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Relic of ancient recombinations in gibbon ABO blood group genes deciphered through phylogenetic network analysis

Takashi Kitano [a,1], Reiko Noda [a], Osamu Takenaka [b,†], Naruya Saitou [a,*]

[a] Division of Population Genetics, National Institute of Genetics, 1111 Yata, Mishima 411-8540, Japan
[b] Primate Research Institute, Kyoto University, Inuyama 484-8506, Japan

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ABSTRACT

The primate ABO blood group gene encodes a glycosyl transferase (either A or B type), and is known to have large coalescence times among the allelic lineages in human. We determined nucleotide sequences of *ca.* 2.2 kb of this gene for 23 individuals of three gibbon species (agile gibbon, white-handed gibbon, and siamang), and observed a total of 24 haplotypes. We found relics of five ancient intragenic recombinations, occurred during *ca.* 2–7 million years ago, through a phylogenetic network analysis. The coalescence time between A and B alleles estimate precede the divergence (*ca.* 8 MYA) of siamang and common gibbon lineages. This establishes the coexistence of divergent allelic lineages of the ABO blood group gene for a long period in the ancestral gibbon species, and strengthens the non-neutral evolution for this gene.

1. Introduction

The human A and B alleles of the ABO blood group gene code for glycosyltransferases, which transfer *N*-acetylgalactosamine and galactose, respectively, to a common precursor (Yamamoto et al., 1990, 1995). Two critical sites for the distinction between A and B activities of the glycosyltransferase have been identified (Yamamoto and Hakomori, 1990) in exon 7, and these nucleotide differences were concordant with serological studies (e.g., Moor-Jankowski et al., 1964). Evolutionary analysis of nucleotide sequences of this gene for great apes and Old World monkeys (Kominato et al., 1992; Martinko et al., 1993) as well as those for human suggested that this gene is under some kind of positive selection (Saitou and Yamamoto, 1997). Further sequence analyses of this gene for great apes and Old World monkeys confirmed the unique feature of this gene (O'hUigin et al., 1997; Kermarrec et al., 1999; Kitano et al., 2000; Noda et al., 2000; Sumiyama et al., 2000).

Lesser apes (gibbons) are also known to be polymorphic at the ABO blood group gene from serological studies (Blancher and Socha, 1997), however, no study was so far conducted at nucleotide sequence level. We thus determined partial sequences of the ABO blood group genes for three gibbon species (agile gibbon [*Hylobates agilis*], white-handed gibbon [*H. lar*], and siamang [*Symphalangus syndactylus*]). Agile gibbon and white-handed gibbon are common gibbons, and occupy parapatric distribution in Malay Peninsula and Sumatra Island. Agile gibbons also distribute in the southwest part of Borneo Island. Siamang is more distantly related from common gibbons, and is sympatric with agile and white-handed gibbons (see http://www.gibbons.de/main/index.html for their geographical distribution map).

The ABO blood group gene is located in autosome (chromosome 9 in human). Recombination or gene conversion events on autosomal DNA sequences may create non-tree structures. We therefore conducted a phylogenetic network analysis so as to detect any reticulations, and found ancient recombinations which is important for inferring the history of genes.

2. Materials and methods

2.1. Genomic DNA sequencing

We sequenced a total of 23 individuals from three lesser ape species (five agile gibbons, 12 white-handed gibbons, and six siamangs) from a part of exon 5 to a part of exon 7 of the ABO blood group genes (*ca.* 2.2 kb). Two primers on exon 5 (forward: 5′-ACC CCC AGC CAA AGG TGC TGA CA-3′) and on exon 7 (reverse: 5′-CGA TGC CGT TGG CCT GGT C-3′) were used for each PCR. PCR products were confirmed by 1% agarose gel electrophoresis, purified using Micro Spin Columns (Amersham Biosciences), and inserted into a vector using TOPO TA Cloning Kit (Invitrogen). At

* Corresponding author. Fax: +81 559 81 6789.
E-mail address: saitounr@lab.nig.ac.jp (N. Saitou).
[1] Present address: Department of Biomolecular Functional Engineering, Ibaraki University College of Engineering, Hitachi 316-8511, Japan.
[†] Deceased.

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least three clones for each allele from each individual were sequenced by using BigDye Terminator Cycle Sequencing Kit and ABI PRISM 377/3100 DNA sequencer (PE Biosystems). Both strands were read using PCR primers and inner primers. A list of primers used in this study is available from T.K. upon request.

