High Order Formation and Evolution of Hornerin in Primates

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Abstract

Genomic duplication or loss can accelerate evolution because the number of repeats could affect molecular pathways and phenotypes. We have previously reported that the repeated region of filaggrin (*FLG*), a crucial component of the outer layers of mammalian skin, had high levels of nucleotide diversity with species-specific divergence and expansion and that it evolved under the birth-and-death model. We focused on hornerin (*HRNR*), a member of the same gene family that harbor similar tandem repeats as *FLG*, and examined the formation process of repeated regions and the evolutional model that best fit the *HRNR* repeated region in the crab-eating macaque (*Macaca fascicularis*), orangutan (*Pongo abelii*), gorilla (*Gorilla gorilla*), and chimpanzee (*Pan troglodytes*) and compared them with the human (*Homo sapiens*) sequence. Paar et al. (2011) and Takaishi et al. (2005) have different theories as to the formation of the repeated region of *HRNR*; both groups share the longest repeat length of 1,404 bp (quartic or longest unit), but they differed in the process. We identified the formation described by Paar et al. {[("39 bp (primary) × 9" × 2 (secondary)) × 2 (tertiary)] × 5 (quartic)} to be conserved in all species except the crab-eating macaque. We detected high nucleotide diversities between the longest repeats, which fits the birth-and-death model. We concluded that the high order repeat formation of *HRNR* was conserved in primates except the crab-eating macaque. As previously identified in *FLG*, the longest repeats have high levels of nucleotide diversity, which could contribute to phenotypic differences between closely related species.

Key words: birth-and-death model, epidermal differentiation complex, hornerin, HRNR, primate, S100 fused type protein.

Introduction

The epidermal differentiation complex (EDC) locus, which is involved in immunity, perception, and epithelia, was identified as one of the most rapidly evolving loci in the human (*Homo sapiens*) genome. EDC on chromosome 1q21 spans ∼1.6 Mb comprising 65 genes represented by four gene families, that is, S100 fused type protein (SFTP), late cornified envelope, small proline repeat-rich, and S100-domain genes. SFTP family members locate in a clustered manner, share a tandem repeat structure on the third exon, and have a similar function in the cornified epithelium (Wu et al. 2009; Henry et al. 2012; Kypriotou et al. 2012). We previously reported that the repeated region of filaggrin, an important component of the outer layers of mammalian skin, has high levels of nucleotide diversity with species-specific divergence and expansion, and evolves under the birth-and-death model (Romero et al. 2017). There is a possibility that all the SFTP family members commonly evolve under the birth-and-death model because their repeated structure rendered the selective advantage and resulted the rapid evolution. Here, we focus on the hornerin gene (*HRNR*), a member of SFTP, which has not been well characterized thus far.

HRNR contains a tandem repeat structure like filaggrin and is expressed in regenerating and normal skin mostly observed on the head, trunk, legs, hands, and feet. *HRNR* is a component of the cornified epithelium of skin and, in conjunction with other members of the SFTP, reinforces the envelopes of the keratinizing epithelia (Henry et al. 2012). The formation of the repeat region of *HRNR* is controversial. Takaishi et al. (2005) described *HRNR* formation as starting with 117 bp, which was later amplified 4 times (468 bp), tripled to form 1,404 bp in tandem, and then further amplified 6-fold to form the repeated region of ∼8,424 bp (fig. 1*A*). On the contrary, Paar et al. (2011) described *HRNR* formation using the Global Repeat Map algorithm as follows: 39 bp, a "primary repeat unit" sequence, was amplified by nine to form a "secondary

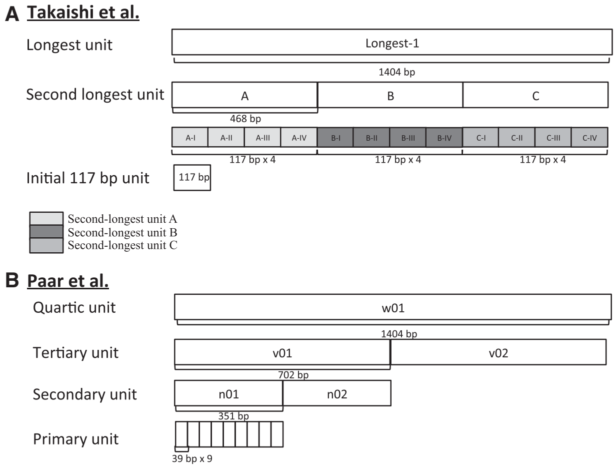


Fig. 1.—Schematic representation of hornerin repeated region formation by Takaishi et al. and Paar et al. (*A*) Takaishi et al. (2005) description starts with 117 bp (Initial 117-bp unit), which amplified 4 times (Second longest unit) and triplicated to form the "Longest unit." Light gray=Second-longest unit type A. Dark gray=Second-longest unit type B. Gray=Second-longest unit type C. (*B*) Paar et al. (2011) description starts with 39 bp to form the primary unit, which amplified 9 times to form the secondary unit (type n01 and n02), the secondary unit duplicated to form the tertiary unit (type v01 and v02) and finally the tertiary unit duplicated again to form the quartic unit (w01).

repeat unit" (351 bp), duplication of the secondary repeat unit forms a "tertiary repeat unit" of 0.70 kb, and finally two tertiary units form a 1.4 kb "quartic repeat unit" (fig. 1*B*). Notably, Paar et al. (2011) detected the higher order repeat structure only in human *HRNR* but not in chimpanzee, which could be a driving force for the evolutionary process. For both proposed formation models, the length of the final formed unit is 1,404 bp; however, the length of the starting unit and the pattern of duplication differ. We searched the best-fit model for the high order repeats contained in *HRNR* using Kimura's two-parameter substitution model. In this study, we scrutinize and compare the high order repeat formation of *HRNR* in several primates, which would facilitate an evaluation of the gene's evolutionary process.

Materials and Methods

HRNR Database Sequences

We obtained full-length *HRNR* DNA sequences from the National Center for Biotechnology Information (NCBI) gene database (http://www.ncbi.nlm.nih.gov/gene/; last accessed October 11, 2018) for the following species: *H. sapiens* (NC 000001.11), *P. troglodytes* (NC 006468.3), *G.* gorilla (NC 018424.1), *P. abelii* (NC 012591.1), and *M. fascicularis* (NC 022272.1).

