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Nucleotide sequence and genomic organization of bird minisatellites

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ABSTRACT

Two minisatellite loci from a Eurasian songbird, the willow warbler (*Phylloscopus trochilus*) were isolated, sequenced and used as probes to detect more than 20 related hypervariable loci. In addition, a sequence flanking one of the minisatellite loci was isolated, and used to study a VNTR locus. The bird minisatellites have a repeat unit of either 12 (AGGGAAGGGCTC) or 17 bp (GGGGACAGGGGACACCC), repeated in tandem 40−100 times per locus, and shows partial similarity to the sequence motifs of human minisatellites. These sequences are among the most variable minisatellites known, with the incidence per gamete of new length alleles estimated from family studies of warblers to about 5.6% per locus. The bird minisatellite alleles show mendelian inheritance and segregation analysis indicates that they are derived from families of sequences with members on several autosomal linkage groups. Some of the warbler core sequences cross-hybridize to hypervariable loci in other species of birds, mammals and fishes.

INTRODUCTION

Vertebrate genomes contain hypervariable loci known as minisatellites (1). Each such locus consist of an array of tandem repeats of a short sequence. A number of minisatellite families have been found, each characterized by a specific sequence (1). Segregation analysis indicates that the different loci within a family generally are distributed over several chromosomes (2).

The extreme variability at these loci renders them particularly suited for use as linkage markers, and for individual identification and paternity determination in forensic medicine (3,4,5,6,7). Some of the human minisatellite sequences cross-hybridize to hypervariable loci in other mammals as well as birds (8,9). The similarities among these sequences and the chi-sequence in *E. coli* has been taken to support the hypothesis that these loci function as universal recombination signals (1).

The minisatellite pattern has been studied in a broad range of species using the human repeats as probes (8,9,10), but sequence information and knowledge of the genomic distribution of minisatellite loci is limited to human (1) and mouse (11). We have therefore cloned songbird minisatellite sequences.

MATERIALS AND METHODS

Blood samples from wild birds

Warblers were caught at two study areas outside Stockholm, Sweden and blood samples were obtained by cutting the claw and collecting 2−10μl either onto filter paper (3MM) or in microcapillaries. The blood samples were stored frozen until the DNA was extracted. Blood samples collected in capillaries gave a more consistent yield of high molecular weight

a

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          10         20         30         40         50
CTCCATCAGCTCCAGGACACCTTCTTCCCCAGGACACGGTTGGTGATTTT
CTGAGGGAAAAACAAAGGCACACAGAGGGGTTTGAGTGCTTTGAGCGTTA
GAGATGCTGAGCACAGGTTTAATTGTTACAGATTCAATTGTTACAGAATC
CCAGGGGGTTTGGGAGGGAAGGGACCCTAAAGCCCACCCAGTGCCACCCC
TGCTGGGCAGACACCTCCCACTGTCCCCCCTGGCTCCAAGCCCAGCGCCC
AGCCTGGCACCAAGGGGCAATAAACAGCACAAAAAAAGGGGAATTTACCC
CTTCGGGCAACTCAAGCAGCTCAGCAGTTGCTGAGTGAGAGCACCAGGAT
ATCCTGACGTGGGAATGGTCAGGAACGGGCAGGGATTGGGAACGGGCTGG
GATTGGGAGCAGGGCTGGGATTGGG(-1Kb-)CTCAGCTA
  1.   AGG------CTCAGCTA
  2.   AGG------CTC
  3.   AGG-AAGGGCTC
  4.   AGG-AAGGGCTC
  5.   AGG-AAGGCAGCAGAGCTCC
  6.   AGG-AAGGGCTC
  7.   AGG-ATGGGCTC
  8.   AGG-AAGGGCTC
  9.   AGG-AAGGGCTC
  10.  AGGGAAGGGCTC
  11.  AG--AAGGGCTC
  12.  AGG-AAGGGCTC
  13.  AGG-AAGGGCTC
  14.  -GGGAAGGGCTC
  15.  -GGGAAGGGCTC
  16.  AGGGAAGGGCTC
  17.  AGG-AAGGCAGCAGAGCTCC
  18.  AGGGAAGGGCTC
  19.  AGGGAAGGGCTC
  20.  AGGGAAGGGCTC
  21.  AGG-AAGGCGGCAGAGAGCTCC
  22.  -GGGAAGGGCTC
  23.  AGG-AAGGGCTC
  24.  AGGGAAGGGCTC
  25.  AG--AAGGGCTC
  26.  AGGGAAGGGCTC
  27.  AGG-AAGG-CACAGAGCTCC
  28.  AGGGAAGGGCTC
  29.  AGGGAAAGGCTC
  30.  AGGGAAGGGCTC
  31.  AGGGAAAGGCTC
  32.  AGGGAAGGGCTC
  33.  -GGGAAGG-CTCAGAGCTC
  34.  AGGGAAGGGCTC
  35.  AGGGAAGGGCTC
  36.  AGGGAAGGGCTC
AGGAAGGCAGTCAGAGCTCCAGCAGATCGCCCAGGCGGGGTGGTAAGGGG
GCTGTCAG

