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Molecular Mapping of Insecticide Resistance Genes in the Yellow Fever Mosquito (Aedes aegypti)

D. W. Severson, N. M. Anthony, O. Andreev, and R. H. ffrench-Constant

Several loci conferring insecticide resistance in the yellow fever mosquito (Aedes aegypti) have previously been mapped by simple recombinational mapping. Here we describe correlation of these resistance phenotypes with molecular gene probes for insecticide target sites by RFLP mapping. The para sodium channel gene homologue and the GABA receptor gene Resistance to dieldrin map to the same genome regions as the DDT/pyrethroid and cyclodiene resistance loci, respectively. Although the acetylcholinesterase (target site of organophosphorus and carbamate insecticides) gene Ace does not map to any known resistance locus, it maps very close to the sex-determining locus. We discuss the possibilities that, if identified, Ace-mediated resistance in A. aegypti will be sex linked or that, as suggested for anopheline mosquitoes, two independent Ace loci may exist, one of which is autosomal. These results support the importance of target site insensitivity as an insecticide resistance mechanism in mosquitoes.

Insecticide resistance is widespread in disease vector mosquitoes and has substan-

Table 1. Segregation of RFLP markers in the F₂ from a cross between Hamburg and Moyo-In-Dry strains

	No. of individuals[a]			
Marker	P₁	H	P₂	χ²[b]
Chromosome 1				
LF188a	24	47	23	0.02
LF217	21	49	24	0.36
D6.12	21	49	24	0.36
LF178	25	49	20	0.70
Ace	26	47	21	0.53
LF397	26	45	22	0.44
Chromosome 3				
LF377	19	47	28	1.72
LF352	17	49	28	2.75
para	16	57	21	4.41
LF386	16	57	21	4.41
LF417	15	56	23	4.81
LF396	17	61	16	8.36[c]
LF347	18	53	23	2.06

[a] Phenotypic designation: P₁ = Hamburg female type; H = heterozygote; P₂ = Moyo-In-Dry male type.
[b] Chi-square tested for an expected 1:2:1 segregation ratio.
[c] $P < .05$.

Table 2. Recombination fractions and standard errors for RFLP marker data

	LF188a	LF217	D6.12	LF178	Ace
Chromosome 1[a]					
LF217	2.15 (1.25)				
D6.12	2.15 (1.25)	0.00			
LF178	20.03 (3.77)	18.81 (3.72)	18.81 (3.72)		
Ace	21.24 (3.82)	20.03 (3.77)	20.03 (3.77)	10.70 (0.88)	
LF397	21.44 (3.93)	20.23 (3.79)	20.23 (3.79)	1.63 (1.09)	0.54 (0.62)

	s	LF169	LF189a	LF98	VCP	Rdl
Chromosome 2[b]						
LF169	1.06 (1.21)					
LF189a	3.30 (2.18)	1.92 (1.32)				
LF98	12.93 (3.07)	12.54 (3.48)	10.13 (3.10)			
VCP	15.57 (4.57)	17.68 (4.09)	15.33 (3.91)	5.61 (2.25)		
Rdl	27.17 (4.39)	26.12 (5.15)	25.10 (5.15)	12.45 (2.59)	15.73 (3.97)	
LF250	29.96 (8.62)	27.78 (7.47)	27.27 (7.75)	15.15 (6.24)	19.44 (6.60)	2.86 (2.82)

	LF377	LF352	para	LF386	LF417	LF396
Chromosome 3[c]						
LF352	5.47 (2.01)					
para	23.13 (4.22)	16.44 (3.64)				
LF386	23.13 (4.22)	16.44 (3.64)	0.00			
LF417	21.01 (4.02)	17.07 (3.69)	1.61 (1.12)	1.61 (1.12)		
LF396	29.57 (4.89)	25.17 (4.67)	10.26 (3.01)	10.26 (3.01)	8.44 (2.69)	
LF347	39.58 (4.94)	38.21 (4.96)	29.57 (4.89)	29.56 (4.89)	27.16 (4.69)	19.41 (4.17)

Standard errors in parentheses.

[a] F_2 from a cross between a Hamburg strain female and a Moyo-In-Dry strain male.

