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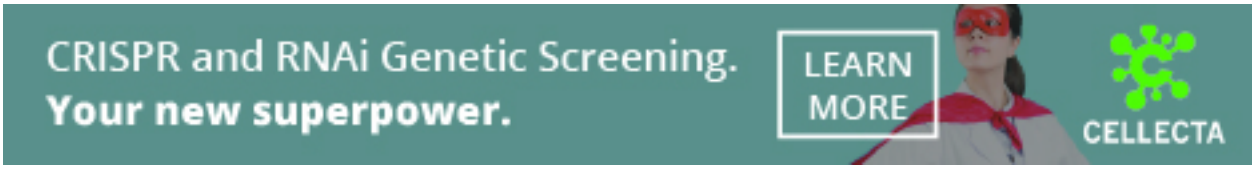
Genome Res. 2000 10: 634-643
Access the most recent version at doi:10.1101/gr.10.5.634



Article

Alu Elements Support Independent Origin of Prosimian, Platyrrhine, and Catarrhine *Mhc–DRB* Genes

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The primate major histocompatibility complex (*Mhc*) genes fall into two classes and each of the classes into several families. Of the class II families, the *DRB* family has a long and complex evolutionary history marked by gene turnover, rearrangement, and molecular convergence. Because the history is not easily decipherable from sequences alone, *Alu* element insertions were used as cladistic markers to support the surmised phylogenetic relationships among the *DRB* genes. Intron 1 segments of 24 *DRB* genes from five platyrrhine species and five *DRB* genes from three prosimian species were amplified by PCR and cloned, and the amplification products were sequenced or PCR–typed for *Alu* repeats. Three *Alu* elements were identified in the platyrrhine and four in the prosimian *DRB* genes. One of the platyrrhine elements (*Alu50J*) is also found in the Catarrhini, whereas the other two (*Alu62Sc*, *Alu63Sc*) are restricted to the New World monkeys. Similarly, the four prosimian elements are found only in this taxon. This distribution of *Alu* elements is consistent with the phylogeny of the *DRB* genes as determined from their intron 1 sequences in an earlier and the present study. It contradicts the exon 2-based phylogeny and thus corroborates the conclusion that the evolution of *DRB* exon 2 sequences is, to some extent, shaped by molecular convergence. Taken together, the data indicate that each of the assemblages of *DRB* genes in prosimians, platyrrhines, and catarrhines is derived from a separate ancestral gene.

[The sequence data described in this paper have been submitted to the GenBank data library under accession nos. AF197226–AF197240.]

The major histocompatibility complex (*Mhc*) is a multicomponent assemblage comprised of genes of different age (Parham 1999). All jawed vertebrates possess two classes of *Mhc* loci and in each class there are several families of genes whose divergence times differ depending on the taxonomical position of the animal (Klein 1986; Klein and Figueroa 1986; Kasahara et al. 1995). In primates, a few of the class I loci diverged prior to the emergence of this order, but most are of much more recent origin (Hughes and Nei 1989a). The primate class II gene families *DO*, *DP*, *DQ*, and *DR*, on the other hand, diverged before the radiation of the eutherian mammals (Carson and Trowsdale 1986). Within the families, the loci can vary considerably in their ages (Satta et al. 1996a,b). Loci of the *DRB* subfamily in particular appear to have undergone frequent expansions and contractions (Klein et al. 1993) that considerably obscured their evolutionary history. The deciphering of their history is further complicated by the fact that parts of the genes are subject to convergent evolution (Andersson et al. 1991; Kriener et al. 2000a,b). Interpreting the evolution of these genes is therefore a daunting task, which can succeed only if based on a combination of different approaches and utilization of a variety of marker systems.

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In earlier publications (Kriener et al. 2000a,b), we provided evidence that exon 2 sequences, on which previous phylogenies of primate *DRB* genes were based (Trtková et al. 1993; Figueroa et al. 1994; Gyllensten et al. 1994), are providing misleading phylogenetic signals. The evolution of the exon is strongly affected by positive selection (Hughes and Nei 1989b), which creates repeatedly and independently similar sequence motifs (O'hUigin 1995; Kriener et al. 2000a,b). These motifs make genes appear more closely related than they are in reality. This, at least, is the message extracted from comparisons of the *DRB* intron with the *DRB* exon 2 sequences. Specifically, whereas the exon 2 sequences suggest that most primate *DRB* genes derive from a common ancestor that existed prior to the divergence of prosimians, Platyrrhini, and Catarrhini, intron sequences support the origin of *DRB* genes in each of the three taxa from a distinct ancestor (Kupfermann et al. 1999; Kriener et al. 2000a,b). Analysis of the exon 2 similarities implies molecular convergence as an explanation and thus indicates that the introns and not exon 2 are reflecting the true *DRB* gene phylogeny. However, as both the exon 2 and intron data are sequence–based, an independent source of information corroborating these conclusions was needed. We sought this source in the *Alu* elements inserted into the *DRB* genes.

10:634–643 ©2000 by Cold Spring Harbor Laboratory Press ISSN 1088-9051/00 $5.00; www.genome.org

The use of short interspersed repetitive elements (SINEs) in phylogenetic analysis is widespread. They have been used successfully to resolve phylogenies of a variety of mammals and other vertebrates (Batzer et al. 1994; Shimamura et al. 1997; Stoneking et al. 1997; Hamdi et al. 1999; Nikaido et al. 1999). They offer the advantages of ubiquity, uniqueness, and stability. Insertion occurs often enough to provide an array of useful cladistic markers. The chance of independent insertions at identical positions is small. Finally, SINEs are rarely removed without leaving evidence of their previous presence.

Alu repeats constitute one of several families of SINEs found in the mammalian genome (Deininger and Batzer 1993; Jurka 1995). They are believed to be derived from the 7SL RNA, which is a part of an 11S cytoplasmic ribonucleoprotein involved in targeting secretory proteins across the membranes of the endoplasmic reticulum (Ullu and Tschudi 1984). The derivation occurred in multiple steps in which two variants were first produced by deletions in different parts of the 7SL RNA gene — the free left *Alu* monomer or FLAM and the free right *Alu* monomer or FRAM — and these monomers then fused to form the dimeric *Alu* elements (Quentin 1992a,b). All three forms are still found in the primate genome. The dimeric family of *Alu* elements is divided into three major subfamilies that are of different age, and which, in the standardized nomenclature of Batzer et al. (1996), are designated J (~80-million-years [my] old), S (~35–44-my old), and Y (< 5-my old). Each subfamily is further divided into sub-subfamilies (e.g., the S subfamily is differentiated into Sx, Sy, Sp, and Sc branches). The subfamilies and the sub-subfamilies are distinguished by diagnostic substitutions shared by all members of a given group. The *Alu* elements are retroposons owing their mobility to the possession of sequences enabling their transcription by RNA polymerase III.