       AGGGAAGGGCTC (Consensus)
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b GGGGACAGGGGACACCC (Consensus)

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1.  GGG-ACAGCCC------
2.  GGGGACAGGG-ACACGGAGA
3.  GGGGACAGGG-ACACCCT
4.  GGGGACAGG--ACACCCT
5.  GGGGACAGGG-ACAGCCC
6.  GGGGACAGGG-ACAGGGAGA
7.  GGGGACAGGG-ACACCCT
8.  GGGGACAGG--ACACCCT
9 .  GGGGACAGG--ACAGCCC
10. GGGGACAGGG-ATACCTG
11. CGGGAAT----------
12. AGGGACACGG-AGAGTGAC
13. AGGGACATGGGGA-----
14. GGGGACA----------
15. GGGACA-CCTCGGGAATA
16. GGGACATGG--AGA----
17. GGGGACAGGG-ACACCCT
18. GGGGACAGG--ACAGCCC
19  GGGGACA----------
20. GGGGACAG-------CCC
21. GGGGACAGGGGATAGCCC
22. GGGGACAGGGGACACGGAGA
23. GGGGACAGGG-ACACCCC
24. GGGGACAGGGGACACCC
25. AGGGACAGGGGACATGGAGA
26. GGGGACAGGG-AGACCCC
27. GGGGAAGTACGAGACCAGGAGCATGGACAGACACCCGAGGACA
28. GGGGACAGGG-ACACCCC
29. GGGGACAGGGGACAC---
30. GGGGACAGGG-ACACCCC
31. GGGGACAGGG-ACAGGCCC
32. GGGGACAGGG-ATACCCCGAGGAC
33. AGGGACACGG-AGA----
34. GGGGACAGGG-ACACCCC
35. GGGGACAGGG-ATACCTCGGGAAT
36. AGGGACATGG-AGAGGGAAT
37. AGGGACACCCC-------
38. GGGGACAGGG-ACACGGAGA
39. GGGGACAGGG-ACACCC

GGGGACACCCCGGCCCACGCTCCCAGCGCGCGCGCCTCACCTGCCTGTATT
GGGGCAGGAGGGTGCCCGGGGGGGCTGCAGGAATTCGA
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Figure 1a. Nucleotide sequence of minisatellite (L18) from willow warbler (*Phylloscopus trochilus*). Individual repeat units are numbered. This alignment of the repeat units emphasizes the differences of individual repeats from the consensus. Dashes indicate gaps.

Figure 1b. Nucleotide sequence of minisatellite (L13) from willow warbler (*Phylloscopus trochilus*). Individual repeat units are numbered. This alignment of the repeat units emphasizes the differences of individual repeats from the consensus. Dashes indicate gaps.