Identification of *HRNR* Repeated Units in Primates

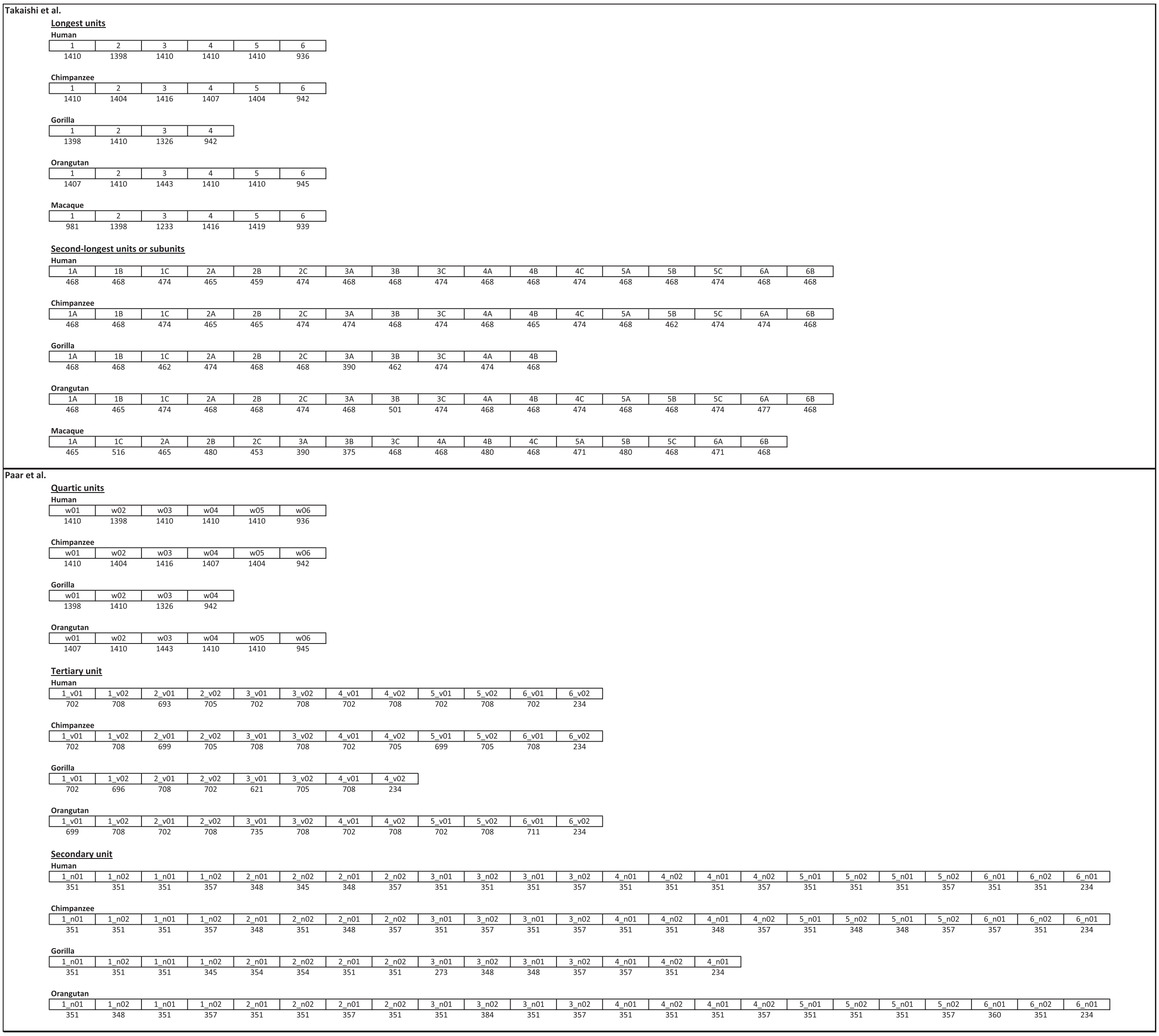
We used the amino acid description of the longest unit given by Takaishi et al. (2005), which was subdivided into three types of the second longest units (A, B, and C). Each was further divided into four initial 117-bp units (I–IV), and the rest of the nucleotide sequence repeats were detected in humans using the Align Sequences Nucleotide Basic Local Alignment Search Tool (BLAST) with an identity >80% (Zhang et al. 2000) (fig. 1 and supplementary fig. 1*A*, Supplementary Material online). As described by Takaishi et al. (2005), the 6th longest unit does not contain the type-C 2nd longest unit (fig. 2). Next, using BLAST (Zhang et al. 2000), we compared the repeated sequences from humans with those in the crab-eating macaque, orangutan, gorilla, and chimpanzee. Finally, the matched sequences were reanalyzed by BLAST and then manually curated (figs. 1 and 2, and supplementary fig. 1*A*, Supplementary Material online). In the case of the crab-eating macaque, we also acquired a dot-matrix plot that compared each initial 117-bp units of humans with the 4th longest unit of the crab-eating macaque (Zhang et al. 2000) (supplementary fig. S2, Supplementary Material online). Each of the identified longest units, second longest, and initial 117-bp units were considered as independent units for the subsequent analyzes. Additionally, we used the 39-bp sequence of the primary unit described by Paar et al. (2011) and detected the primary units for human, chimpanzee, gorilla, orangutan, and crab-eating macaque and then reconstructed the secondary, tertiary, and quartic units using BLAST (supplementary fig. 1*B*, Supplementary Material online).

The multiple alignment viewer Mview (Brown et al. 1998) allowed us to observe the alignment location between each of the initial 117-bp units of humans with the 4th longest unit of the crab-eating macaque, orangutan, and chimpanzee and the 2nd longest unit of the gorilla (supplementary fig. S3, Supplementary Material online).

The percentage of similarity by a pairwise comparison between the longest units was conducted using the percent identity matrix included in the web services of Clustal Omega at the European Molecular Biology Laboratory-European Bioinformatics Institute (McWilliam 2013) (http://www.ebi.ac.uk/Tools/msa/clustalo/; last accessed October 11, 2018).