2.2. Sequence analyses

CLUSTAL W ver.1.8 (Thompson et al., 1994) was used for multiple alignments. Because phylogenetic network analysis was shown to be quite effective from our previous studies (Saitou and Yamamoto, 1997; Kitano and Saitou, 1999; Kitano et al., 2000; Noda et al., 2000; Sumiyama et al., 2000), this analysis was used. A phylogenetic network based on a distance matrix was first constructed by using the neighbor-net method (Bryant and Moulton, 2004; Huson and Bryant, 2006), then phylogenetic networks based on multiple alignment of nucleotide sequence data were constructed manually following the procedure of Bandelt (1994) and Saitou and Yamamoto (1997). Phylogenetic trees were constructed by using the neighbor-joining method (Saitou and Nei, 1987). Kimura's (1980) two-parameter method was used to estimate numbers of nucleotide substitutions. MEGA version 4 was used for tree construction (Tamura et al., 2007).

3. Results and discussion

3.1. Nucleotide sequences of ABO blood group genes for three gibbon species

Genotypes and inferred allele types of each individual determined in this study are listed in Table 1. We found a total of ten, seven, and seven haplotypes for agile gibbon (AG-1–AG-10), white-handed gibbon (WH-1–WH-7), and siamang (SI-1–SI-7), respectively. Three agile gibbon haplotypes (AG-1, AG-9, and AG-10) and four white-handed gibbon haplotypes (WH-4–WH-7) were A type, while all others were B type. Haplotype WH-3 had a seven-base insertion in exon 7 that produces a frame-shift, and its protein product is probably nonfunctional O type, though the O phenotype has not been observed for white-handed gibbon (Blancher and Socha, 1997).

Table 1
Individuals, genotypes, and ABO blood types.

Individual	Genotype	Inferred allele type
Agile gibbon		
S-001	AG-1(2)/AG-2(6)	AB
S-002*	AG-3(5)/AG-4(6)	BB
S-003†	AG-5(6)/AG-6(6)	BB
S-004†	AG-7(6)/AG-8(6)	BB
S-005†	AG-9(1)/AG-10(1)	AA
White-handed gibbon		
S-006*	WH-1(5)/WH-2(5)	BB
S-007**	WH-1(5)/WH-1(5)	BB
S-008**	WH-1(5)/WH-1(5)	BB
S-009*	WH-1(5)/WH-3(5)	BO
S-010**	WH-4(4)/WH-4(4)	AA
S-297*	WH-7(3)/WH-6(3)	AA
S-298*	WH-6(3)/WH-1(5)	AB
S-299**	WH-1(5)/WH-1(5)	BB
S-300*	WH-7(3)/WH-1(5)	AB
S-301*	WH-7(3)/WH-2(5)	AB
S-302*	WH-7(3)/WH-1(5)	AB
S-303*	WH-7(3)/WH-5(4)	AA
Siamang		
S-128*	SI-1(7)/SI-2(7)	BB
S-129*	SI-2(7)/SI-3(7)	BB
S-130*	SI-1(7)/SI-4(7)	BB
S-131†	SI-5(7)/SI-6(7)	BB
S-132*	SI-2(7)/SI-7(7)	BB
S-133**	SI-2(7)/SI-2(7)	BB

Numbers in parentheses for each haplotype designate the cluster ID shown in Fig. 2. Individuals with double asterisks (**) are homozygotes, while those with single asterisk (*) are heterozygotes with both haplotypes confirmed to be authentic through coexistence of haplotypes (see text). Individuals with dagger (†) consist of haplotypes from the same cluster.

A multiple alignment of variant sites of these sequence data is shown in Fig. 1. We constructed a phylogenetic network of these gibbon haplotypes and a human A type allele (DDBJ/EMBL/GenBank accession No. AJ536122) as an outgroup, and classified a total of 24 haplotypes into seven clusters as shown in Fig. 2. Clusters 1, 2, and 6 consist of only agile gibbon haplotypes, and clusters 3 and 4 consist of only white-handed gibbons. Cluster 5 is mainly formed with haplotypes observed in white-handed gibbon, but one haplotype (AG-3) was found in agile gibbon. Haplotype AG-3 was identical to WH-1, suggesting hybridization between agile and white-handed gibbons. In fact, hybrids between these two gibbon species have been reported in the wild (Brockelman and Gittins, 1984). Cluster 7 consists of only siamang haplotypes, and it is rather distant from the remaining 6 clusters. We also constructed a distance-based phylogenetic network, and the network showed a similar pattern (Supplementary Fig. 1).

Because the human A sequence is outgroup to all gibbon sequences, we expect it to be connected to the branch between the two clusters (7 for siamangs and 1–6 for common gibbons), according to the established phylogenetic relationship of gibbon species (Sibley and Ahlquist, 1984). However, the human A sequence is connected to a node inside the common gibbon group (see Fig. 2). This implies some unusual history on the ABO blood group gene in gibbons, as we will see later.

