -0.17)
WC	30.7 (29-32)	9.0 (4-15)	4.2 (2.5-7.1)	0.87 (0.79-0.94)	0.72 (0.61-0.87)	-0.25 (-0.42 to -0.01)
XM	28.3 (23-32)	10.7 (5-15)	5.8 (2.7-11.8)	0.83 (0.72-0.90)	0.74 (0.64-0.94)	-0.16 (-0.41 to 0.05)

"*n*" = number of genotyped individuals

Table 1: Standard statistics of genetic diversity for the nine geographic samples (Figure 1). These statistics correspond to the arithmetic means and ranges (in parentheses) for the total loci of each sample.

A



B

Geographic sample	LY	RZ	QD	TZ	ND	BH	SW	WC	XM
LY									
RZ									
QD									
TZ									
ND									
BH									
SW									
WC									
XM									

Figure 2: (A) Unrooted NJ tree for the pairwise F_{ST} of the nine geographic samples (Figure 1). Expected bootstrap scores, as calculated with the exact approach in the Appendix, are presented next to each internal branch. Branch lengths are drawn proportional to their tree-based estimates of evolutionary distance. For illustrative purposes only, this tree is drawn as midpoint rooted, which thereby means that the bootstrap score of 59 for the BH, ND, SW, WC, and XM group is actually for the internal branch that bipartitions them from RZ, LY, QD, and TZ.
(B) G-tests of the between-sample genotype frequencies for A4 and TCA160 (above and below the black diagonal, respectively). Gray cells refer to tests that are significant after Bonferroni correction. Unlike the results for A4 and TCA160, those for TCA31 are not presented as none are significant.

Phylogeographic group	SCS	ECS	YS
SCS (118.7, 104-133)		0.029 [0.029]	0.062 [0.064]
ECS (41.0, 25-51)	290-2900		0.052 [0.053]
YS (101.6, 70-123)	640-6400	530-5300	

Table 2: Pairwise regional F_{ST}, *d* (in brackets), and divergence times (ranges, in years ago) for the three phylogeographic groups. Regional F_{ST}/*d* and divergence times are presented above and below the diagonal, respectively. Sample sizes for the three groups (mean and range of *n*) are given in parentheses. TZ is not included in these estimations, because of its uncertain group placement.

between the SCS and ECS populations during the last glacial period when sea levels fell by >100 m) [7]. Such dispersal may have also led to admixture in TZ (as implied by STRUCTURAMA), and thereby, to a reduction in its genetic signal for an SCS phylogeographic connection.

Conclusions

Clearly, our current findings must be regarded as preliminary, since they are based on only three microsatellite loci and nine geographic samples from China. Still, the fact that our phylogeographic structure and its low/shallow divergence are congruent with the findings of the COI study [4] and with those for other sympatric marine species [5-7] illustrates the potential utility of microsatellite markers for resolving the recent phylogeography of the rock shell throughout its range. Thus, we now call for a comprehensive microsatellite investigation of the rock shell to complement its detailed COI study. Towards this goal, although some refinement of their laboratory procedures may be needed, obvious candidates for this research are the six other loci that were reported by Li et al. [12], but not used here.

Appendix

Expected bootstrap scores for each group of the original NJ tree were exactly determined as follows. All possible triplets of A4, TCA31, and TCA160 (i.e., "A", "B", and "C", respectively) and their associated absolute frequencies were calculated with the polynomial: $(A + B + C)^3 = A^3 + 3A^2B + 3A^2C + B^3 + 3AB^2 + 3B^2C + C^3 + 3AC^2 + 3BC^2 + 6ABC$. Thus, there are 10 possible bootstrap datasets for A4, TCA31, and TCA160 and their absolute counts sum up to 27 (i.e., 3^3). Correspondingly, the relative frequencies for all ten bootstrap datasets are 1/27 for AAA, 3/27 for AAB, 3/27 for AAC, 1/27 for BBB, 3/27 for ABB, 3/27 for BBC, 1/27 for CCC, 3/27 for ACC, 3/27 for BCC, and 6/27 for ABC.

To determine exactly the expected scores, the ten bootstrap datasets for A4, TCA31, and TCA160 were generated and their NJ trees were inferred. Each group of the original NJ tree was scored for its presence among the ten bootstrap NJ trees. For each original group, the relative frequencies of the bootstrap datasets for its presences were summed to obtain (without the use of *n* replications) its expected score.

In a bootstrap analysis with three loci, the expected relative frequency of the ABC dataset (i.e., the original matrix) is 6/27. Thus, in our bootstrap analysis of A4, TCA31, and TCA160, each group of the original NJ tree is expected to receive a bootstrap score of at least 22%.

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