As *Alu* elements are ubiquitous in the primate genome, they can be identified relatively easily in any genomic region of interest. In earlier studies, we identified a series of > 60 *Alu* elements in the catarrhine *Mhc–DRB* genes (Schönbach and Klein 1991; Mňuková et al. 1994; Satta et al. 1996a) and designated them *Alu1–Alu61*. The aim of the present study was to identify platyrrhine and prosimian DRB gene-associated *Alu* elements and use them to resolve the incongruences between the exon 2- and intron-based phylogenies.

RESULTS

In our search for *Alu* markers suited to the stated purpose, we focused on intron 1 because of its proximity to exon 2, which is the most variable of all the *DRB* exons, because of its length of several kilobase pairs (kb), which increases the likelihood of repeats' presence, and because several *Alu* elements were identified in it in catarrhine *DRB* genes (Andersson et al. 1987; Mňuková et al. 1994; Satta et al. 1996a), including one old element (*Alu50J*). To identify *Alu* elements in platyrrhine *DRB* genes, we selected seven genomic New World Monkeys (NWM) DNA samples bearing previously identified *DRB* exon 2 sequences (Trtková et al. 1993). Using exon 1- and exon 2-based primers, we then attempted to amplify the entire intron 1 and most of exon 2 of the different *DRB* genes by PCR. We succeeded in amplifying segments from 24 different *DRB* genes and failed with 5, perhaps because of a too large intron length. We confirmed the identity of the 24 amplification products by cloning them and sequencing their ends, including exon 2. For 23 of the 24 clones, the exon 2 sequences have already been described (Trtková et al. 1993; Gyllensten et al. 1994; Antunes et al. 1998; Kriener et al. 2000a, b), whereas one sequence identified a new exon 2, which we designate *Sasc–DRB*W3401*. Ten of the 24 amplification products were chosen for restriction digest and hybridization analysis.

Samples of each of the 10 clones were divided into three parts and each part was digested with a different pair of restriction enzymes (*Bam*HI–*Hin*dIII, *Hin*dIII–*Hin*cII, and *Bam*HI–*Eco*RI). The digests were separated by gel electrophoresis, blotted, and the blots were hybridized with an *Alu*-specific probe (Fig. 1). The probe was obtained by PCR amplification of human genomic DNA using primers *Alu1* and *Alu2* (Table 1). It was ~ 250-bp long and was shown in control experiments to hybridize with members of the J, Sb, Sc, and Sq subfamilies of *Alu* elements. By use of this probe, one or two hybridizing fragments could be identified on the blots of the digested NWM clones. The positive fragments were then subcloned and sequenced. The sequences were aligned and the *Alu* elements in them identified (Fig. 2).

This approach revealed the presence of three distinct *Alu* elements in platyrrhine *DRB* intron 1 sequences. One of these three elements, *Alu50*, was identified previously in catarrhine *DRB* genes (Mňuková et al. 1994), the other two are new and so we designated them *Alu62* and *Alu63*. The identification of the first element as *Alu50* is based on sequence similarity and sharing of flanking direct repeats, as well as its position and orientation in intron 1 (Figs. 2 and 3). By some of the same criteria, *Alu62* and *Alu63* are distinct from all other *Alu* elements identified thus far, which means that they are absent in all of the analyzed catarrhine *DRB* genes. The *Alu50* element was found to be present in all 10 clones; *Alu62* and *Alu63* were present in some of them and absent in others (Fig. 2).

The presence or absence of the three *Alu* elements among the remaining 14 of the 24 clones (i.e., those not subjected to restriction enzyme analysis) was es-

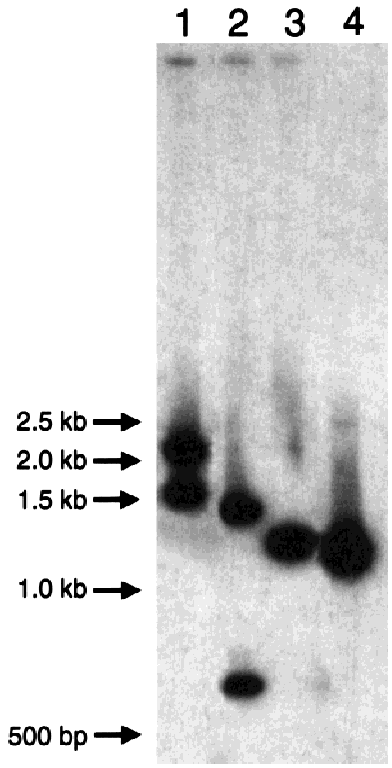


Figure 1 Southern blot analysis of a *Saoe-DRB11*0102* intron 1 clone. The clone was digested with different pairs of restriction enzymes. The digests were separated by gel electrophoresis, blotted, and the blot hybridized with an *Alu*-specific probe. (*Lanes* 1–3) Digests obtained after treatment with the *Bam*HI/*Hin*dIII, *Hin*dIII/*Hin*cII, and *Bam*HI/*Eco*RI enzymes, respectively.(*Lane* 4) Positive control. A 1.2-kb fragment containing *Alu50J* was amplified from human genomic DNA and blotted.

tablished by PCR typing. To this end, the DNA isolated from the individual clones was PCR amplified by using different combinations of primers specific for each of the three *Alu* elements (Table 1; Fig. 4). The specificity of the primers was based on the uniqueness of the sequences flanking the individual *Alu* elements. The typing identified *Alu50* in all 14 clones; *Alu62* in *Saoe-DRB11*0105, Caja-DRB1*0307, Caja-DRB*W1602, Caja-DRB*W1603, Caja-DRB*W1605, Caja-DRB*W1612, Sasc-DRB*W1401, Sasc-DRB*W1901, Sasc-DRB*W3401, Ceap-DRB*W1301*, and *Ceap-DRB*W1502*; and *Alu63* in *Saoe-DRB11*0105, Caja-DRB1*0307, Caja-DRB*W1602, Caja-DRB*W1603, Caja-DRB*W1605, Caja-DRB*W1612, Sasc-DRB*W1901*, and *Ceap-DRB*W3201*. [The *Alu50* element is present in all catarrhine *DRB* genes tested except *Maar-DRB1*0301*, in which the *Alu50* region of intron 1 has been deleted (K. Kriener unpubl.). The *Alu62* and *Alu63* elements, as already mentioned, are absent in all identified catarrhine *DRB* genes.]