There are some large reticulations in this network, such as shown with orange and blue lines. These reticulations suggest recombination events. However, there is a possibility of artificial recombination during PCR. We therefore examined each haplotype. First of all, there are four and one homozygous individuals in white-handed gibbon and siamang, respectively (those with double asterisks in Table 1). Therefore, haplotypes WH-1, WH-4, and SI-2 are considered to be authentic sequences under no possibility of artificial recombination. These three haplotypes were also found in heterozygous individuals S-002 (note that haplotype AG-3 is identical with WH-1), S-006, S-009, S-298, S-300, S-302, S-128, S-129, and S-132. We thus confirm authenticity of another haplotypes found in these individuals: AG-4 in S-002, WH-2 in S-006, WH-3 in S-009, WH-6 in S-298, WH-7 in S-300 and S-302, SI-1 in S-128, and SI-7 in S-132 (see Table 1). In this fashion, two haplotypes (AG-3 and AG-4) of agile gibbons, all seven haplotypes (WH-1–WH-7) of white-handed gibbons, and five haplotypes (SI-1–SI-4 and SI-7) of siamangs were confirmed to be authentic haplotypes. The remaining ten haplotypes were found in four agile gibbon individuals (S-001, S-003, S-004, and S-005) and one siamang individual (S-131). Since we will consider recombinations only between seven clusters shown in Fig. 2 in later sections, individuals with two haplotypes from the same cluster (those with † in Table 1) are not scrutinized more. This leaves us only one agile gibbon individual, S-001, for this individual is heterozygous for haplotypes AG-1 and AG-2 belonging to clusters 2 and 6, respectively (see Fig. 2).

We therefore carried out an independent PCR for the individual S-001 using the same PCR primers and direct-sequencing using haplotype specific primers. Haplotypes AG-1 and AG-2 were confirmed in this individual (data not shown). Thus, we conclude that artificial recombinant sequences were not included in this study. If recombinations are detected from these sequences, these are natural ones.

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank International Nucleotide Sequence Database under accession numbers AB196674–AB196697.

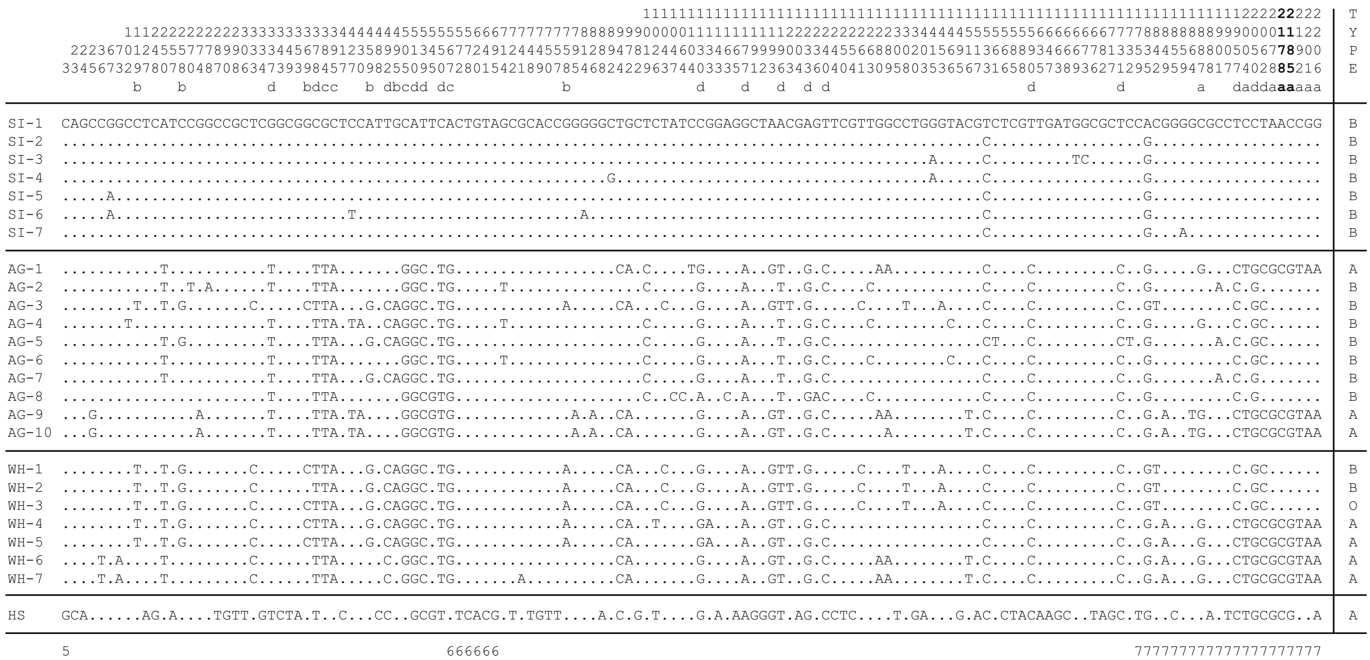

Fig. 1. Variant positions excluding gaps of ABO blood group genes from exon 5 to exon 7 for three gibbon species. Dots denote identical nucleotides with the first sequence. Haplotype names are AG (agile gibbon), WH (white-handed gibbon), SI (siamang), and HS (human, AJ536122). "a", different sites between A and B alleles among gibbons (two sites in bold [2178 and 2185] are responsible for two amino acid changes that distinguish the A and B glycosyltransferases), "b", sites mostly shared by WH-1–WH-5, and AG-3, "c" sites mostly shared by human and siamangs, and "d" sites shared mostly only by siamangs. Numbers below the human sequence indicated each exon number.