[b] JoinMap-derived composite data from Severson et al (1993, 1994a)

CHROMOSOME 1

Figure 1. RFLP genetic linkage map for the Ace locus in a Hamburg × Moyo-In-Dry F_2 intercross population and marker positions relative to the composite linkage map for chromosome 1 in *A. aegypti*. Map distances are indicated in Kosambi centiMorgans.

tially hindered their control. The yellow fever mosquito (*Aedes aegypti*), an important disease vector, has developed resistance to a wide range of insecticides. These resistance phenotypes have subsequently been mapped to their respective linkage groups via simple recombinational mapping (Hitchen and Wood 1974, 1975a,b; Lockhart et al. 1970; Malcolm 1983; Malcolm and Wood 1982).

Target site insensitivity, as opposed to insecticide metabolism, is one of the major causes of insecticide resistance, and the three major insecticide target site genes have recently been cloned from the genetic model *Drosophila melanogaster*

Table 3. Recombination fractions and standard errors for phenotypic marker data

	DDT	s	Si
Chromosome 2[a]			
s	8.35 (0.30)		
Si	12.89 (1.01)	7.33 (0.22)	
DI	—[b]	27.14 (0.37)	21.28 (0.36)

	blt	DDT[py]
Chromosome 3[c]		
DDT[py]	15.47 (0.38)	
min	30.70 (1.00)	20.17 (0.54)

Standard errors in parentheses.

[a] JoinMap-derived composite data from Hitchen and Wood (1975b) and Lockhart et al. (1970).

[b] Two-point comparisons not available.

[c] JoinMap-derived composite data from Hitchen and Wood (1974, 1975a) and Malcom and Wood (1982).
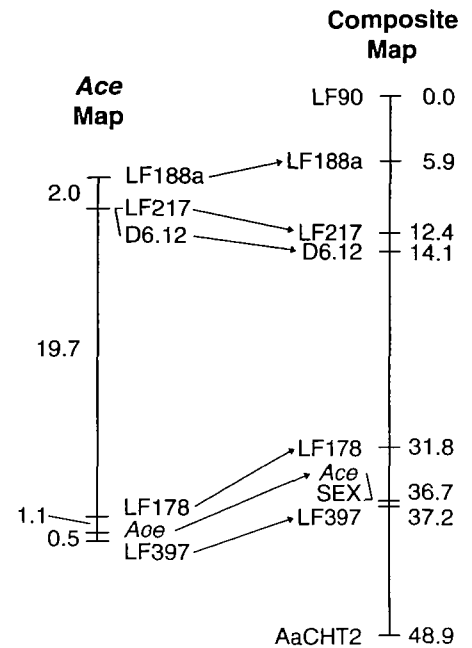
(ffrench-Constant et al. 1992). The three main insecticide targets are (1) acetylcholinesterase or AChE, the target for organophosphorus (OP) and carbamate insecticides; (2) the γ-aminobutyric acid (GABA) receptor gene *Resistance to dieldrin* or *Rdl*, the site of action of cyclodiene insecticides; and (3) the *para* sodium channel gene, the target for DDT and pyrethroids. In all three of these genes, single- or multiple-point mutations appear to cause target site insensitivity. A number of point mutations in the *Drosophila Ace* gene have been shown to affect the sensitivity of AChE inhibition by OP and carbamate insecticides (Mutero et al. 1994). In contrast, a single amino acid replacement in the GABA receptor gene *Rdl* causes resistance to cyclodienes in a wide range of insects (ffrench-Constant et al. 1993; Thompson et al. 1993b). A single amino acid replacement in *para* gene homologues also appears to cause *knockdown resistance* (*kdr*) to DDT (Reenan R, Ganetzky BS, Pittendrigh BR, and ffrench-Constant RH, unpublished data). The role of these target site genes in *A. aegypti* resistance has however not been investigated.