The *Alu50*, *Alu62*, and *Alu63* elements are located in the same region of intron 1, arranged in this order in the 5′ to 3′ direction, ~1.2 kb downstream from the 5′ end of intron 1 (Fig. 3). Where all three elements are present on the same clone, *Alu62* is immediately adjacent to *Alu50* in a head-to-head orientation and *Alu63* is ~270 bp downstream of *Alu62* in the same orientation as *Alu62*. In the same region, different *Alu* elements are found in both catarrhini (*Alu29*; Satta et al. 1996a) and prosimians (see below). The region therefore appears to be highly prone to *Alu* insertions. Sequence comparisons (Fig. 2) and analysis of diagnostic sites (Jurka and Milosavljevic 1991), as well as phylogenetic analysis of the sequences (Fig. 5), assign both *Alu62* and *Alu63* to the *Sc* subfamily.

To investigate *Alu* elements of prosimian *DRB* genes, we used the exon 1- and exon 2-based primers on genomic DNA isolated from three prosimian species to amplify intron 1 of these genes by PCR. The amplification products, which ranged in length from 3.5 to 10 kb, depending on the species and the gene, were cloned, the clones digested with restriction enzymes, the fragments separated by electrophoresis and blotted, and the blots hybridized with an *Alu*–specific probe. The enzymes and the probe used were the same as those used in the study of the platyrrhine *DRB* genes. The weakly hybridizing fragments of five clones (three from *Galago moholi* and one each from *Tarsius syrichta* and *T. bancanus*) were subcloned and sequenced. The sequences revealed the presence of four different *Alu* elements. Three of them, found in the *Galago,* could be identified by sequence comparisons as dimeric type II repeats characteristic for galago genomes (Daniels and Deininger 1985, 1991). Because the three elements are distinct from each other and from all previously identified, *DRB*-associated *Alu* repeats, we designate them *Alu64*, *Alu65*, and *Alu66*. *Alu64* is located in a region corresponding to that occupied in the platyrrhini by *Alu50*, *Alu62*, and *Alu63*; *Alu65* is found ~700 bp downstream of *Alu64*; *Alu66* is located at the 3′ end of intron 1. The *Alu64* and *Alu65* elements are present in the *Gamo-DRB*W301* gene; the *Alu66* element in the *Gamo-DRB*W401* and **W501* genes. The fourth element, designated *Alu67*, was found in the two tarsier species; it resembled the recently described tarsier-specific type of elements (Zietkiewicz et al. 1999). It occurred at the 3′ end of intron 1 in the *Tasy-DRB*W101* and *Taba-DRB*W201* genes. Neither *Alu50*, which is present in virtually all platyrrhine and catarrhine *DRB* genes, nor any of the other *Alu* elements identified previously in *DRB* genes could be found in any of the prosimian genes. The presence in the prosimian genes of a single copy of the sequence that flanks both ends of the *Alu50* element (data not shown) indicates that *Alu50* was apparently never present in these genes. The prosimian genes possess an entirely different set of *Alu* elements than the *DRB* genes in Platyrrhini and Catarrhini.

To determine the relationship of the *Alu*-element

Table 1. Oligonucleotide Primers Used for PCR Amplification

Designation	Sequence (5′ to 3′)	Specific for
Exon 1 primers		
DRB1L	CCTGTTCTCCAGCATGGTGTG	Generic
K3	GTGACACTGATGGTGCTGAGCTGG	Generic
R1	GCATCTCTGACCAGCAACTGATGA	Generic
Exon 2 primers		
K5	CTCTCCGCGGCACTAGGAACCTCTC	Generic
K8	TCCGGGTGTTGAAGTGCTCTGC	*Saoe-DRB1*03*
K9	CACCTGGCCCCGCTTCTGCTC	Generic
K10	AAGCGCAAGTTCTCCTCCTGATTA	*Caja-DRB1*03*
K13	TCTCCCAGTCCCGTAGTTGTGTC	Generic
K50	CCAGCGCGATTACGCAC	*Saoe-DRB3*05*
K60	CACCGCGGCCCGCTCGTC	*Camo-DRB*W17*
K61	GCTCTCACCAACCCCGTAGT	*Ceap-DRB*W13*
PefuL2	AACCCCGTAGTTGTGTCTGCA	Generic
P9	CGCTGTCTGCTCCCTCTTCA	*Gamo-DRB*W501*
Primers used for amplification of the hybridization probe		
Alu1	GGGCGGATCACGAGGTCAGG	Generic
Alu2	CAGTGGCGGGATCTCGGCTC	Generic
Primers flanking *Alu50J, Alu62Sc,* and *Alu63Sc*		
A50-I	ATTTCATAGGTTCCCCCATCA	Generic
A50-II	CATGTCTGCTTATCTCTTCCTC	*Saoe-DRB*
A50-III	GCGACATCTGCTTACCTCTCTC	*Camo-DRB*
A50-IV	CACACTCTCCCCCTTCTC	*Saoe-DRB*
A50-V	CCAACCTCTCTGTCTTAGTC	*Saoe-DRB*
A50-VI	GAATAGAGCCCAGTAAACAG	*Camo-DRB*
A50-VII	AGGGTTCTGGGTTCTGATTG	*Camo-DRB*
ASc-I	ATCAAAATGAAAGGGTGCTG	*Saoe-DRB*
ASc-II	TGGAGCTTCAGCCCCTTC	*Saoe-DRB*
ASc-III	CCCAGAACCCTGGTTTATTC	Generic
ASc-IV	CTGTTTACTGGGCTCTATTC	*Camo-DRB*
ASc-V	ACTGTGGTGTAACTTCCTTGAG	*Saoe-DRB*
ASc-VI	TGGTGTGTGACTTCCATGAGA	*Camo-DRB*

distribution to the phylogeny of the *DRB* genes, we superimposed the former on the latter in Figure 6. The tree in Figure 6 is based on ~600 bp of sequence at the 5′ end of intron 1 (Kriener et al. 2000a; Fig. 3), in addition to ~500 bp of sequence flanking the *Alu* elements. The sequences of the Alu region were submitted to GenBank (accession nos. AF197226–AF197240). The presence or absence of the *Alu* elements shows no correlation with either the species of origin of the DNA or the exon 2-based *DRB* gene classification as reflected in the gene designations. In contrast, the *Alu* element distribution correlates with the intron 1 phylogeny, which also correlates with the presence or absence of a previously described 870-bp deletion at the 3′ end of the platyrrhine intron 1 (Kriener et al. 2000a,b; Fig. 3). Because the deletion is absent in all tested catarrhini *DRB* genes, it apparently arose in an ancestral gene within the platyrrhine clade.