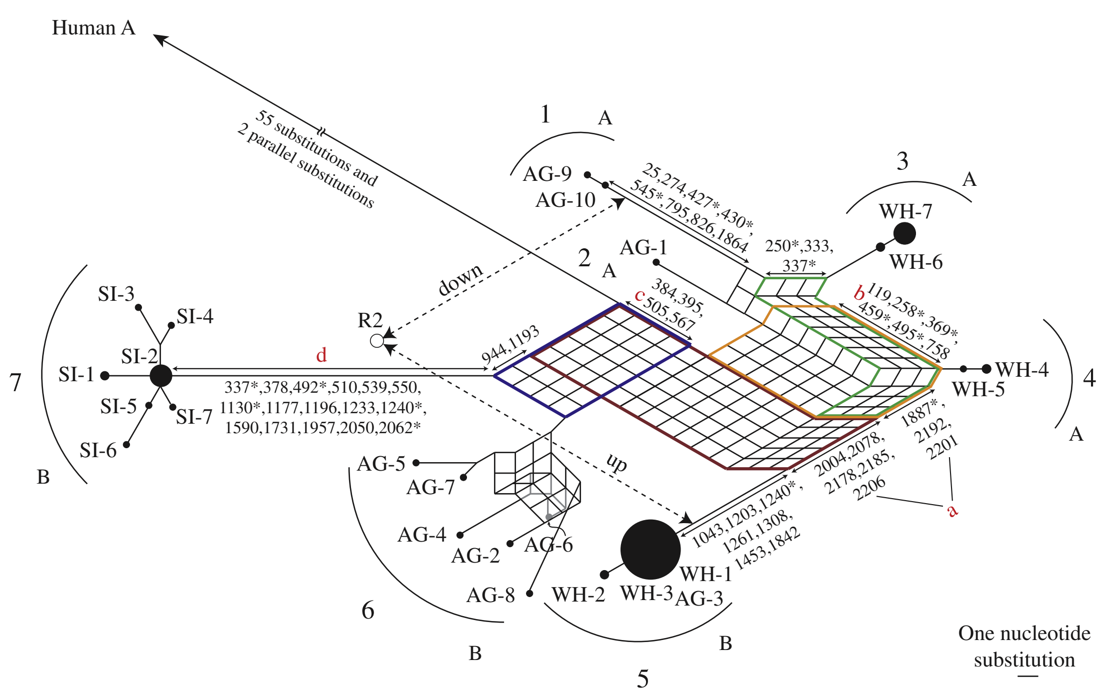

Fig. 2. A phylogenetic network of the ABO blood group genes for three gibbon species and one human allele (DDBJ/EMBL/GenBank Accession No. AJ536122). Numbers on branches are nucleotide positions responsible for those branches. Numbers with asterisks signify nucleotide positions in which parallel substitutions are inferred. A full circle shows one haplotype, and its size indicates the number of haplotype copies. R2 is the missing recombinant. The clusters of gibbons are numbered from 1 to 7. See legend of Fig. 1 for meanings of sites a-d. Each color corresponds to a recombination (see text). Note that a reticulation to show a recombination producing the cluster 2 could not be observed in the phylogenetic network.

3.2. Polymorphisms in gibbon ABO blood group genes

Nucleotide diversities of agile gibbons, white-handed gibbons, and siamangs were 0.0090, 0.0062, and 0.0010, respectively. It should be noted that the nucleotide diversities for the two common gibbon species, having both A and B type alleles, were extremely high compared to that for the corresponding region of chimpanzee ABO blood group gene (Kitano et al., 2000; Sumiyama et al., 2000) and to the overall genomic diversity for the human population (Sachidanandam et al., 2001). This high diversity of

the ABO blood group gene in gibbons suggests the existence of positive selection on this gene.

The average nucleotide difference (d) between siamangs and two common gibbon species was estimated to be 0.0156. The value was estimated excluding two sites (2178 and 2185) which are responsible for two amino acid changes that distinguish the A and B glycosyltransferases, because there is a possibility that some kind of selection operated on the sites. When we estimated d using synonymous and intron data, similar values (0.0276 for synonymous sites, 0.0169 for intron, and 0.0178 for synonymous sites and intron combined) were obtained. If we assume that the divergence time (T) between siamang and common gibbons is 8 MYA according to Sibley and Ahlquist (1984), the evolutionary rate (d/2T) for this gene region becomes 9.8×10^{-10}/site/year. This happens to be quite similar to the one (1.03×10^{-9}/site/year) for human and chimpanzee where the substitutional difference is 0.0123 (Fujiyama et al., 2002) and divergence time was assumed to be *ca.* 6 MYA.