In order to investigate the importance of target site insensitivity in *A. aegypti* we were interested in attempting to correlate the recombinational map position of previously identified resistance loci with molecular probes for these three target site
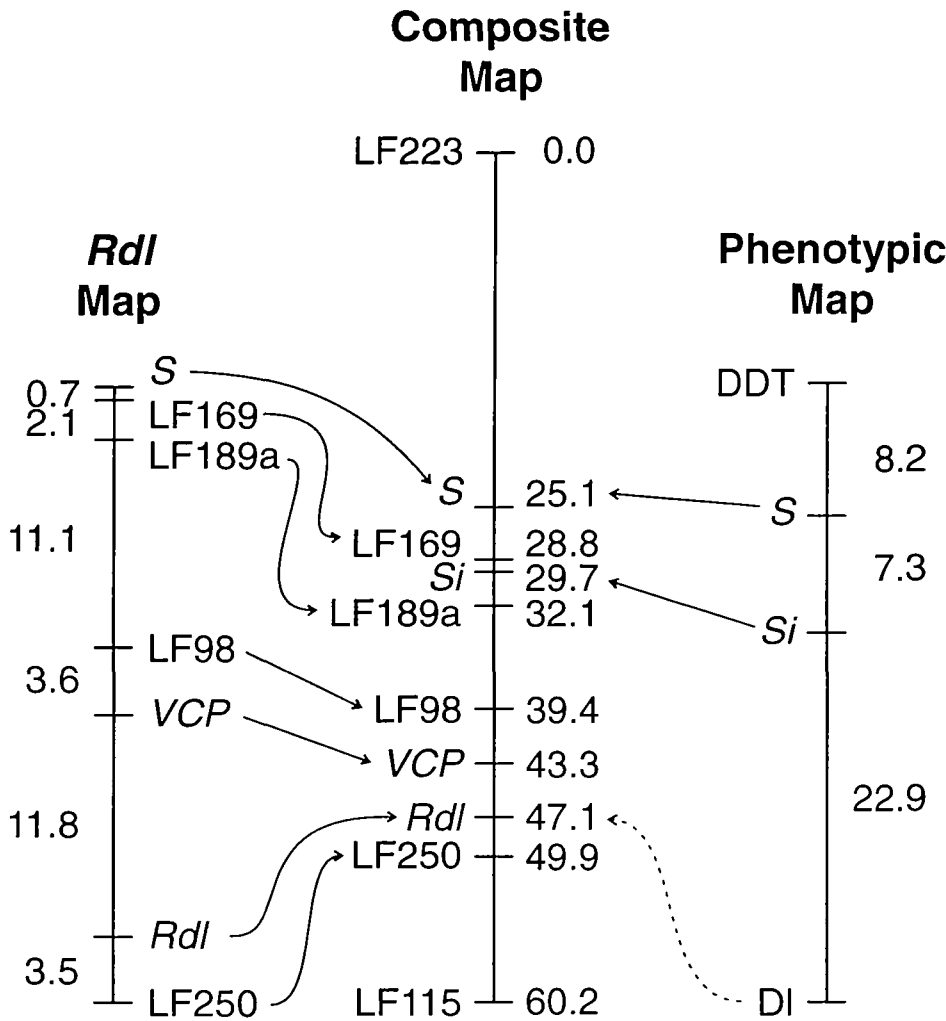
genes. In this study molecular probes for these three genes were used as RFLP markers to determine their genome positions and subsequently to correlate the location of the insecticide target genes with the resistance phenotypes previously mapped by simple recombinational mapping. Here we report that the map positions of both the *Rdl* and *para* gene homologues correlate with the previously described locations for the cyclodiene and DDT/pyrethroid resistance loci, respectively. Interestingly, although insensitivity to AChE has not been reported for *A. aegypti*, the *Ace* gene maps as a single locus in close proximity to the sex-determining locus. We discuss the implications of these results for the relative importance of target site insensitivity in mosquito insecticide resistance.

Table 4. Segregation of the Ace locus relative to sex determination in the F_2 from a cross between Hamburg and Moyo-In-Dry strains

Sex	No. of individuals[a]		
	P_1	H	P_2
Female	26	21	0
Male	0	26	21

[a] Phenotypic designation: P_1 = Hamburg female type; H = heterozygote; P_2 = Moyo-In-Dry male type.

CHROMOSOME 2

Composite Map

LF223 ─ 0.0

Rdl Map

Phenotypic Map

Figure 2. RFLP genetic linkage map for the *Rdl* locus based on recombination fractions observed in two mapping populations (Severson et al. 1993, 1994a), a phenotypic linkage map for the DI locus and a DDT locus based on recombination fractions extracted from the literature (Hitchen and Wood 1975b; Lockhart et al. 1970) and marker positions relative to the composite linkage map for chromosome 2 in *A. aegypti*. Map distances are indicated in Kosambi centiMorgans.

blotting, and hybridizations were performed as previously described (Severson et al. 1993, 1994a).