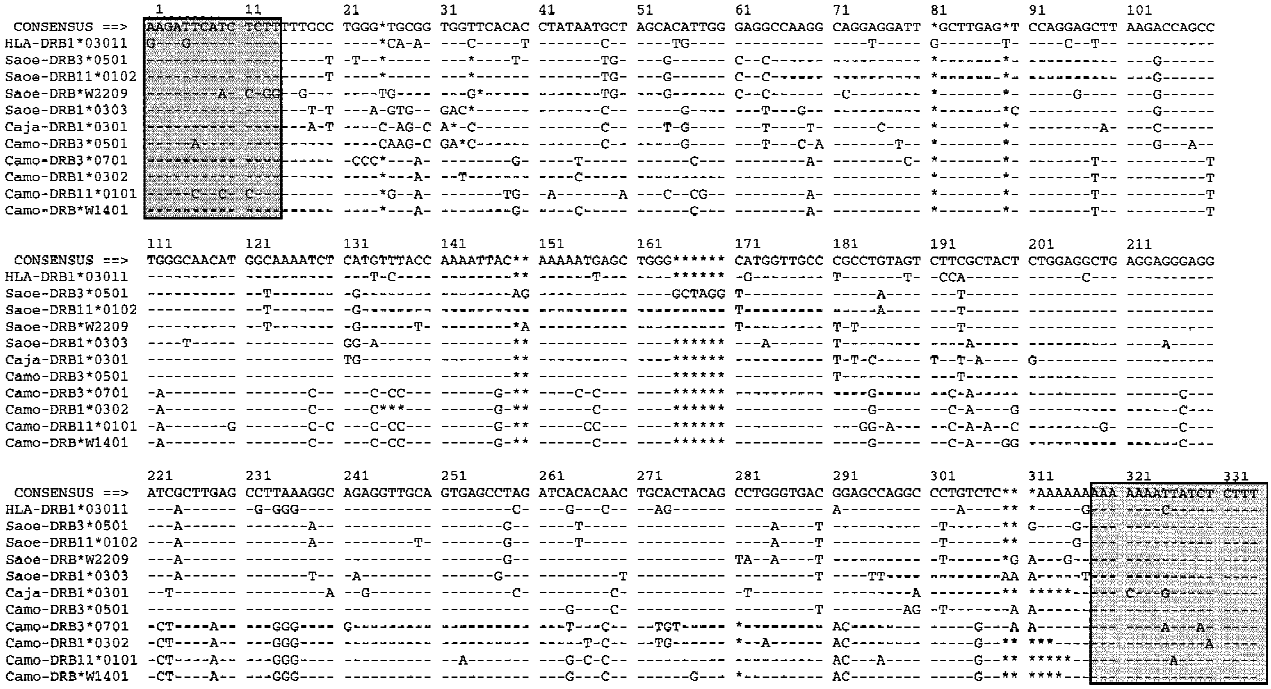
DISCUSSION

In the preceding text, we regarded the primate order as consisting of three monophyletic groups, the prosimians, the platyrrhines, and the catarrhines. Although the monophyly of the last two groups has never been seriously contested, that of the prosimians *is* contentious (Martin 1990), and we used the traditional designation merely as a convenient way of referring to non-anthropoid primates. Recent molecular evidence, in fact, strongly bolsters the splitting of prosimians into Strepsirrhini and Haplorrhini, the latter being a sister group of Anthropoidea (Goodman et al. 1998). The Strepsirrhini include lemurs and galagos, the Haplorrhini the tarsiers. The existence of separate sets of *Alu* elements in galagos and tarsiers (Daniels and Deininger 1985, 1991; Zietkiewicz et al. 1999; the present report) provides additional evidence for this split. The apparent polyphyly of the prosimians must be taken into account when interpreting the results of the present study.