Multiple Alignment and Phylogenetic Analyzes

We performed the multiple nucleotide sequence alignment using the profile alignment in ClustalW implemented in MEGA 6.06 (Tamura et al. 2011) using the longest human unit described by Takaishi et al. (2005) as a reference (supplementary table S1, Supplementary Material online). We then constructed neighbor-joining trees and maximum likelihood trees. Neighbor-joining trees were constructed by considering pairwise deletions for an average of 1,200 nucleotides, proportional nucleotide differences (*p*-distance), and 1,000 bootstrap resampling. We compared 24 DNA/Protein models in MEGA 6.06 and the best-fit model was Kimura's two-parameter substitution model assuming that the rate of



Takaishi et al.

Longest units

Human

1	2	3	4	5	6
1410	1398	1410	1410	1410	936

Chimpanzee

1	2	3	4	5	6
1410	1404	1416	1407	1404	942

Gorilla

1	2	3	4
1398	1410	1326	942

Orangutan

1	2	3	4	5	6
1407	1410	1443	1410	1410	945

Macaque

1	2	3	4	5	6
981	1398	1233	1416	1419	939

Second-longest units or subunits

Human

1A	1B	1C	2A	2B	2C	3A	3B	3C	4A	4B	4C	5A	5B	5C	6A	6B
468	468	474	465	459	474	468	468	474	468	468	474	468	468	474	468	468

Chimpanzee

1A	1B	1C	2A	2B	2C	3A	3B	3C	4A	4B	4C	5A	5B	5C	6A	6B
468	468	474	465	465	474	474	468	474	468	465	474	468	462	474	474	468

Gorilla

1A	1B	1C	2A	2B	2C	3A	3B	3C	4A	4B
468	468	462	474	468	468	390	462	474	474	468

Orangutan

1A	1B	1C	2A	2B	2C	3A	3B	3C	4A	4B	4C	5A	5B	5C	6A	6B
468	465	474	468	468	474	468	501	474	468	468	474	468	468	474	477	468

Macaque

1A	1C	2A	2B	2C	3A	3B	3C	4A	4B	4C	5A	5B	5C	6A	6B
465	516	465	480	453	390	375	468	468	480	468	471	480	468	471	468

Paar et al.

Quartic units

Human

w01	w02	w03	w04	w05	w06
1410	1398	1410	1410	1410	936

Chimpanzee

w01	w02	w03	w04	w05	w06
1410	1404	1416	1407	1404	942

Gorilla

w01	w02	w03	w04
1398	1410	1326	942

Orangutan

w01	w02	w03	w04	w05	w06
1407	1410	1443	1410	1410	945

Tertiary unit

Human

1_v01	1_v02	2_v01	2_v02	3_v01	3_v02	4_v01	4_v02	5_v01	5_v02	6_v01	6_v02
702	708	693	705	702	708	702	708	702	708	702	234

Chimpanzee

1_v01	1_v02	2_v01	2_v02	3_v01	3_v02	4_v01	4_v02	5_v01	5_v02	6_v01	6_v02
702	708	699	705	708	708	702	705	699	705	708	234

Gorilla

1_v01	1_v02	2_v01	2_v02	3_v01	3_v02	4_v01	4_v02
702	696	708	702	621	705	708	234

Orangutan

1_v01	1_v02	2_v01	2_v02	3_v01	3_v02	4_v01	4_v02	5_v01	5_v02	6_v01	6_v02
699	708	702	708	735	708	702	708	702	708	711	234

Secondary unit

Human

1_n01	1_n02	1_n01	1_n02	2_n01	2_n02	2_n01	2_n02	3_n01	3_n02	3_n01	3_n02	4_n01	4_n02	4_n01	4_n02	5_n01	5_n02	5_n01	5_n02	6_n01	6_n02	6_n01
351	351	351	357	348	345	348	357	351	351	351	357	351	351	351	357	351	351	351	357	351	351	234

Chimpanzee

1_n01	1_n02	1_n01	1_n02	2_n01	2_n02	2_n01	2_n02	3_n01	3_n02	3_n01	3_n02	4_n01	4_n02	4_n01	4_n02	5_n01	5_n02	5_n01	5_n02	6_n01	6_n02	6_n01
351	351	351	357	348	351	348	357	351	357	351	357	351	351	348	357	351	348	348	357	357	351	234

Gorilla

1_n01	1_n02	1_n01	1_n02	2_n01	2_n02	2_n01	2_n02	3_n01	3_n02	3_n01	3_n02	4_n01	4_n02	4_n01
351	351	351	345	354	354	351	351	273	348	348	357	357	351	234

Orangutan

1_n01	1_n02	1_n01	1_n02	2_n01	2_n02	2_n01	2_n02	3_n01	3_n02	3_n01	3_n02	4_n01	4_n02	4_n01	4_n02	5_n01	5_n02	5_n01	5_n02	6_n01	6_n02	6_n01
351	348	351	357	351	351	357	351	351	384	351	357	351	351	351	357	351	351	351	357	360	351	234

Fig. 2.—Longest, second-longest, quartic, tertiary, and secondary units of hornerin in primates by Takaishi et al. and Paar et al. formation. By Takaishi et al. formation, the number of longest-units was six for human, chimpanzee, orangutan, and crab-eating macaque and four for gorilla. Each longest-unit comprising second-longest units (*A*–*C*) varied from 981 to 1,443 bp and the last longest-unit lacking the second-longest unit C ranged from 936 to 945 bp. By Paar et al. formation, the number of quartic units was six for human, chimpanzee, orangutan, and crab-eating macaque and four for gorilla and ranged from 936 to 1,410 bp. The number of tertiary units was 12 for human, chimpanzee, orangutan, and crab-eating macaque and 8 for gorilla and the length varied from 234 to 711 bp. The number of secondary units was 23 for human, chimpanzee, orangutan, and crab-eating macaque and 15 for gorilla and the length varied from 234 to 357 bp.

substitution for each site follows a Gamma distribution with a BIC of 16041.42 (supplementary table S2, Supplementary Material online). Maximum likelihood trees were constructed using the following predefined parameters: partial deletions, Kimura's two-parameter substitution model assuming that the rate of substitution for each site follows a Gamma distribution, the nearest-neighbor-interchange heuristic method, and 1,000 bootstrap resampling. A cutoff value of 50% was set for the condensed tree (Romero et al. 2017).