3.3. Explanation of recombination and phylogenetic network by using model data

Let us explain how to infer recombination events from the phylogenetic network using one example shown in Fig. 3. An ancestral sequence (Anc) produced two descendant sequences (P1 and P2) by accumulating five and four substitutions in each lineage, respectively (Fig. 3A and B). A recombination between variant sites 6 and 7 then produced two recombinants, R1 and R2 (Fig. 3C). The phylogenetic network for these five sequences is shown in Fig. 3D. It should be noted that the horizontal edge of the rectangle in Fig. 3D corresponds to upstream sequence difference (sites 1–5), while the vertical edge to downstream differences (sites 7, 8, 9, and 12) accumulated from the ancestral sequence to two parental ones. Because five substitutions at sites 2, 3, 4, 8, and 12 occurred in the lineage from the ancestor to parental allele P1 and four substitutions occurred at sites 1, 5, 7, and 9 in the lineage from the ancestor to parental allele P2, the location of the ancestral sequence is in the middle of the rectangle (see Fig. 3D). After the recombination, three nucleotide substitutions (sites 6, 10, and 11) accumulated to produce present-day sequences P1′, P2′, and R1′ from P1, P2, and R1, respectively (Fig. 3E), while the R2 lineage became extinct. The phylogenetic network shown in Fig. 3F represents the relationship of the three present-day sequences, an outgroup sequence, and the ancestral one. Two parental lineages (P1′ and P2′) are located on a diagonal with long external branches, while R1′ has a short external branch and is located on another diagonal with the outgroup sequence. This is the pattern we should expect if a recombination happened in some ancient time for any autosomal genes.

3.4. Recombinations in gibbon ABO blood group genes

We are now ready to interpret the phylogenetic network of Fig. 2 following the scheme shown in Fig. 3F. There are seven clusters and one outgroup (human) in the network of Fig. 2. Because the recombinant lineage R1′ is located opposite to the outgroup sequence in Fig. 3F, cluster 4 (WH-4 and WH-5) may correspond to recombinant lineage in Fig. 2. If so, the two parental lineages should be clusters 1 (AG-9 and AG-10) and 5 (WH-1, WH-2, WH-3, and AG-3), because of their long branch lengths, resembling P1′ and P2′ lineages of Fig. 3F. Under this interpretation, the location of the missing (or probably extinct) recombinant lineage R2 is shown in Fig. 2. If so, two edges of the parallelogram formed with clusters 1, 4, 5, and R2 should correspond to upstream and downstream of the crossing over point which created recombinants. In fact, eight downstream sites designated as "a" in Fig. 2 indicate the allelic difference between A and B alleles of gibbons, while six upstream sites "b" correspond to the split separating clusters 4 and 5 from the others (see Figs. 1 and 2). Interestingly, not only "a" sites but also all sites on the branch connecting to cluster 5 were in downstream, and not only "b" sites but almost all sites on the branch connecting to cluster 1 were in upstream. This strongly indicates occurrence of recombination between "a" and "b" sites. If there was no recombination, the distribution of substi-

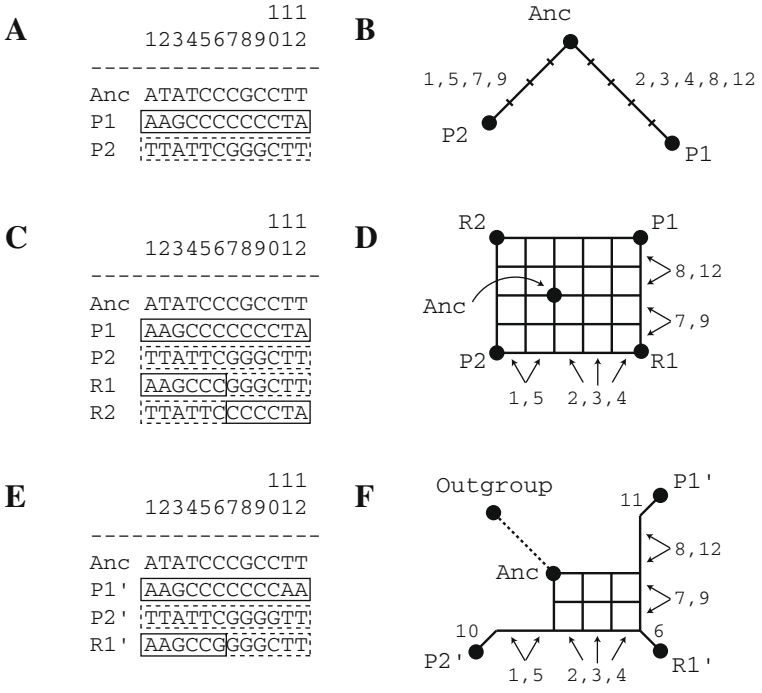

Fig. 3. Explanation of a recombination in a phylogenetic network by using model data (A–F). A, C, and E are nucleotide sequences, and B, D, and F are corresponding phylogenetic networks, respectively. See text for the details.