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         10         20         30         40         50
CCACAGCTCATCTACTCCGACTCCTGGCTCGTACTTCCAGGAGTGGTGCG
TGTGTCTCCCGTGGTTATCTCAAGTCCTATTCCTCGCACATGTTGTACGT
TCGAGAGTGGATGTAGCATGTTCCTGCAAGACATCTTGGACGTCACTATC
AGTCGAGCTCCACTCACGACATAGCTCTTCCTTCCGCTTGTGCGCACATA
TGCTAGTTCTTACTCTCGCATCCATCCACATGTCACACCTACCATACGCA
ATTCTGACGTCCGCGAGTACATGCCATAGCATCGCTCGTTGACACACGTC
GCCTAGCGCTGGCTCACACTCGTAGGCTCGCATGCATATACTCATCGACT
ATACATTTCTGCGCGTGTCACTCATTCCTCTGCCAACTCTCATCGTGTCG
CCATAGCTCGCTTGGCGTCACATGCGCGTCGGAATTCGTCGTCGCGGGAG
TCCTTCCTTGCCGTCCTCACTGCTCACGCCTCGACGACTCGTGCCGTAGA
CAAGCGCGTGCCATGTTGCCTCGCTAGCACGCGCTGCTCTCATACGCTTG
CGAGTACATCCGCTCGCAAGTGCGTCTTTGCCTCCACGCTCATCGCGTAC
GCGTCTTGCTACACATGACAGATCGTGTCTCGACAGGTCGGAATTGGCTT
AGAGTGACGAAGCGCTCAACTAACGCTAGGGCGTGCACATGTGGCAGAGG
GCCTACGCAAGCAGGCTAGGCGTGCCAGCATACGTGAGGAATCTGTTACG
CTCGGAAGGTCACACACG
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Figure 2. Nucleotide sequence (L17) of a region flanking a minisatellite locus in the willow warbler (*Phylloscopus trochilus*).

DNA. The blood was resuspended in 50μl STE (50mM NaCl, 10mM Tris HCl pH 7.5, 1mM EDTA), 250 μl buffer (10mM Tris pH=7.5, 15 mM EDTA, 100mM NaCl, 2% SDS), 10μl 1M dithiothreitol (DTT) and 10 μl Proteinase K (10mg/ml) was added, and the digest incubated for 5 hrs at 65°C. It was then extracted with phenol once, followed by an extraction with choroform and the DNA precipitated by ethanol or dialyzed against water using the microconcentrator Centricon 30 (Amicon).

Table 1. Comparison of the consensus sequence of minisatellites from various sources (Refs 1,14,15).

Minisatellite	*Consensus sequence*
Bird	
L18 (willow warbler)	AGGGAAGGGCTC
L13 (willow warbler)	GGGGACAGGGGACACCC
Human	
33.1	AAGGTGGGCAGGAAGT
33.3	CCGGGAGGTGGGCAGGAAX
33.5	GGAGGYGGGCAGGAGG
33.6	AGGGCTGGAGG
33.11	GGTGGTGGGCAGGAAGC
33.15	AGAGGTGGGCAGGTGG
plg3	GGAGGTGGGCAGGARG
myoglobin	GGAGGTGGGCAGGAAC
E. coli	
Chi	GCTGGTGG

Table 2. Mutational changes between minisatellite repeats.

Minisatellite (no repeats)	Mutations per repeat (d)		Mean per cent divergence due to unique substitutions (e)
	Point mutations	Length mutations (c)	
Bird			
L13 (n=39)	1.4	0.72	4.0
L18 (n=36)	0.78	0.28	2.8
Human (a)			
33.15 (n=29)	0.34	0.10	1.1
33.6 (n=11)	0.27	0.09	0.7
33.5 (n=14)	2.85	0.50	5.5
g (n=25) (b)	1.89	0.28	1.6

a, From Jeffreys et al. (1).
b, From Wong et al. (6).
c, Deletions and additions.
d, Total number of mutations divided by the number of repeats sequenced.
e, Number of repeat unit variants divided by the total number of nucleotides in the repeat units sequenced. For instance, in the L18 clone there are 36 repeats whose average length is 12 bases ($36\times12=432$bp) and 12 variant repeats were found, giving a mean percent unique substitution divergence of $12/432=2.8\%$.