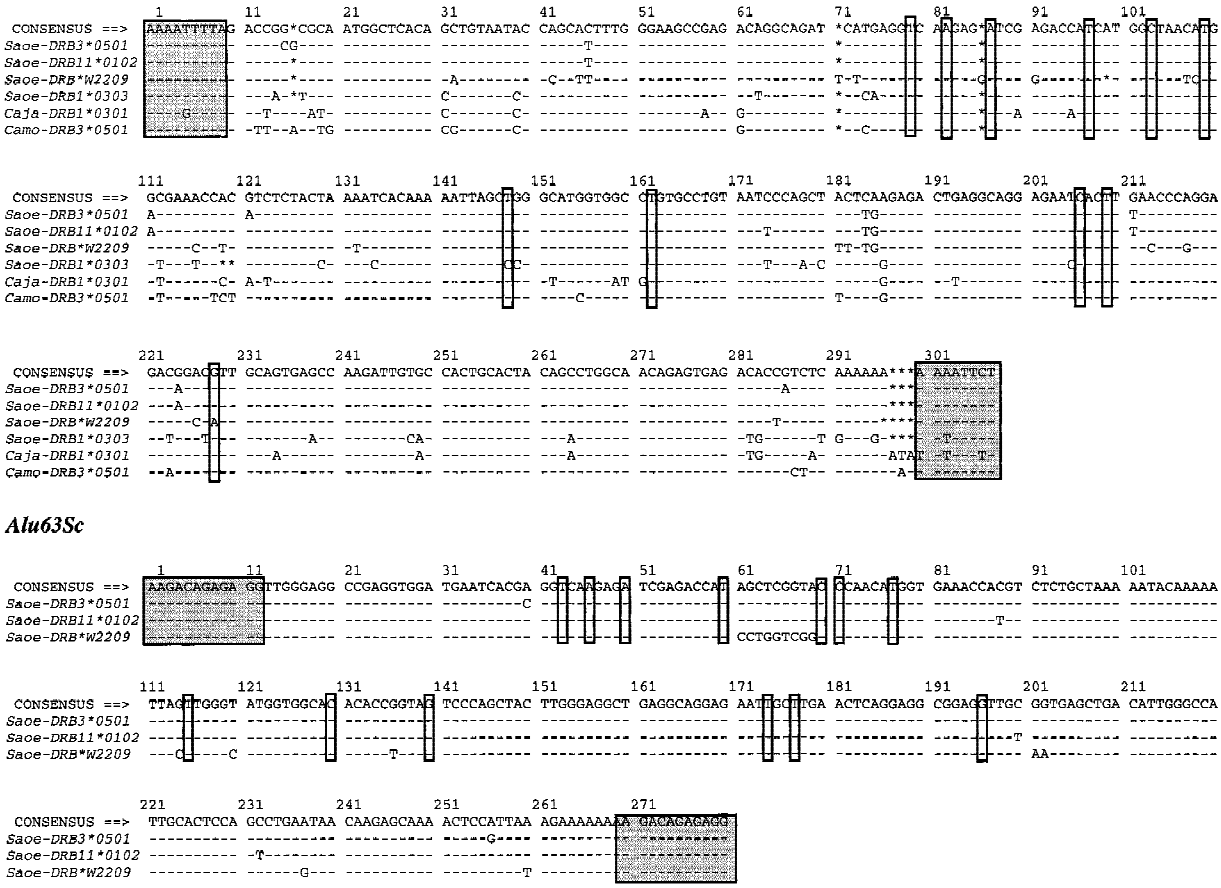
Considered in the context of previous work on primate *DRB* genes in our laboratory (Trtková et al. 1993, 1995; Figueroa et al. 1994; Kriener et al. 2000a,b; Kupfermann et al. 1999) and in other laboratories (Slierendregt et al. 1992; Gyllensten et al. 1994; Knapp et al 1997; Antunes et al. 1998), the results described here lead us to two conclusions. The first conclusion is that in each of the four main primate groups (Strepsirrhini,

Platyrrhini

Alu50J



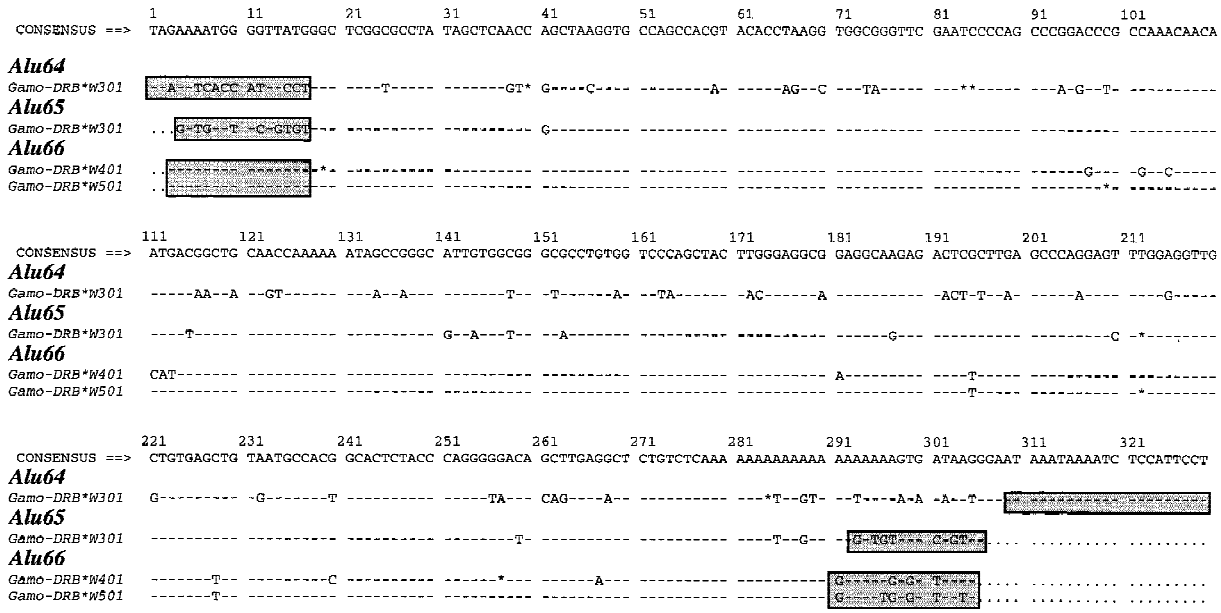
Alu62Sc



Alu63Sc

Figure 2 (*See facing page for legend.*)

Strepsirrhini



Haplorrhini

Alu67

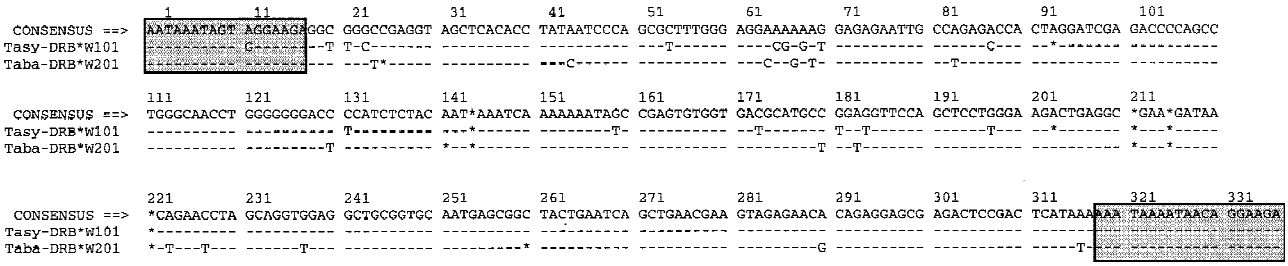


Figure 2 Nucleotide sequence alignments of the *Alu* elements identified in the *DRB* genes of platyrrhini, strepsirrhini, and haplorrhini. The shaded boxes represent the flanking direct repeats that are created during the insertion of the *Alu* element. In *Alu62* and *Alu63*, the diagnostic positions used for the subfamily classification are highlighted. A simple majority consensus sequence is given at the top. A dash (-) indicates identity with the consensus, an asterisk (*) an indel, and a dot (.) unavailability of sequence information. Numbering above the sequences starts with the first nucleotide of the alignment.