Linkage Analysis

Chi-square goodness-of-fit values for the HAM × MOY F_2 intercross population were calculated for segregation and independent assortment ratios for all pairs of loci. Multipoint linkage analyses were performed using the JoinMap computer program (Stam 1993). A minimum LOD threshold of 3 was used to identify linkage between markers. Recombination frequencies were converted into map distances (cM) using the Kosambi (1944) function. Composite linkage maps also were constructed using the JoinMap program. A composite RFLP map was prepared from all available RFLP mapping data; for reference purposes we included data for RFLP markers that define the ends of each linkage group. The *Rdl* locus composite map included previously published RFLP genetic data involving this locus (Severson et al. 1993, 1994a). The composite DDT and DI loci maps included phenotypic marker data extracted from the literature (Hitchen and Wood 1974, 1975a,b; Lockhart et al. 1970; Malcolm and Wood 1982). Because cross-resistance to DDT and pyrethroids is provided by a single locus on chromosome 3 (Malcolm 1983), we combined the independently reported mapping data for this locus.
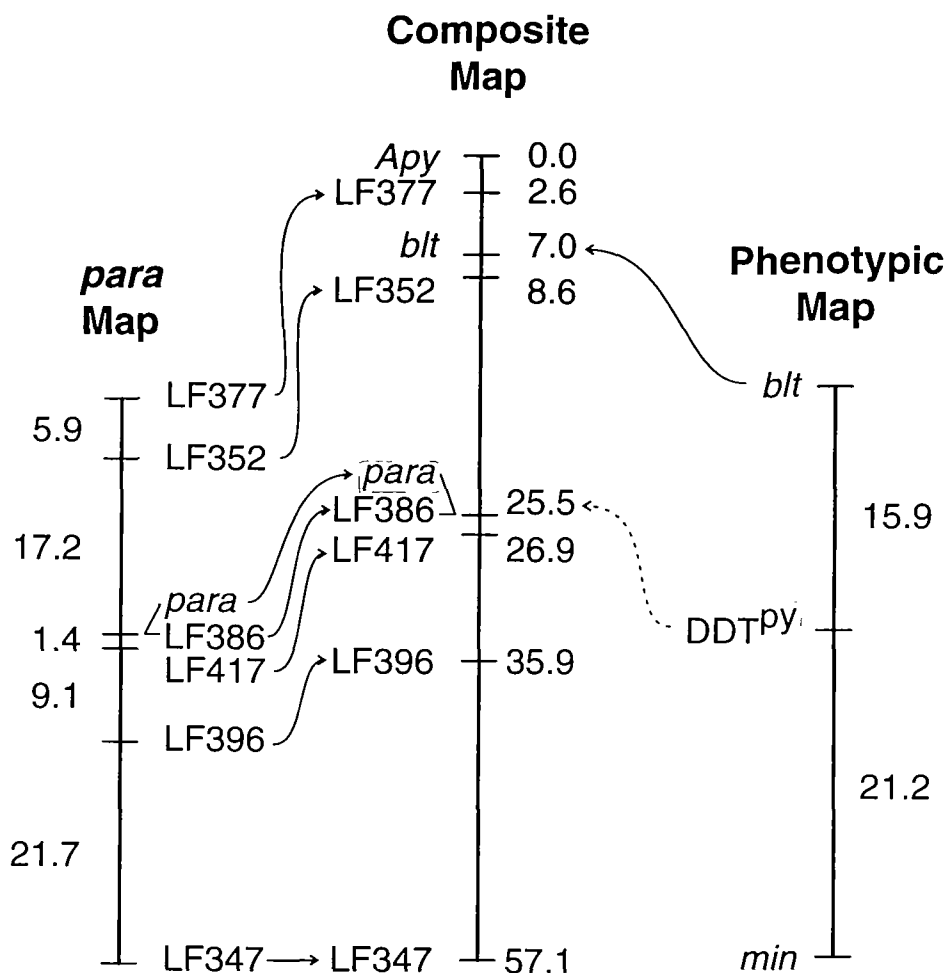
Results

Segregation Ratios and Recombination Fractions

Segregation ratios for the HAM × MOY F_2 population are shown in Table 1. The RFLP loci generally fit the expected 1:2:1 segregation ratios, with the exception that a slight excess of heterozygotes was obtained for the LF396 locus on chromosome 3. Recombination fractions and standard errors for the three insecticide resistance genes and RFLP markers are shown in Table 2. JoinMap-derived recombination fractions and standard errors for insecticide resistance phenotypes and some mutant marker phenotypes are shown in Table 3.