Cloning of minisatellites

Genomic DNAs extracted from skeletal muscle of one willow warbler (*Phylloscopus trochilus*), was digested with *Eco* RI, ligated into the arms of lambda gt10 and packaged as described (12). About 5×10^4 plaque forming units (pfu's) were plated and plaque hybridization performed using radioactively labeled fragments of human minisatellite 6:3 and 15:1 (1) (probes kindly provided by Dr Alec J. Jeffreys). Filters were hybridized at 42°C for 5 hrs in 50% formamide, 5 × SSPE (1×SSPE= 0.18M NaCl, 0.01M NaH_2PO_4, pH=7.4, 0.001M EDTA, pH=7.4), 2.5×Denhardt (12), 5% dextran sulfate, 0.2% sodium dodecyl sulphate (SDS), 0.1μg salmon sperm DNA per ml and washed for 30 minutes in 2×SSPE, 0.1%SDS at 65°C. A total of 22 pfu's hybridized to the human probes under these conditions. Three sequences (L13, L17, L18) derived from two phage clones will be described.

The recombinant phage inserts were 3.5−4.5 kb and very unstable, with deletions reducing their size during plaque purification (data not shown). Two restriction fragments (L17 (750bp) and L18 (2000bp)) hybridizing to the human minisatellite probes were isolated by Southern blotting from one phage and one fragment from each of the other two (L13 (750bp). The fragments were cloned into Bluescript and sequenced from both ends by the chain termination method.

Southern blotting

2−5 μg of genomic DNA was digested with *Hinf* I and electrophoresed through a 24 cm long 0.7% agarose gel to display a multi-locus pattern. To study the VNTR pattern (for variable number of tandem repeat) a 1.2% agarose gel was used. The buffer contained 1×TBE (0.089 M Tris-borate, 0.089M boric acid, 0.002M EDTA). The gels were run at 25mA and 40V until DNA fragments smaller than about 1.5kb (for multi-locus pattern), or 0.5kb (for VNTR), had electrophoresed off the gel. The gel was then denatured in 0.5M NaOH, 1.5M NaCl, neutralized in 0.5M Tris HCl pH=7.5, 1.5M NaCl and

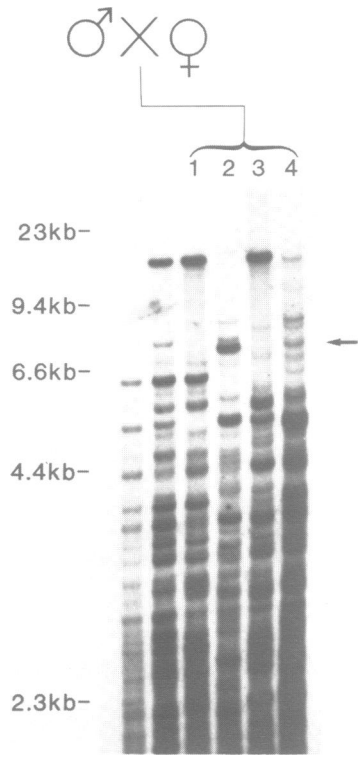

Figure 3. Segregation of minisatellite fragments in a family of the willow warbler. Southern blot of *Hinf* I digested DNA of the male, female and three offspring (1–3) were hybridized with the L17 probe. The arrow indicates a fragment in individual 2 not present in either parent and presumably representing a newly arisen allele.

transferred overnight in 5×SSPE onto a nylonmembrane (Nytran). The membrane was hybridized in 4×SSPE, 3×Denhardt, 5% dextran sulfate, 50% formamide, 0.1% SDS at 42°C and probed with the cloned radioactively labeled birdminisatellite fragments. The membrane was washed at room temperature in 5×SSPE, 0.1% SDS for 10 min, then transferred either to a low stringency wash to obtain a multi-locus pattern (1×SSPE, 0.1% SDS at 55°C for 10 min) or to a high stringency wash to obtain VNTR pattern (0.1×SSPE, 0.1% SDS at 65°C for 20 min).