Estimation of Polymorphic/Variant Sites, Nucleotide Diversity, and Ratio of Synonymous and Nonsynonymous Sites Using the DNAsp5 Program

The number of nonsynonymous and synonymous substitutions between repeats within a species was calculated using DNA polymorphism and estimating the synonymous and nonsynonymous change options in DNAsp5 (Librado and Rozas 2009). DNAsp5 estimated the nucleotide diversity (π), which is the average number of nucleotide differences per site between sequences. DNAsp5 also calculated the ratio of the number of nonsynonymous nucleotide substitutions per total number of nonsynonymous sites for each codon (*Ka*) to the number of synonymous nucleotide substitutions per total number of synonymous sites for each codon (*Ks*) (Hu and Banzhaf 2008; Librado and Rozas 2009). We estimated the *Ka*/*Ks* ratio for each pair of within-species repeats including gaps using the program DNAsp5. The level of purifying selection was then calculated as (1 – *Ka*/*Ks*) × 100.

Inference of Gene Duplication and Loss in the Species Tree

We used the NOTUNG program to reconcile the species tree that had the best fit for duplication, transfer, loss, and incomplete lineage sorting events from the maximum likelihood tree described earlier (Durand et al. 2006; Vernot et al. 2008; Stolzer et al. 2012).

Results

HRNR Sequences in Primates

The hornerin gene *HRNR* is localized on chromosome 1 of the crab-eating macaque, orangutan, gorilla, chimpanzee, and human. *HRNR* comprises three exons with a conserved exon/intron composition between the mouse and human. The repeat region is embedded in the third exon and does not include introns (Henry et al. 2012; Kypriotou et al. 2012). The DNA sequences of *HRNR* were obtained from the NCBI gene database for human, chimpanzee, gorilla, orangutan, and crab-eating macaque. The length of the longest repeat formed was 1,404 bp and shared both formations proposed by Paar et al. (2011) and Takaishi et al. (2005) (fig. 1). In the subsequent analyses, each of the repeats was considered as an independent unit.

The number of the longest units was six for human, chimpanzee, orangutan, and crab-eating macaque and four for gorilla (fig. 2). Each longest unit comprising second longest units (A, B, and C) varied from 981 to 1,443 bp, and the last longest unit lacking the second longest unit C ranged from 936 to 945 bp (fig. 2). The similarity between the longest units within a species ranged from 75.8% for the crab-eating macaque to 98.3% for human (supplementary table S3, Supplementary Material online).

Phylogenetic Analysis of Longest Units across Species

Based on the results of the multiple sequence alignment of the longest unit (supplementary table S1, Supplementary Material online), we constructed phylogenetic trees using the neighbor-joining and maximum likelihood methods with a cutoff value of 50% due to the high similarity of the repeats, as described in Materials and Methods. The crab-eating macaque was used as an outgroup (fig. 3 and supplementary fig. S4*A*, Supplementary Material online).

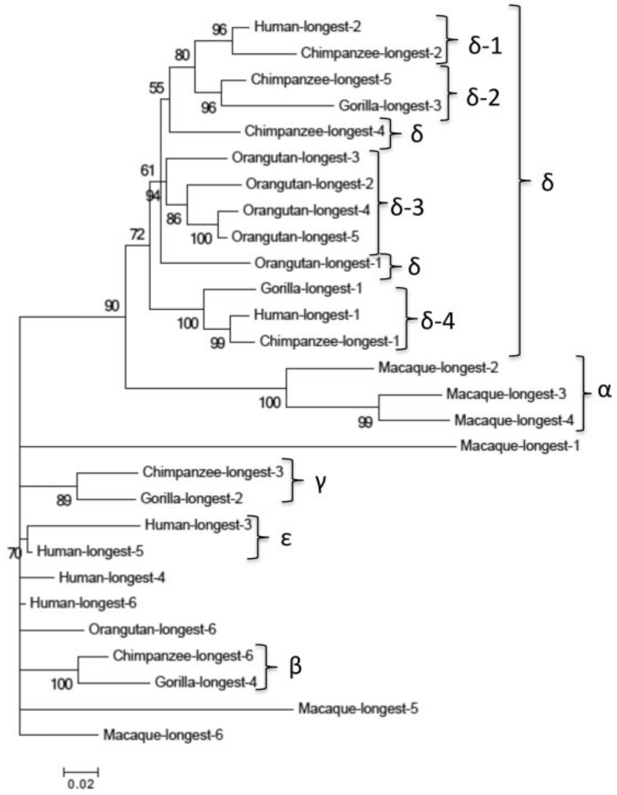


Fig. 3.—Maximum likelihood tree reconstruction using hornerin longest-units in these primates and the following parameters: partial deletions, Kimura's two-parameter substitution model assuming that the rate of substitution for each site follows Gamma distribution, the nearest-neighbor-interchange heuristic method, and 1, 000 bootstrap resampling. A cutoff value for the condensed tree was set at 50%. Crab-eating macaque was used as an outgroup. The longest-units that clustered within the species are found in clusters "α," "δ-3," and "ϵ." Some of gorilla and chimpanzee repeats clustered across species in clusters "β" and "γ." The rest of the repeats formed a big cluster "δ" across species, which was further subdivided into clusters from "δ-1" to "δ-4." Chimpanzee 4th longest-unit and orangutan 1st longest-unit were in cluster "δ." Finally, human 4th longest-unit and 6th longest-unit, orangutan 6th longest-unit, and crab-eating macaque 1st longest-unit, 5th longest-unit, and 6th longest-unit did not cluster with any repeat.

Both methods gave similar phylogenetic trees (fig. 3 and supplementary fig. S4*A*, Supplementary Material online). In the maximum likelihood tree, the longest units, from 2 to 4 for crab-eating macaque (cluster "α"), from 2 to 5 for orangutan (cluster "δ-3"), and 3 and 5 units for human (cluster "ε") clustered within the species. Some of gorilla and chimpanzee repeats clustered across species as follows: chimpanzee 6th longest unit and gorilla 4th longest unit (cluster "β") and chimpanzee 3rd longest unit and gorilla 2nd longest unit (cluster "γ"). The rest of the repeats formed a big cluster "δ" across species, which was further subdivided; chimpanzee and human 2nd longest units ("δ-1"), chimpanzee 5th longest unit, and gorilla 3rd longest unit ("δ-2"), orangutan repeats from 2 to 5 ("δ-3"), and gorilla, chimpanzee, and human 1st longest units ("δ-4") (fig. 3). Chimpanzee 4th longest unit and orangutan 1st longest unit were in cluster "δ." Finally, human 4th and 6th longest units, orangutan 6th longest unit, and crab-eating macaque 1st, 5th, and 6th longest units did not cluster with any repeat. The neighbor-joining tree further grouped the human 4th longest unit into cluster "ε," the crab-eating macaque 1st longest unit into cluster "α," and the orangutan 6th longest unit and crab-eating macaque 5th and 6th longest units into cluster "β" (supplementary fig. S4*A*, Supplementary Material online).