Haplorrhini, Platyrrhini, and Catarrhini), there are multiple *DRB* loci present. This claim is best supported by data available for the Catarrhini, in which multiple loci have actually been assigned to their respective positions on genetic maps (Klein et al. 1991). In humans, for example, nine *DRB* loci are known to exist, although they never occur all together on one chromosome (Klein et al. 1991). For the Platyrrhini, Haplorrhini, and Strepsirrhini the claim is largely based on the detection of more than two *DRB* sequences per individual (Trtková et al. 1993; Figueroa et al. 1994; Gyllensten et al. 1994; Antunes et al. 1998; the present study). The organization of the loci is not known in any of these groups. The observation that different numbers of sequences are amplified from various individuals can, however, be taken as a hint that the number of loci per haplotype varies in the same manner as it does in human *DRB* haplotypes.

The second conclusion is that in each of the four primate groups, the *DRB* genes derive from a separate ancestral gene. The four ancestors all in turn derive from a single gene that was the last common ancestor of all primate *DRB* genes. The existence of an ancestral primate gene separate from the ancestral genes of *DRB* loci in other orders of eutherian mammals is suggested first, by intron sequence-based phylogenies (Kriener et al. 2000a,b; Kupfermann et al. 1999; Fig. 6), and second, by the types and distribution of the *DRB*-associated *Alu* elements (Schönbach and Klein 1991; Mňuková et al. 1994; Satta et al. 1996a; the present

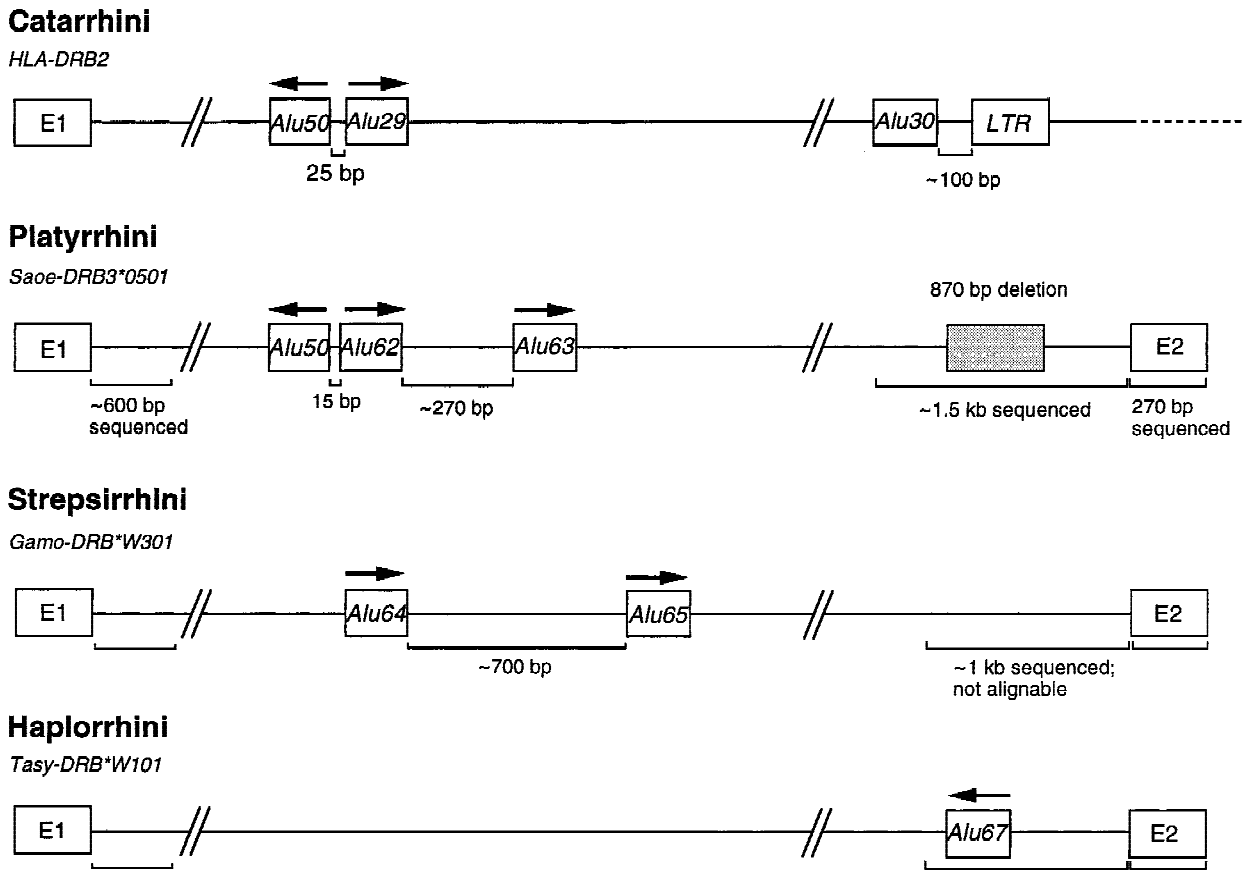


Figure 3 Diagram of intron 1 of selected catarrhine, platyrrhine, and prosimian *DRB* genes. The *Alu* elements identified in the intron are shown and their orientation is indicated by arrows. The distances between the *Alu* elements and the length of sequenced fragments in the 5′ and the 3′ ends of intron 1 are indicated by brackets. The shaded box represents a 870-bp deletion in the 3′ end of intron 1. The drawing is not to scale.

study). The intron sequence data are fully congruent with the *Alu* distribution results. Each of the four primate groups has a monophyletic set of *DRB* intron sequences and each has a distinct set of *Alu* elements. The only shared element is *Alu50* (a member of the oldest subfamily of elements), which is found in both the Platyrrhini and Catarrhini; all of the others are restricted in their distribution to only one of the four groups. The sharing of *Alu50* supports the grouping of Platyrrhini and Catarrhini into Anthropoidea. The group restriction of the other *Alu* elements supports the monophyly of each of these groups and of the *DRB* genes in the Strepsirrhini, Haplorrhini, Platyrrhini, and Catarrhini. The group restriction is also found in the shorter downstream introns surveyed by Kupfermann et al. (1999). This distribution pattern is consistent with the assumption that new elements became inserted into the *DRB* genes in each of the four primate groups as they diverged from each of their common ancestors.