RESULTS AND DISCUSSION

Organization of the minisatellite loci

Two (L18 and L13) of the three cloned sequences contain short tandem repeats (Fig 1a,b). The L18 clone contains approximately 100 copies of a repeat unit of 12 bases, 36 of which were sequenced, while L13 contain about 45 copies of a unit of 17 bases, 39 of which were sequenced.

The other sequence (L17), derived from the same phage clone as L18, show no simple repeat structure (Fig 2.), yet it does resemble L13 and L18 in being rich in guanine and tend to have runs of guanines. In Southern blot analysis of genomic DNA cut with *Hinf*

Table 3. Segregation of minisatellite fragments in a total of 11 warbler families. The expected distribution of fragments is given for a transmission frequency of 50% with no linkage between minisatellite loci and no new mutations. In addition, the expected distribution is indicated for cotransmission of fragments when the transmission ratio is reduced to 30%. The minisatellite fragments were detected using the L17 probe.

Number of offspring receiving the fragment (s)	Father					Mother				
	Single fragment		Pairs of fragments			Single fragment		Pairs of fragments		
	Obs.	Exp.	Obs.	Exp.		Obs.	Exp.	Obs.	Exp.	
		50%		30%	50%		50%		30%	50%
0	0	2.4	60	59	11.7	1	2.2	72	73.1	14.9
1	10	10.6	151	128	51.8	10	10.7	184	161.5	65.5
2	20	19.9	118	121.7	101	27	21.5	156	154.2	126.3
3	30	22.2	54	67	112.3	23	22.7	71	85.2	142.2
4	11	15.7	17	22.6	81	13	15.9	25	28.7	102.5
5	6	7.2	3	4.7	36.3	6	7.1	1	5.8	46.2
6	2	1.8	0	–	8.9	2	1.7	0	0.5	11.4
7	1	0.2	0	–	–	0	0.2	0	–	–
Transmission frequency	42%					40%				

I, L17 detects the identical set of minisatellite fragments as L18 (data not shown), indicating that it is derived from a sequence flanking the cloned minisatellite locus (L18).

Sequence and variability of the repeat unit

The consensus sequences of the bird minisatellites show partial identity to each other as well as to the consensus for other minisatellites (Table 1). Notable is the frequent occurrence of mirror repeats of homopurine runs, such as GGGGACAGGGG in L13 and GGGAAGGGCTCGGGAAGGG. Sequences like these probably are capable of forming triplex structures that could have regulatory or recombinational roles (13).

The variability of the bird repeat units is compared with that of human minisatellite repeat units in Table 2 (1). About 67–74% of the total number of differences between repeat units are point mutations in L13 and L18, respectively. The mutations are scattered over most of the repeat unit, although streches of G's seems more conserved. In the frequency of point mutational differences and number of length differences, the bird minisatellites appear less homogeneous than three of the four previously described human minisatellites (Table 2).

Genomic distribution of minisatellite loci

The inheritance of minisatellite alleles and the independent segregation of fragments was studied using the distribution of minisatellite fragments detected by probe L17 in 7 families of willow warbler and 4 families of wood warbler (*Phylloscopus sibilatrix*) (2). An example of the segregation of minisatellite fragments in a family of willow warblers is shown in Fig 3. The segregation of single fragments in individual families conformed to the expected binomial proportions, assuming a 50% transmission frequency (probability of transmission of a fragment from parent to offspring). To increase the number of data the different families