Additionally, we constructed a species-gene tree and identified the duplications and losses in the phylogeny. Clusters "α," "δ-3," and "ε" were the results of duplications within the crab-eating macaque, orangutan, and human, respectively. Five losses within cluster "δ" and six losses on the remaining branches were identified between the orangutan, gorilla, chimpanzee, and human (supplementary fig. S4*B*, Supplementary Material online).

HRNR Formation in Primates

Primary Units (39 bp) Described by Paar et al. (2011)

The proposed formation by Takaishi et al. (2005) was {[(117 bp × 4) × 3 subunits] × 6 units} (fig. 1*A*), whereas the formation by Paar et al. (2011) was {[("39 bp (primary) × 9" × 2 (secondary)) × 2 (tertiary)] × 5 (quartic)} (fig. 1*B*). We examined both possibilities and clarified which structure was detectable in primates.

We started with the primary unit (39 bp) sequence and estimated the primary units for all primates using BLAST (fig. 4*A* and *B*). The phylogeny of the primary units divided them into nine clusters (order, 1–9) in each species tree by Paar et al. (2011). This pattern was observed for human, chimpanzee, gorilla, and orangutan (fig. 4*A* and supplementary fig. S4*A*–*D*, Supplementary Material online). However, the branches had low bootstrap values, and when adjusted to a cutoff value of 50%, the nodes were lost probably due to the short length of the unit and low similarity across the primary units (~30% in human). The primary units of the crab-eating macaque did not divide into the nine clusters and were placed into a different order unit (supplementary fig. S5*E*–*G*, Supplementary Material online). The primary units of the crab-eating macaque suggest a different duplication pattern from those of large primates with independent duplications and losses, resulting in the length variation observed in the longest unit (fig. 4*B* and supplementary fig. S5*E*–*G*, Supplementary Material online).

Initial 117-bp Unit and Second Longest Unit Described by Takaishi et al. (2005)

We focused on the starting unit proposed by Takaishi et al. (2005) and estimated the initial 117 bp for all primates except the crab-eating macaque using BLAST (fig. 1*A*). As described in the previous section, the crab-eating macaque had a low conservation to human at 117-bp units and was excluded from further analyzes (supplementary fig. S2*A*, Supplementary Material online and fig. 4*A* and *B*).

According to the *HRNR* formation proposed by Takaishi et al. (2005), the phylogenetic tree of the initial 117-bp units groups them into I–IV clusters for each of the A to C types of second longest units (figs. 1*A* and 5). Taking this into consideration, we constructed a phylogenetic tree for the initial 117-bp units for human, chimpanzee, gorilla, and orangutan. The clustered pattern described by Takaishi et al. (2005) was not detected; we observed the following overall clusters: A-I, A-IV, B-III, and C-II ("Group-1st"); A-II, B-I, B-IV, and C-III ("Group-2nd"); and A-III, B-II, C-I, and C-IV ("Group-3rd"), with the exception of a few initial 117-bp units in human B-III, chimpanzee A-I and IV, B-I to III, and C-III and IV, gorilla B-I and II and C-I, orangutan A-I and III, B-I to IV, and C-I and III, and all orangutan C-II and IV (fig. 5 and supplementary figs. S3 and S5*A*–*C*, Supplementary Material online). The phylogenic clustering implies the following sequential pattern: "Group-1st," "Group-2nd," and "Group-3rd," which fit the length of the secondary unit (351 bp) described by Paar et al. (2011) (figs. 1*B* and 5).

Next, we used the second longest units to construct a phylogenetic tree (figs. 1*A* and 2). Indeed, the tree clustered together each of the second longest unit types A to C (supplementary fig. S7, Supplementary Material online); however, the phylogeny of the initial 117-bp unit was not consistent with the duplication pattern described by Takaishi et al. (2005). We concluded the proposed pattern by Takaishi et al. unlikely (fig. 5 and supplementary fig. S6*A*–*C*, Supplementary Material online).

Secondary Unit (351 bp) by Paar et al. (2011)

We reconstructed the secondary units organized by nine repeats of the primary unit as described before, with the exception of the crab-eating macaque, in which the order of the primary units was not sequential (fig. 2). We constructed a phylogenetic tree for the secondary units across species (supplementary fig. S8, Supplementary Material online). The phylogeny clustering for the secondary units gathered an n01 type, n02 type, and the last units for gorilla (except for w04-v01-n01 and w04-v02-n01), orangutan, chimpanzee, and human secondary units (supplementary fig. S8, Supplementary Material online).

Tertiary Units (702 bp) by Paar et al. (2011)

We next focused on tertiary units proposed by Paar et al. (2011). We used the secondary units to reconstruct the tertiary units (fig. 2). Paar et al. (2011) divided tertiary units into "v01" and "v02" types. We constructed a phylogenetic tree