tutions on the branch connecting to cluster 1 (seven and one for upstream and downstream regions, respectively) and on the branch connecting to cluster 5 (zero and seven upstream and downstream regions, respectively) should be expected to have the same proportion. However, the difference between these two distributions is highly significant ($P < 0.001$, Fisher's exact test). We also constructed phylogenetic trees for the upstream and the downstream regions (Supplementary Fig. 2), and they clearly

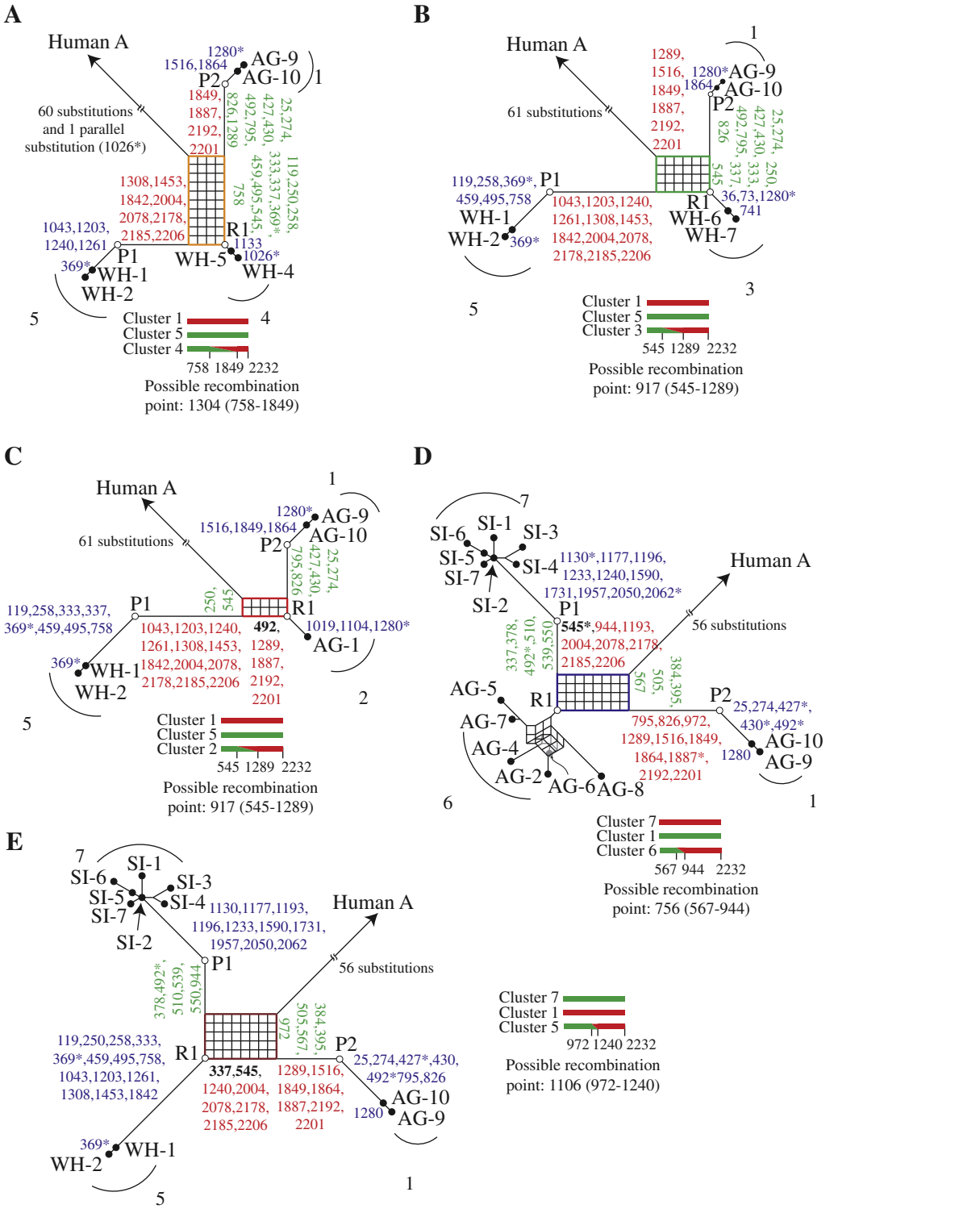

Fig. 4. A phylogenetic network of the clusters 1, 4, 5 for gibbon ABO blood group genes with the human A allele (A). P1 and P2 show parental nodes of the recombination, and R1 shows a recombinant of P1 and P2. Green and red colored numbers indicate nucleotide changes in the upstream and downstream regions, respectively. Blue colored numbers indicate nucleotide changes after the recombination. The diagram in the bottom shows the estimated location of the recombination. Phylogenetic networks of the clusters 1, 3, 5 of gibbon ABO blood group genes with the human A allele (B), of the clusters 1, 2, 5 of gibbon ABO blood group genes with the human A allele (C), of the clusters 1, 6, 7 of gibbon ABO blood group genes with the human A allele (D), and of the clusters 1, 5, 7 of gibbon ABO blood group genes with the human A allele (E). Each diagram shows the estimated location of the recombination.

Table 2
Recombinant, parental allele, and range of recombination.

Recombinant	Parental allele	Range of recombination (midpoint)
Cluster 4	Clusters 1 and 5	758–1849 (1304 ± 546)
Cluster 3	Clusters 1 and 5	545–1289 (917 ± 372)
Cluster 2	Clusters 1 and 5	545–1289 (917 ± 372)
Cluster 6	Clusters 1 and 7	567–944 (756 ± 189)
Cluster 5	Clusters 1 and 7	972–1240 (1106 ± 134)

Note: Locations of exon 6, intron 6, and exon 7 are 563–697, 698–1749, and 1750–2232, respectively.

showed different branching patterns, confirming the recombination event.

We thus chose clusters 1, 4, and 5 of gibbon ABO blood group genes and human A allele as an outgroup and constructed their phylogenetic network (see Fig. 4A). Nucleotide differences in the upstream region (sites 119–758) divided clusters 4 and 5 from cluster 1 and human A, while nucleotide differences in the downstream region (sites 1849–2201) divided clusters 1 and 4 from cluster 5 and human A. The midpoint (site 1304) was assumed to be the recombination point. One and two substitutions between R1 and WH-5 and between R1 and WH-4, respectively, accumulated after the recombination (Fig. 4A). These substitutions were on intron 6, and the recombination time was estimated to be 0.7 MYA [=(((1 + 2)/2)/2232)/9.8×10^{-10}] from the branch R1-cluster 4. Four