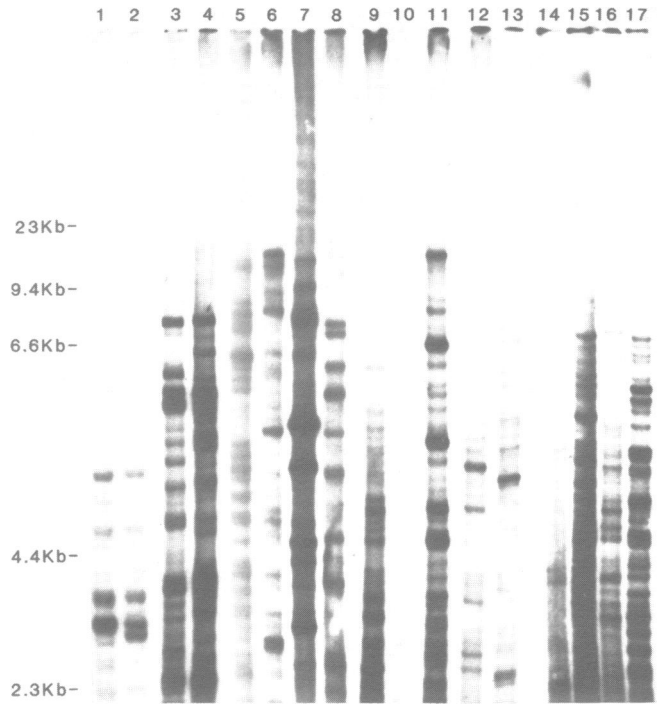

Figure 4. Evolutionary conservation of minisatellite sequences. Hybridization pattern of the L17 probe to DNA from human (lanes 1,2); house mouse (*Mus musculus*) (3,4); fin whale (*Balaenoptera physalus*) (5); sperm whale (*Physeter macrocephalus*) (6); whitebeak dolphin (*Lagenorhyncus albirostris*) (7); beluga (*Delphinapterus leucas*) (8); wood warbler (*Phylloscopus sibilatrix*) (9); great tit (*Parus major*) (10); willow warbler (*Phylloscopus trochilus*) (11); brown trout (*Salmo trutta*) (12); and Arctic charr (*Salvelinus alpinus*) (13). Hybridization pattern of the L13 probe to DNA from human (14); willow warbler (15); great tit (16) and wood warbler (17).

were then pooled; the distribution of single fragments in the pooled data also conformed to the expected binomial proportions (Table 3).

The cotransmission of fragments (or lack of fragments) in the 11 families was compared to the expected distribution for unlinked loci (removing completely linked fragments) and a transmission ratio of 50%. The observed distribution deviated from that expected by an excess of fragments not cotransmitted to any, or very few, offspring. Significant linkage between loci would result in an excess of fragments cotransmitted *both* to very few and all offspring. Fragments that failed to be cotransmitted may represent allelic fragments and, occasionally, new length variants (offspring 2, Fig 3). Indirect support for that allelic fragments and new variants could give rise to the observed distribution of pairs can be obtained by examining the expected distribution for a lower probability of transmission of fragments. For example, a transmission ratio of 30% (unlinked loci) gives a better fit to the observed distribution (Table 3).

The segregation analysis thus indicates that L17 hybridizes to a number of unlinked or loosely linked loci. To estimate the number of different minisatellite loci detected (L) we used the method of Jeffreys et al (2),

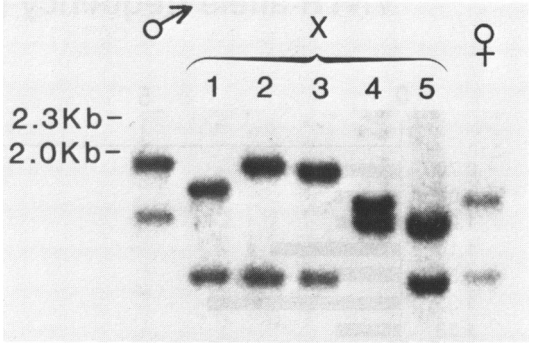

Figure 5. Segregation of alleles at a locus containing a variable number of tandem repeats (VNTR) in a family of willow warblers detected with the L17 probe. Offspring 1 has a new length variant not present in either parent.

$$L=n-a-b,$$

where n is the number of fragments scored (in either the father of the mother), a the number of allelic pairs and b the number of linked pairs. Using the data from the different families we estimate that the L17 probe detects alleles from on the average 23 loci in the males and 20 loci in the females. On average, however, only 17 loci (with the longest minisatellite alleles) were routinely scored in the segregation analysis.