The above interpretation is seemingly contradicted by the exon 2-based phylogenies of the *DRB* genes (Trtková et al. 1993; Gyllensten et al. 1994; Figueroa et al. 1994). These phylogenies lead to the conclusion that separate allelic lineages at *DRB* loci diverged before the divergence of primates into the four

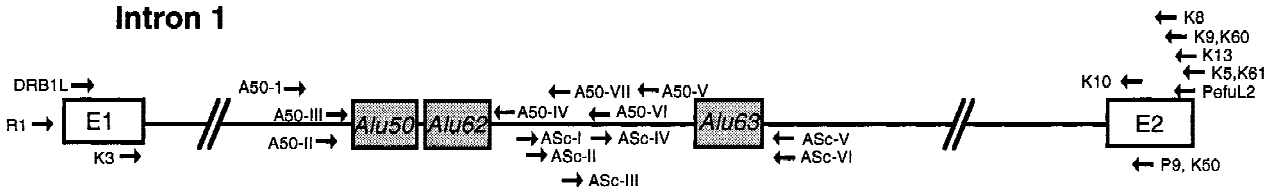


Figure 4 Location of the primers used for PCR amplifications. Primers (arrows) located in exon 1 and exon 2 were used to amplify the entire intron 1 of *DRB* genes. The cloned products were typed for the presence of *Alu50*, *Alu62*, and *Alu63* with combinations of the primers Alu50-I–Alu50-VII and AluSc-I–AluSc-VI. The sequences of the primers are given in Table 1. (E) Exon, the shaded boxes represent *Alu* elements.

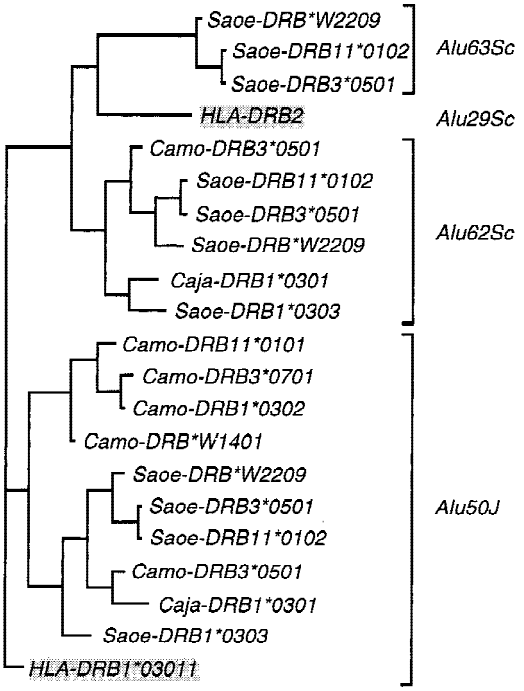


Figure 5 Maximum likelihood tree based on the sequences of human and NWM *Alu* elements (Fig. 2). The classification of *Alu62* and *Alu63* in the Sc subfamily is supported by their grouping with *Alu29* of the *HLA-DRB2* gene. Sequences of *Alu50* from the *HLA-DRB1*03011* gene and of *Alu29* from the *HLA-DRB2* gene are highlighted.

taxonomical groups and have persisted to the present day. Responsible for the phylogenies are sequence motifs shared between exon 2 segments of not only primates, but often also different orders of eutherian mammals (Andersson et al. 1987; Gustafsson and Andersson 1994). However, as discussed elsewhere (Kriener et al. 2000a, b), there is now compelling evidence available in support of the notion that the sharing of motifs is the result of convergent evolution driven by positive selection on exon 2, which codes for the main functional part of the class II Mhc molecules. Exon 2 sequences are therefore not suitable for phylogenetic analyses of *DRB* genes. True phylogenies of these genes can be revealed by the intron sequences and corroborated by the distribution and identities of the *Alu* elements inserted into them.

METHODS

Source of DNA

Genomic NWM DNA was isolated from peripheral blood leukocytes of one cotton-top tamarin (*Saguinus oedipus*, *Saoe*; Universität Bielefeld, Germany), two common marmosets (*Callithrix jacchus*, *Caja*), one common squirrel-monkey (*Saimiri sciureus*, *Sasc*; both TNO Institute of Applied Radiobiology and Immunology, Rijswijk, The Netherlands), one black-capped capuchin (*Cebus apella*, *Ceap*), and two dusky titis (*Callicebus moloch*, *Camo*; both Universität Kassel, Germany). [The species abbreviations listed in parentheses are in accordance with the rules for standardized *Mhc* nomenclature (Klein et al. 1990)]. Prosimian DNA was prepared from one Philippine tarsier (*Tarsius syrichta*, *Tasy*), one Horsfield's tarsier (*Tarsius bancanus*, *Taba*; both CNRS Paris, France), and one moholi bushbaby (*Galago moholi*, *Gamo*; Universität Kassel). The DNA was extracted according to the protocol of Blin and Stafford (1976).

PCR

Fifty to one hundred nanograms of genomic DNA were amplified with 0.5 µM of each of the two primers (Table 1; Fig. 4), 200 µM of each of the four deoxyribonucleotide phosphates, and 1.5 mM $MgCl_2$ in the form of Hot Wax Mg^{2+} beads (Invitrogen, Leek, The Netherlands) using the GeneAmp XL PCR Kit (Perkin Elmer Applied Biosystems, Foster City, CA). The amplification was performed in the Gene Amp PCR System 9600 (Perkin Elmer Cetus, Norwalk, CN) and consisted of 12 cycles of denaturation at 94°C for 30 sec, followed by annealing and extension at 64°C for 8 min, then 24 cycles, in which the annealing temperature was raised by 0.15°C in every cycle. The reaction was completed by a final primer extension for 10 min at 72°C. The amplification products were purified and cloned in the *Sma*I site of the pGEM-3Zf(+) (Promega, Madison, WI) or the pUC18 plasmid vector (Amersham Pharmacia Biotech, Freiburg, Germany).

DNA Sequencing

Double-stranded DNA was prepared with the QIAgen Plasmid Kit (Qiagen, Hilden, Germany) and sequenced by using the AutoRead Sequencing Kit (Amersham Pharmacia Biotech). Five microliters of each sequencing reaction mixture were loaded on a 6.6% acrylamide gel and run in the Automated Laser Fluorescent DNA sequencer (Amersham Pharmacia Biotech). Cycle sequencing reactions were performed with the 7-deaza-dGTP Kit (Amersham Pharmacia Biotech) and run in the LiCor sequencer (MWG Biotech, Ebersberg, Germany).