Linkage Analysis

The map position of the *Ace* locus in the HAM × MOY F_2 population and the marker positions relative to the composite RFLP linkage map for chromosome 1 are shown in Figure 1. No evidence for recombination between the *Ace* locus and sex determi-

Materials and Methods

Cloning of Insecticide Resistance Genes

The cloning of the *Rdl* homologue from *A. aegypti* via the low stringency screening of an adult cDNA library has been previously reported (Thompson et al. 1993a). The *Ace* gene homologue was cloned from *A. aegypti* cDNA via the use of degenerate primers in the polymerase chain reaction (Anthony et al. 1995). The 89 bp *para* gene homologue probe was cloned from genomic DNA using degenerate PCR primers in the procedure of Knipple et al. (1991).

Mosquito Crosses, DNA Isolation, and Southern Blotting

RFLP genetic data for the *Ace* and *para* loci are based on F_2 intercross progeny from a pairwise mating between the *A. aegypti* Hamburg (HAM) and Moyo-In-Dry (MOY) strains. The origins of these strains and RFLP-based estimates of genetic diversity between them are described elsewhere (Severson et al. 1994b). Mosquitoes were reared as previously described (Christensen and Sutherland 1984). DNA isolation from individual mosquitoes, digestion with *Eco*RI, Southern

CHROMOSOME 3

Composite Map

para Map (left) | Composite Map (center) | **Phenotypic Map** (right)

Composite Map (center):

Apy	0.0
LF377	2.6
blt	7.0
LF352	8.6
para	
LF386	25.5
LF417	26.9
LF396	35.9
LF347 → LF347	57.1

para Map (left):

LF377
5.9
LF352
17.2
para
1.4 LF386
LF417
9.1
LF396
21.7
LF347

Phenotypic Map (right):

blt — 15.9
DDT^{py}
21.2
min

Figure 3. RFLP genetic linkage map for the *para* locus in a Hamburg × Moyo-in-Dry F_2 intercross population, a phenotypic linkage map for the DDT^{py} locus based on recombination fractions extracted from the literature (Hitchen and Wood 1975a, Malcolm and Wood 1982), and marker positions relative to the composite linkage map for chromosome 3 in *A. aegypti*. Map distances are indicated in Kosambi centiMorgans.

nation was observed (Table 4), indicating that the *Ace* and sex-determination loci are tightly linked in *A. aegypti*.

The map position of the *Rdl* locus, based on two independent RFLP mapping populations, and the marker positions relative to the composite RFLP linkage map for chromosome 2 are shown in Figure 2. Also included are the map positions of the dieldrin resistance (DI) and a DDT resistance (DDT) phenotypic loci and two mutant marker loci, *spot-abdomen* (s) and *Silver-mesonotum* (Si). Comparative map positions indicate that the GABA receptor gene *Rdl* and the DI resistance phenotype loci map to the same chromosome region on chromosome 2. This suggests that the *Rdl* gene is responsible for cyclodiene resistance in *A. aegypti*.

The map position of the *para* locus in the HAM × MOY F_2 population and the marker positions relative to the composite RFLP linkage map for chromosome 3 are shown in Figure 3. Also included is the map position of the DDT with cross-resistance to pyrethroids locus (DDT^{py}) relative to two mutant marker loci, *black-tarsus* (blt) and *miniature-body* (min). Comparative map positions indicate that the *para* gene and the DDT^{py} resistance locus map to the same general chromosome region on chromosome 3. This suggests that DDT^{py} resistance in *A. aegypti* is determined by the *para* gene.