Evolutionary conservation of minisatellites

The L17 probe hybridize to hypervariable regions of the DNA of other birds, some mammal

Table 4. Segregation of alleles at a VNTR locus in 11 warbler families. Alleles are denoted by their approximate length in kilobases. Underlined alleles are mismatched relative to those present in the parents and are presumably new length variants. Dashes indicate total loss of alleles. The mismatched alleles were in three cases (Family 1,3,4) of paternal origin, three cases (Family 3,7) of maternal origin, and in one case (Family 3) of either origin.

Family Number	Parental genotypes Male	Female	Offspring genotypes
1.	2.1/1.7;	1.8/1.5	1.9/1.5; 2.1/1.5; 2.1/1.5; 1.7/1.8; 1.7/1.5
2.	2.2/1.4;	1.85/1.6	2.2/1.6; 2.2/1.6; 2.2/1.6; 2.2/1.4; 2.2/1.6; 2.2/1.6
3.	2.2/1.55;	2.2/1.4	2.2/1.55; 1.55/-; 1.4/1.65; 2.2/1.55; 1.4/1.55; 2.7/2.2
4.	1.6/1.3;	1.4/1.45	1.3/1.4; 1.45/1.6; 1.3/1.45; 1.4/1.6; 1.45/1.6; 1.45/1.6; 1.4/1.8
5.	1.4/1.45;	2.3/1.9	1.4/1.9; 2.3/1.4; 2.3/1.4; 2.3/1.4; 1.9/1.45; 1.9/1.45; 2.3/1.45
6.	1.3/1.5;	2.0/2.1	1.5/2.1; 1.5/2.0
7.	2.05/1.7;	1.9/1.95	2.05/-; 2.05/-; 2.05/1.95; 1.95/1.7; 1.95/2.05; 2.05/51.95
8.	1.9/1.5;	1.65/1.65	1.5/1.65; 1.65/1.9; 1.65/1.9; 1.5/1.65; 1.5/1.65
9.	2.3/1.9;	2.1/1.45	2.3/2.1; 2.3/1.45; 2.3/1.45; 2.1/2.3; 1.9/1.45;2.1/1.9
10.	2.5/1.9;	1.7/2.0	2.5/1.7; 2.5/2.0; 2.5/2.0; 1.9/1.7; 1.9/2.0; 2.5/1.7; 2.5/1.7
11.	2.3/1.9;	2.4/1.6	2.3/2.4; 1.9/1.6; 1.9/1.6; 1.9/2.4; 2.3/1.6