and five substitutions in the upstream region (1304 bp) between P1 and WH-1 and between P1 and WH-2, respectively, accumulated after the recombination. These substitutions were on introns 5 and 6, and the recombination time was estimated to be 3.5 MYA [=(((4 + 5)/2)/1304)/9.8×10^{-10}] from the branch P1-cluster 5. Three and two substitutions in the downstream region (928 bp) between P2 and AG-9 and between P2 and AG-10, respectively, accumulated after the recombination. A substitution on site 1864 was a nonsynonymous substitution in exon 7. We excluded this substitution to eliminate the effect of selection. The recombination time was estimated to be 1.7 MYA [=(((2 + 1)/2)/928)/9.8×10^{-10}] from the branch P2-cluster 1. By averaging those three estimates, the time of the recombination that produced the cluster 4 became 2.0 [=(0.7 + 3.5 + 1.7)/3] MYA.

Since cluster 4 was judged as the recombinant lineage, we eliminated the cluster 4 from the phylogenetic network of Fig. 2 and reconstructed the new phylogenetic network (see Supplementary Fig. 3A). When we constructed a phylogenetic network involving only the clusters 1, 3, and 5 of gibbon ABO blood group genes with human A allele as an outgroup (Fig. 4B), the midpoint (site 917) was assumed the recombination point. Three and four substitutions (on introns 5 and 6) between R1 and WH-6 and between R1 and WH-7, respectively, accumulated after the recombination (Fig. 4B), and the recombination time was estimated to be 1.6 MYA [=(((3 + 4)/2)/2232)/9.8×10^{-10}] from the branch R1-cluster 3. Six and seven substitutions (on introns 5 and 6) in the upstream region (917 bp) between P1 and WH-2 and between P1 and WH-1, respectively, accumulated after the recombination, and the recombination time was estimated to be 7.3 MYA [=(((6 + 7)/2)/917)/9.8×10^{-10}] from the branch P1-cluster 5. Two and one substitutions in the downstream region (1315 bp) between P2 and AG-9 and between P2 and AG-10, respectively, accumulated after the recombination. A substitution on site 1864 was a nonsynonymous substitution, and was excluded. Therefore, the recombination time was estimated to be 0.4 MYA [=(((0 + 1)/2)/1315)/9.8×10^{-10}] from the branch P2-cluster 1. By averaging those three estimates, the time of the recombination that produced the cluster 3 became *ca.* 3.1 [(1.6 + 7.3 + 0.4)/3] MYA.

Since cluster 3 was also judged as the recombinant lineage, we further eliminated cluster 3 and reconstructed the new phylogenetic network (Supplementary Fig. 3B). In the same way, three additional recombination events were observed (Figs. 4C–E), and the time of recombination was calculated for each event. All recombinations were estimated to occur on the first half of intron 6 (Table 2).