Restriction Enzyme Digestion and Southern Blotting

Clones were digested with the restriction enzyme combinations *Bam*HI/*Hin*dIII, *Hin*dIII/*Hin*cII, and *Bam*HI/*Eco*RI (NEB, Beverly, MA; Boehringer, Mannheim, Germany) according to the manufacturer's instructions. After 1 hr of incubation, the digestion products were separated on a 1% agarose gel and transferred to positively charged nylon membranes (Hybond N^+, Amersham Pharmacia Biotech or Gene Screen Plus, NEN, Boston, MA) by alkaline vacuum blotting with 0.4 N NaOH as a transfer solution.

Hybridization

A hybridization probe was obtained by PCR amplification of human genomic DNA using primers Alu1 and Alu2. The primers bound to the 5′ and 3′ ends of a dimeric Alu element, respectively, and amplified a 250-bp fragment. The PCR product was labeled with $\alpha[^{32}PdCTP]$ by using Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech). Prehybridization was carried out for 1 or 2 hr at 42°C in a solution containing 50% formamide, 5x SSPE, 5x Denhardt's solution, 0.1% SDS, and 100 µg/ml sonicated salmon sperm DNA. The hybridization probe was denatured and added to a fresh hybridization solution. Hybridization was carried out overnight at 42°C. The membranes were washed twice for 15 min at room temperature in a solution containing 2x SSPE and 0.2% SDS, and once for 15 min at 50°C in a solution containing

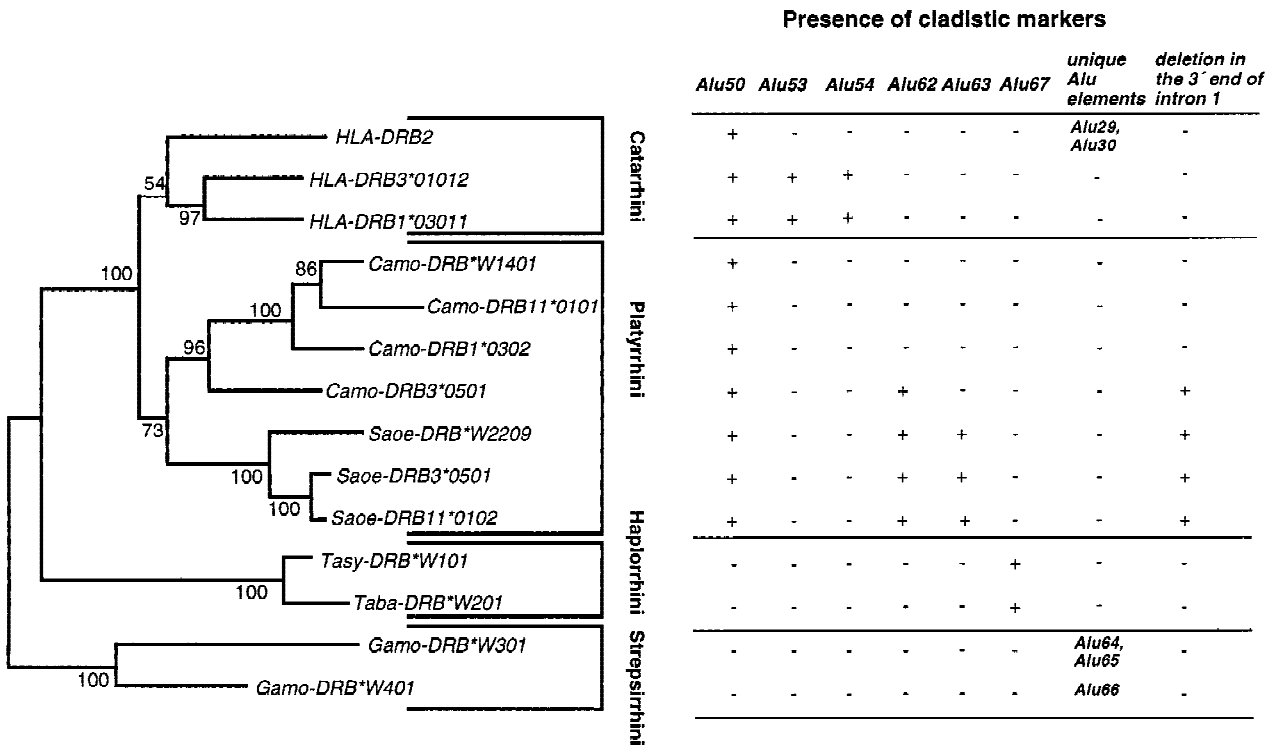


Figure 6 Comparison of a phylogeny obtained by sequence data with the distribution of cladistic markers. The neighbor-joining tree is based on the sequence of the 5′end of intron 1 in addition to the sequence from the region surrounding the *Alu* elements. Major primate groupings (Catarrhini, Platyrrhini, Haplorrhini, and Strepsirrhini) correspond to clades indicated on the tree. The numbers at nodes indicate the percentage of recovery of that node in 500 bootstrap replications. The presence and absence of *Alu* elements and of an 870 bp-deletion at the 3′end of intron 1 are indicated by + and − symbols, respectively. The names of unique *Alu* elements are given where they occur.

0.5x SSPE and 0.2% SDS. The filters were then used to expose X-ray film (XAR5; Kodak, Stuttgart, Germany). The hybridization-positive restriction fragments thus identified were subcloned and sequenced.

Sequence Analysis and Classification of *Alu* Elements

Sequences were scanned by using the program Dotty Plot, version 1.0c (Gilbert 1995a) and aligned with the help of the program SeqPup version 0.4 (Gilbert 1995b). Genetic distances were calculated by the two-parameter method (Kimura 1980). Phylogenetic trees were drawn by the neighbor-joining method (Saitou and Nei 1987) in the version specified by the program MEGA (Kumar et al. 1993) and by the maximum likelihood method using the program PHYLIP (Felsenstein 1993). *Alu* elements were aligned to the consensus sequence of *Alu* elements and their flanking direct repeats were identified. They were classified in subfamilies according to Jurka and Milosavljevic (1991).

ACKNOWLEDGMENTS

We thank Ms. Jane Kraushaar for editorial assistance, Dr. Herbert Tichy, MPI für Biologie, Tübingen for the NWM and prosimian DNA samples, and Dr. Philippe Dijan, CNRS, Paris for providing us with tarsier DNA. The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

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Received October 4, 1999; accepted in revised form January 18, 2000.