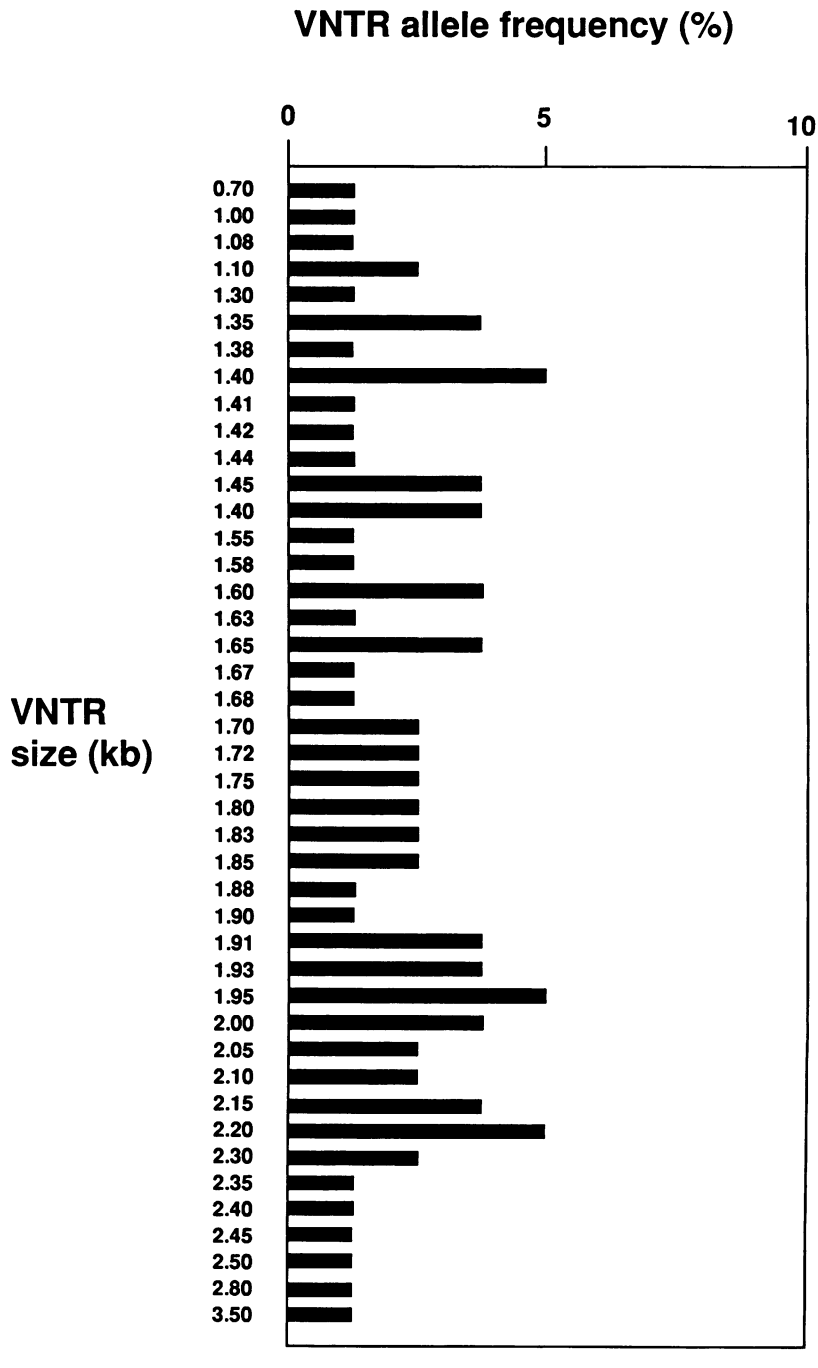

Figure 6. Frequencies of allelic variants at a VNTR locus detected by the L17 probe in 40 willow warblers (22 parents and 18 individuals from different regions).

and fish species (Fig 4). The evolutionary cross-hybridization indicates that the bird sequences can be used to study genetic relationships among individuals in a wide range of species. Thus, they may complement the human minisatellite sequences and extend the range of species whose genetic structure can be explored by minisatellite analysis.

Mutation rate of minisatellite sequences

To estimate the rate at which new length variants arise we used the L17 probe, containing a sequence from a region flanking one of the cloned minisatellite loci (L18). Using a high stringency wash, the L17 probe hybridizes only to a single highly variable (VNTR) locus (14) (Fig. 5).

The segregation of alleles at this locus was followed in 11 families of warblers (Table 4). The correct paternity in these families was first determined using the full minisatellite pattern (data not shown). The fraction of shared minisatellite fragments between warblers from different localities is about 12% (Gyllensten et al. in prep.). Since about 17 fragments were scored in an individual, on the average half of which are expected to be of paternal origin, the probability of inferring an incorrect paternity using this probe is on the order of $0.12^{8.5}=1.5\times10^{-9}$. Further, the allele frequency distribution at the VNTR locus in the willow warbler (based on analysis of 80 alleles from parental birds and additional males from various localities) indicates that the average allele frequency is about 2.3%, giving a probability of incorrect paternity using the VNTR locus of 0.045 (6) (Fig. 6).

Of a total of 62 offspring, 7 were observed to carry an allele at the VNTR locus not present in either the father or the mother (Table 4), suggesting that they represent newly arisen length mutations. One such new length mutation detected within a family is shown in Fig. 5, offspring 1. In none of the 11 families did analysis of the minisatellite pattern indicate illegitimacy. In addition, three of the new length variants must have arisen in the male germline, three in the female germline and one could have arisen in either. Based on these analysis, the apparent frequency of new length variants in these families is $7/62\times2=5.6\%$ per gamete. This estimate is similar to that for the most unstable human minisatellite sequence family (7).

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