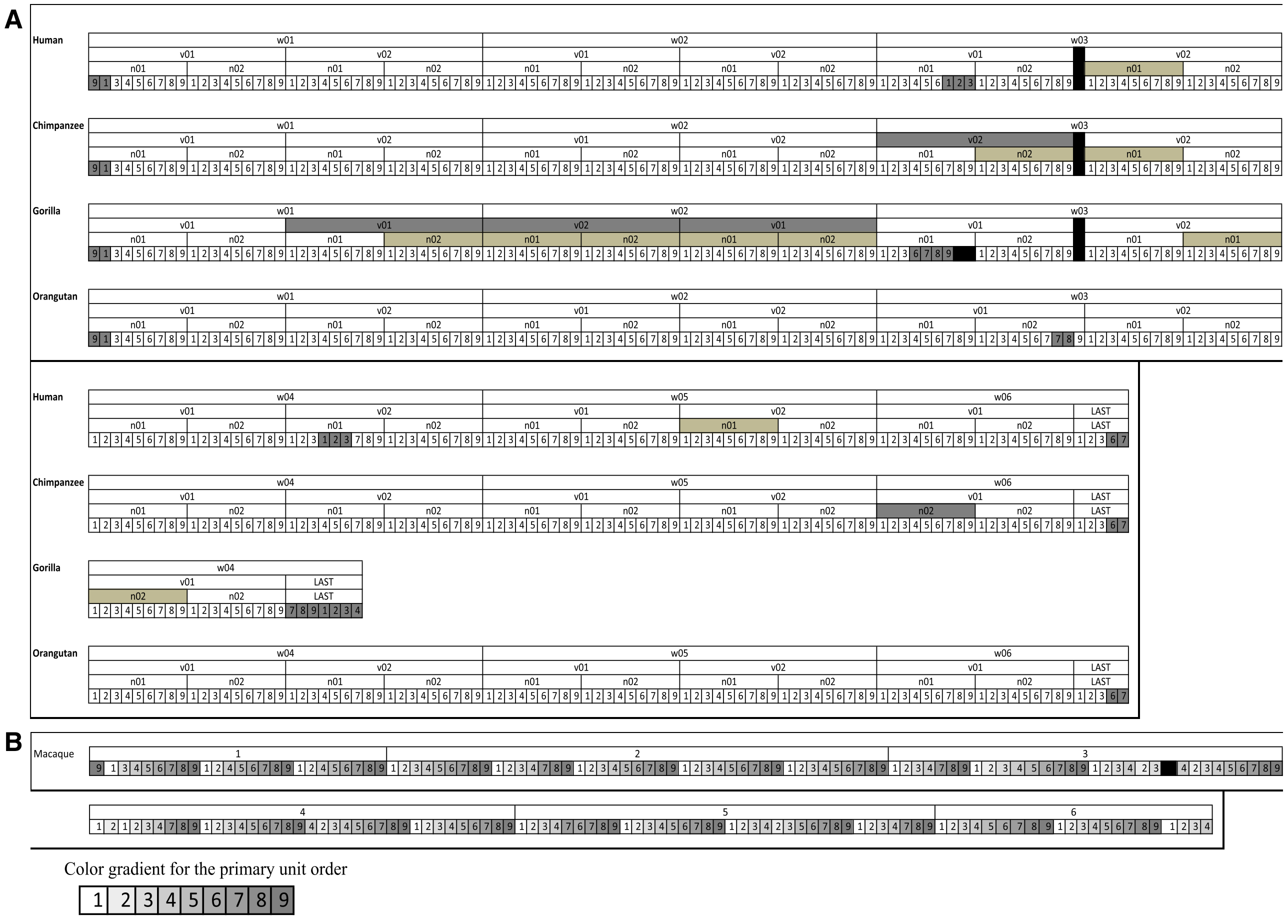


FIG. 4.—Quartic, tertiary, secondary, and primary units reconstructed by using the clusters from the 39-bp units' phylogenetic tree analysis. (*A*) Quartic, tertiary, secondary, and primary units in human, chimpanzee, gorilla, and orangutan. First line represents quartic units from w01 to w06, second line shows tertiary units type v01, v02, and last unit, third line shows secondary units type n01, n02, and last unit and the fourth line are primary units ranging from type 1 to 9. Black=absent unit. Second line dark gray=tertiary unit not in order. Third line light brown=secondary unit not in order. Fourth line dark gray=primary unit not in order. (*B*) Primary units in crab-eating macaque ranging from type 1 to 9. White to dark gray=color gradient for the primary unit. Black=absent unit.

for the tertiary units across species and identified clusters for the v01 type, v02 type, and the last units for chimpanzee (except for w03-v01), gorilla (except for w01-v02, w02-v01, w02-v02, and w03-v01), orangutan, and human (supplementary fig. S9, Supplementary Material online). The tertiary units were clustered similar to the phylogeny of the secondary units. In the phylogeny of the secondary and tertiary units, we also observed duplications within species, more commonly in orangutan and human (supplementary figs. S7 and S8, Supplementary Material online).

Quartic Units (1404 bp) by Paar et al. (2011) or the Longest Unit by Takaishi et al. (2005)

As for the quartic unit repeat, Takaishi et al. (2005) described six longest units and Paar et al. (2011) described five. Because the last unit contains only the second longest units of type A and B, it was not detected by the Global Repeat Maker algorithm developed by Paar et al. (2011) (fig. 1*B*). Multiple alignment and phylogenetic analyses for the longest unit were described in the previous section.

Taking into consideration our results from the analyses of both Takaishi et al. (2005) and Paar et al. (2011), we concluded that the secondary units duplicated to form the tertiary units and then again duplicated to form quartic units. According to the description by Paar et al. (2011), this organization was only detected in human but not in chimpanzee. However, our analyses detected the organization to be conserved in chimpanzee, gorilla, and orangutan by the longest unit clustering found in the phylogenetic analysis (fig. 3) using the latest version of the NCBI gene database.

Nucleotide Diversity in the Longest Units of Hornerin

The concerted and birth-and-death models have been proposed to explain the nondivergent evolutionary pattern found in repeated gene families. In the concerted model, variations in one repeat spread to the others by unequal crossover or gene