We present a complex evolutionary scenario based on the above results as shown in Fig. 5. There are five recombination events during the evolution of gibbon ABO blood group genes, spanning

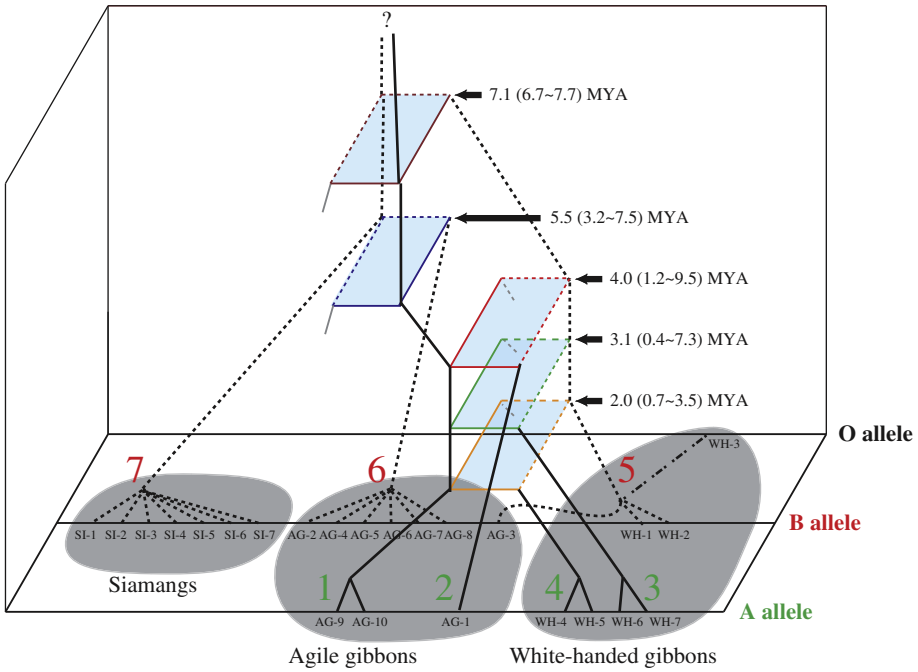

Fig. 5. An evolutionary scenario of the ABO blood group genes for three lesser ape species. Solid, dotted, and dashed and double-dotted lines indicate A, B, and O type allelic lineages, respectively. Numbers 1–7 in the current plane designate seven clusters of Fig. 2. Five recombination events, scattering 2–7 MYA, are shown by parallelograms. Each color correspond each recombination of Fig. 2 and Fig. 4. Grey truncated lines indicate undetected or extinct lineages.

ca. 2–7 MYA. These times were estimated excluding nonsynonymous substitutions. When all substitutions were used to estimate times, however, similar values were obtained (data not shown), because about 70% of the analyzed region in this study was introns. Although recombinations in human populations have been reported (e.g., Roubinet et al., 2004), this is probably the first report on very ancient recombinations in primates. Two most ancient ones precede speciation of agile gibbons and white-handed gibbons, while they were after speciation (ca. 8 MYA [Sibley and Ahlquist, 1984]) of siamang and common gibbon lineages. Because estimated times have ranges and hybridizations among gibbons are not rare, it is not easy to say that these recombinations occurred before or after the speciations, but at least we can conclude that these recombinations occurred in very ancient times.

If we accept the evolutionary scenario shown in Fig. 5, the divergence or coalescence time between A and B alleles precede the divergence (*ca.* 8 MYA) of siamang and common gibbon lineages. Let us estimate the coalescence time. It becomes 9.6 (range: 8.1–11.2) MYA ($=0.0188 \pm 0.0030/2[9.8 \times 10^{-10}]$), based on the average nucleotide difference (excluding sites 2178 and 2185) between the clusters 1 and 7, since the clusters are not recombinant alleles in this data (see Fig. 5). When we estimated the average nucleotide difference between the clusters 1 and 7 using intron data (0.0182 ± 0.0033), similar value [9.3 (range: 7.6–11.0) MYA] was obtained. When we estimated the average nucleotide difference using synonymous data (0.0469 ± 0.0177), a much longer time 24.1 MYA (range: 15.0–33.1) was obtained. This suggests that the coexistence of A and B type alleles in the common ancestral species of siamang and common gibbons, and that A type alleles might be lost from the siamang lineage.

All these observations indicate existence of positive selection for the gibbon ABO blood group gene. Saitou and Yamamoto (1997) suggested the existence of some kind of positive selection at the ABO blood group gene through sequence analysis of human, chimpanzee, gorilla, orangutan, and some Old World monkeys. This study on lesser apes further strengthens the positive selection as common feature of the primate ABO blood group genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.02.023.

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