Discussion

Cyclodiene resistance is inherited as a single gene with a semidominant resistance

phenotype in *A. aegypti* (Lockhart et al. 1970). RFLP mapping of the *Rdl* cDNA shows a good correlation with the localization of the resistance locus DI on chromosome 2. This result is consistent with our previous observation that cyclodiene-resistant *A. aegypti* carry the same alanine to serine amino acid replacement in the *Rdl* GABA receptor gene as other cyclodiene/picrotoxin-resistant insects (Thompson et al. 1993a). Further, *A. aegypti Rdl* GABA receptors mutated to the resistance associated serine show insensitivity to picrotoxin when expressed in baculovirus-infected insect cells (Shotkoski et al. 1994). There is therefore considerable evidence that the originally mapped cyclodiene locus DI is indeed the *Rdl* GABA receptor gene.

The *para* sodium channel gene was originally cloned from *D. melanogaster* (Loughney et al. 1989). Linkage between *para* gene homologues and *knock down resistance* (kdr) to pyrethroids has been shown in both houseflies (Knipple et al. 1994; Williamson et al. 1993) and cockroaches (Dong and Scott 1994). Resistance is associated with a single-point mutation in kdr-type alleles in houseflies (Williamson et al. 1996) and cockroaches (Miyazaki et al. 1996), and with an additional point mutation in super-kdr houseflies (Williamson et al. 1996). The *A. aegypti para* gene homologue probe we used maps to chromosome 3, in the same location as a locus conferring resistance to DDT and pyrethroids. That this locus confers cross-resistance to both DDT and pyrethroids (Malcolm 1983) is especially interesting because this cross-resistance spectrum is also characteristic of kdr resistance in houseflies. We can therefore infer that the DDT/pyrethroid locus on chromosome 3 is associated with target site insensitivity and that an additional locus associated with DDT resistance on chromosome 2 in *A. aegypti* is probably associated with increased metabolic activity, probably mediated by glutathione-S-transferase or cytochrome P450 activity.

Finally, although the *Ace* gene homologue did not map to any known resistance locus, it did map very close to the sex-determining locus. Insensitivity to AChE has been reported in a number of different mosquito species (ffrench-Constant and Bonning 1989), but not in *A. aegypti*. To date however, no sex-linked insensitive AChE locus has been reported for any mosquito species. There are two hypotheses to explain these observations: (1) there are two *Ace* loci in *A. aegypti*, as

suggested for anopheline mosquitoes (Banks et al. 1996), but only the autosomal copy of *Ace* is potentially associated with resistance, or (2) *A. aegypti* only carries a single *Ace* locus, and if detected, resistance associated with insensitivity to AChE will be sex linked, in contrast with other mosquito species. At present our data support the second hypothesis, because examinations of Southern blots probed with the *A. aegypti Ace* gene only reveals evidence for a single sex-linked *Ace* locus in this species.

This study suggests that of the resistance traits mapped in *A. aegypti*, two of the most widespread resistance mechanisms, cyclodiene and DDT/pyrethroid resistance, are associated with target site insensitivity. Knowledge of the mechanisms of insecticide resistance is useful to the formulation of control strategies. Thus insensitivity of the *para* target site to DDT will confer cross-resistance to pyrethroids. The spraying of DDT may, therefore, have reduced the potential efficacy of pyrethroids even before they had been introduced. Finally, identification of the point mutations conferring target site insensitivity, which therefore delimit insecticide binding sites themselves, may aid in the rational design of new insecticides to overcome existing resistance mechanisms.

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Comparative Mapping of Anchor Loci from HSA19 to Cattle Chromosomes 7 and 18

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Six loci—CALR, EPOR, JUNB, JUND, CEA, and PRKCG—were assigned to bovine chromosomes using PCR-based hybrid somatic cell analysis. The five genes other than CALR are comparative mapping anchor loci. This study, together with the previous assignment of three anchor loci—INSR, LDLR, APOE—and four other genes—AMH, GPI, RYR1, LHB—defines the conserved synteny relationship between human chromosome 19 and cattle chromosomes 7 and 18. Genes on HSA19p13.3-13.2 are conserved in cattle chromosome 7, while those on HSA19-q13.1-13.4 are conserved in cattle chromosome 18. In contrast, homologous genes from HSA19 are located on four different mouse chromosomes, namely MMU10, MMU8, MMU9, and MMU7. This is further evidence that syntenic conservation between cattle and human generally exceeds that observed between human and mouse.

The rapid development of gene maps in various species has made comparative gene mapping a valuable tool for the study of genome organization and chromosome evolution. Comparative genome analysis is primarily based on the mapping of individual conserved loci in different spe-