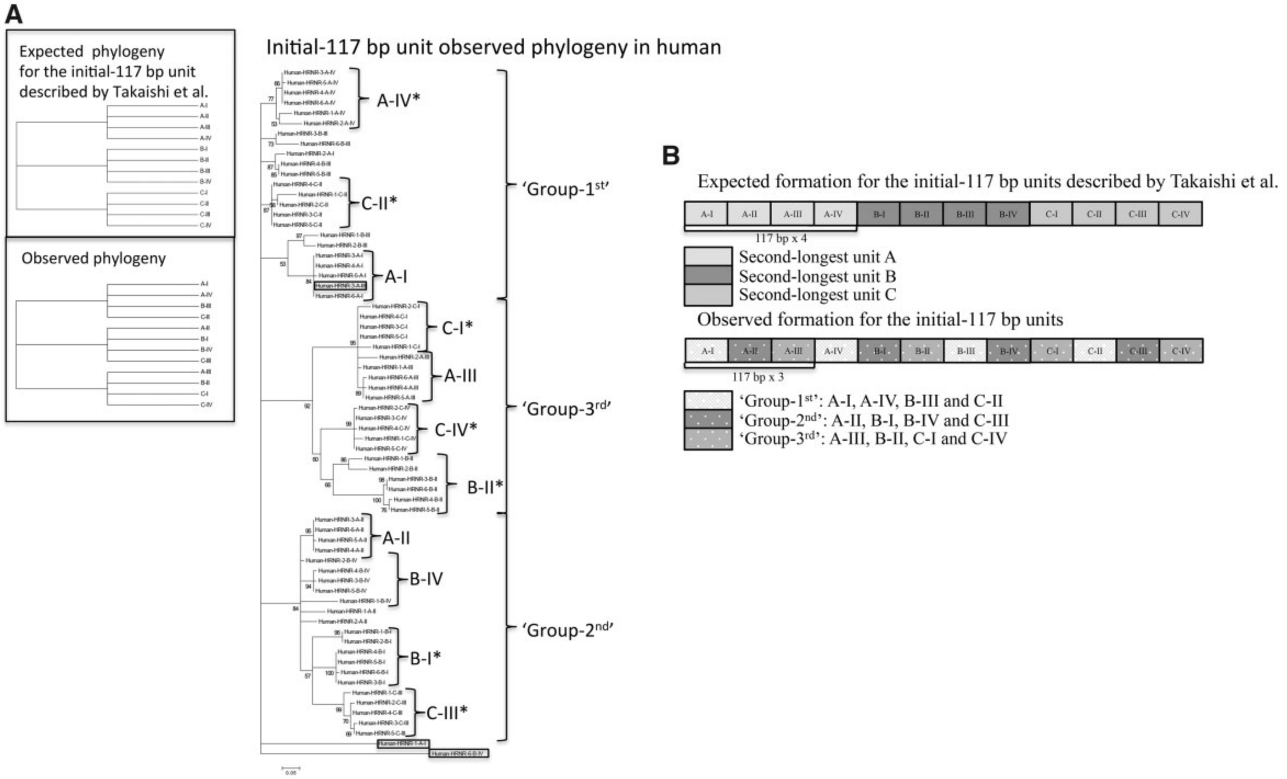

A
Expected phylogeny for the initial-117 bp unit described by Takaishi et al.
Observed phylogeny
Initial-117 bp unit observed phylogeny in human
A-IV*
C-II*
A-I
'Group-1st'
C-I*
A-III
C-IV*
'Group-3rd'
B-II*
A-II
B-IV
'Group-2nd'
B-I*
C-III*
B
Expected formation for the initial-117 bp units described by Takaishi et al.
A-I A-II A-III A-IV B-I B-II B-III B-IV C-I C-II C-III C-IV
117 bp x 4
Second-longest unit A
Second-longest unit B
Second-longest unit C
Observed formation for the initial-117 bp units
A-I A-II A-III A-IV B-I B-II B-III B-IV C-I C-II C-III C-IV
117 bp x 3
'Group-1st': A-I, A-IV, B-III and C-II
'Group-2nd': A-II, B-I, B-IV and C-III
'Group-3rd': A-III, B-II, C-I and C-IV

Fig. 5.—Maximum-likelihood tree analyses for human initial-117-bp units and schematic representation. (*A*) Maximum-likelihood tree using the following parameters: partial deletions, Kimura's two-parameter substitution model assuming that the rate of substitution for each site follows Gamma distribution, the nearest-neighbor-interchange heuristic method, and 1,000 bootstrap resampling. A cutoff value for the condensed tree was set at 50%. According to the *HRNR* formation proposed by Takaishi et al. the expected phylogenetic tree should group I–IV clusters for each of the A–C types of second-longest units. The observed phylogeny detected the following clusters; A-I, A-IV, B-III, and C-II ("Group-1st"), A-II, B-I, B-IV, and C-III ("Group-2nd"), and A-III, B-II, C-I, and C-IV ("Group-3rd") with the exceptions of human B-III. Black rectangle=initial-117-bp unit clustered on a different numerical group. Asterisk=initial-117-bp units that clustered on their proper group. (*B*) Schematic representation from the Maximum-likelihood tree previously described. The cluster pattern follows, "roup-1st," "Group-2nd," and "Group-3rd," which fits the length of the secondary unit described by Paar et al. Light gray=second-longest unit A, dark gray=second-longest unit B, gray=second-longest unit C. Light gray with white dots=A-I, A-IV, B-III, and C-II ("Group-1st"). Dark gray with white dots=A-II, B-I, B-IV, and C-III ("Group-2nd"). Gray with white dots=A-III, B-II, C-I, and C-IV ("Group-3rd").

conversion, maintaining homogeneity among the repeats (Nei et al. 2000). In the birth-and-death model, new repeat genes diversify by silent nucleotide substitutions, which, with enough divergence time, can lead to lineage-specific expansions (Nei et al. 2000). The main difference between these two models is the high levels of intragenic nucleotide diversity that are only found in the birth-and-death model (Nei et al. 2000).

We calculated the total number of sites showing variation and nucleotide diversity (π) between the longest units of *HRNR* in each primate using the DNAsp5 program, as described in Materials and Methods. The estimated π of human, chimpanzee, gorilla, orangutan, and crab-eating macaque was 1.1×10^{-2}, 1.4×10^{-2}, 1.6×10^{-2}, 1.4×10^{-2}, and 1.7×10^{-2}, respectively (table 1). We then compared π of *HRNR* with that of the polyubiquitin gene and filaggrin repeats (Romero et al. 2017), and rDNA genes, which are representative multigene families that have evolved under birth-and-death evolution and concerted evolution, respectively (Nei et al. 2000; Ganley and Kobayashi 2007). The π of *HRNR* was comparable with that of polyubiquitin genes (range $= 8.9 \times 10^{-2}$ to 2.0×10^{-1}) and filaggrin repeats (range $= 3.2 \times 10^{-2}$ to 7.7×10^{-2}), and was much larger than those of rDNA genes (range $= 1.0 \times 10^{-5}$ to 1.8×10^{-4}) (Nei et al. 2000; Ganley and Kobayashi 2007). This suggests that *HRNR* longest units have evolved under the birth-and-death model.

Selection within the Repeats of Each Species

The birth-and-death model maintains repeat similarity by purifying selection. Under purifying selection, the number of nonsynonymous variations in a gene is expected to be smaller than the number of synonymous variations. Purifying selection can be measured by making pairwise comparisons [$(1 - Ka/Ks) \times 100$], which indicate the percentage of synonymous mutations (Nei 2007).

Table 1
Nucleotide Variation, Average Synonymous Variations, Average Nonsynonymous Variations and Average Ka/Ks from All Repeats Within Five Primate Species

	Nucleotide Variation	Average Ks	Average Ka	Average Ka/Ks	Average (1−Ka/Ks)×100
Human	0.13	0.22	0.12	0.54	46.30
Pan troglodytes	0.14	0.23	0.14	0.63	36.84
Gorilla gorilla	0.16	0.28	0.16	0.56	44.16
Pongo abelii	0.14	0.2	0.11	0.71	29.05
Macaque	0.17	0.32	0.26	0.51	49.47

We found that the average percentage of synonymous substitutions for human, chimpanzee, gorilla, orangutan, and crab-eating macaque was 46.30%, 36.84%, 44.16%, 29.05%, and 49.47%, respectively (table 1). Although nucleotide substitutions were generally synonymous, we found three pairs of slightly higher nonsynonymous variations in the longest units within species; the 3rd and 2nd longest units, 3rd and 4th longest units, and 3rd and 5th longest units. None of the pairs of repeats in the crab-eating macaque, gorilla, chimpanzee, or human had $Ka/Ks > 1$ (supplementary table S4, Supplementary Material online). We concluded that the high synonymous variation detected in the longest units fits the birth-and-death model.

Discussion

Hornerin has a unique and complex duplicate formation that is different from other SFPTs. The hornerin repeat formations proposed by Takaishi et al. (2005) {[(117 bp × 4 initial) × 3 subunits] × 6 units} and by Paar et al. (2011) {[("39 bp × 9 primary" × 2 secondary) × 2 tertiary] × 5 quartic} were examined and compared among several primates (fig. 1).

In the repeat organization by Paar et al. (2011), 39 bp of the primary unit was detected in chimpanzee, gorilla, orangutan, and crab-eating macaque (fig. 2). The phylogeny of the primary units per species divided them into nine clusters in chimpanzee, gorilla, and orangutan but not the crab-eating macaque (fig. 4 and supplementary fig. S5*E*–*G*, Supplementary Material online). We did not observe the expected phylogeny for the initial 117-bp unit with a 4-fold duplication pattern as described by Takaishi et al. (2005) (fig. 5). The phylogeny for most human, chimpanzee, gorilla, and orangutan secondary units clustered in n01, n02, and the last units and the tertiary units clustered in v01, v02, and the last units (supplementary fig. S8, Supplementary Material online). We observed clustering within human and orangutan, suggesting unique duplications in these species (supplementary fig. S8, Supplementary Material online). We concluded that the primary unit is conserved and detectable in all primates but the duplication clustering order from 1 to 9 was not found in the crab-eating macaque (supplementary fig. S5*E*–*G*, Supplementary Material online). We confirmed that in all primates except the crab-eating macaque the formation model started with the primary units which duplicated to make the secondary units, and the secondary units duplicated twice again to form the tertiary structure, as described by Paar et al. (2011) (fig. 1*B*).

The longest units or quartic units, common to both Takaishi et al. (2005) and Paar et al. (2011), were used to understand hornerin evolution. Two models have been proposed to explain the nondivergent evolutionary pattern found in repeated gene families; concerted and birth-and-death (Nei et al. 2000). In the concerted model, the low level of diversity of repeats within a species is the results from unequal crossover or gene conversion, and the expected phylogeny clusters together the more similar repeats within a species. In the birth-and-death model, there is a high level of diversity between repeats, and genes have a close interspecies pattern in the phylogeny (Nei et al. 2000). Similar to filaggrin, hornerin units have high synonymous nucleotide diversity together with various lineage-specific expansions that fit the birth-and-death model (Nei et al. 2000; Nei and Rooney 2005; Nei 2007; Eirín-López et al. 2012; Sabbagh et al. 2013) (table 1 and supplementary tables S3 and S4, Supplementary Material online). The birth-and-death model has been used to explain most of multigene families evolution. One example is the Odorant-Binding Protein (OBP) family of Drosophila species (Vieira et al. 2007). All members from the OBP family show tandem repeat duplications evolving under purifying selection with different functional constraints per gene and a progressive nucleotide divergence, and likely rapidly affecting the sensitivity or specificity in detecting odorants under different environments (Eirín-López et al. 2012). Another example is the fatty acid reductase (FAR) multigene family. The number of FAR genes per genome can vary greatly between organisms and phylogenetic relationships of its representatives show a pattern of between-species gene clustering, including some relative long branches. Most of the expansions occurred in plants and insects compared with other taxa and is the underlying cause for their ability to synthesize and utilize a wide variety of fatty alcohol-based or derived compounds for a number of highly specialized functions (Eirín-López et al. 2012). As for the OBP and FAR families, the gene divergence found in different species result in rapid functional changes. It has previously been reported that gene-associated tandem repeats act as an accelerator of evolution by generating variation in structure and functionality (Fondon and Garner 2004). Under the birth-and-death model, duplicates vary by silent nucleotide variations, which, with enough divergence time, can lead to lineage-specific expansions, especially observed in multigene families evolution. The similar evolutionary pattern for hornerin and filaggrin could be an indication that members of the SFTP family also evolve under the birth-and-death model (Vieira et al. 2007). The possibility of all the members of SFTP evolving under this model could explain the advantage of their repeated structure resulting in the rapid evolution required by genes from skin.

Hornerin variation within a species has not been reported; however, the biological importance of tandem repeats was mentioned by Paar et al. (2011) as a rapidly evolving type of DNA sequences that could contribute to phenotypic differences, even between closely related species such as humans and chimpanzees. The Global Repeat Map of Paar et al. (2011) failed to find the high order repeat of human in chimpanzee; however, our analysis detected the 39-bp length to be conserved in all primates except the crab-eating macaque, probably due to differences in the database version. Our analysis also detected unique duplications for all primates, especially for human and orangutan in the secondary and tertiary phylogeny. Neuroblastoma break-point family (*NBFP*) copy number variability is a dramatic example of high order repeat in human and chimpanzee (Paar et al. 2011). The NBFP repeat is related to the evolutionary level of higher primates and the high order repeat pattern shows a discontinuous jump in the evolutionary step from 48 monomers in chimpanzee to 165 monomers in human, possibly related to a regulatory function of high order repeats (Paar et al. 2011). Indeed, there was no change in the number of hornerin copies between chimpanzees and humans; however, the duplications found in the phylogeny could possibly contribute to a difference in phenotype. Although the functional significance of copy number variation of *HRNR* repeats in primates requires further studies, we suggest that, like *NBFP* and *FLG*, *HRNR* high repeat order pattern might contribute to a different phenotype.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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