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A first-generation microsatellite-based integrated genetic and cytogenetic map for the European rabbit (*Oryctolagus cuniculus*) and localization of *angora* and *albino*

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Summary

Although the European rabbit (*Oryctolagus cuniculus*) is used both in agronomics and in research, genomic resources for this species are still limited and no microsatellite-based genetic map has been reported. Our aim was to construct a rabbit genetic map with cytogenetically mapped microsatellites so as to build an integrated genetic and cytogenetic map. A reference population of 187 rabbits comprising eight three-generation families with 10–25 offspring per family was produced. One hundred and ninety-four of 305 previously identified microsatellites were included in this study. Of these, 158 were polymorphic with two to seven alleles. The map reported here comprises 111 markers, including 104 INRA microsatellites, five microsatellites from another source and two phenotypic markers (*angora* and *albino*). Ninety markers were integrated into 20 linkage groups. The remaining 21 microsatellites mapped to separate linkage groups, 19 with a precise cytogenetic position and two with only a chromosomal assignment. The genetic map spans 2766.6 cM and covers 20 rabbit chromosomes, excluding chromosomes 20, 21 and X. The density of this map is limited, but we used it to verify the location of *angora* and *albino* on chromosomes 15q and 1q, respectively, in agreement with previously published data. This first generation genetic/cytogenetic map will help gene identification and quantitative trait loci mapping projects in rabbit.

Keywords *albino*, *angora*, chromosomes, cytogenetic map, genetic map, linkage, microsatellites, rabbit.

Introduction

The European rabbit (*Oryctolagus cuniculus*) is a valuable mammalian species to humans both as a domestic and wild animal (Lebas *et al.* 1997). Wild *O. cuniculus* is a popular game animal, especially in Europe, and many domestic *O. cuniculus* breeds and strains are raised commercially for meat, wool and fur and as pets. Rabbit breeding corresponds to a small but active economic sector; i.e. the worldwide production of meat reaches 1 million tons in carcass weight per year, with 50% produced in Europe and 30% in China (Magdelaine 2003). Rabbit skins and pelts, by-products of meat, are used as fur and for the manufacture of felt, with global production estimated at one billion per year (Lebas *et al.* 1997). In addition, the domestic rabbit is used as a laboratory animal and contributes greatly to biological and medical research. Given the importance of rabbits in many domains, it is surprising how slow the development of genomic resources has been for this species. A first step was taken in August 2004 with the announcement by the National Human Genome Research Institute (USA) of a program for the partial sequencing of the rabbit genome, together with that of eight other mammals (http://www.nih.gov/news/pr/aug2004/nhgri-04.htm). A genome sequence with threefold coverage is now available at

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(http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=geno meprj&cmd=Retrieve&dopt=Overview&list_uids=12818) and will help future mapping projects.

The genetic map is still very limited in this species, with only one partial map (Fox 1994; Korstanje *et al.* 2001, 2003) comprising 77 morphological, biochemical and molecular makers distributed in 15 linkage groups, among which only seven are anchored onto chromosomes. In addition, amplified fragment length polymorphism (AFLP) markers were used to identify a quantitative trait locus (QTL) for serum high density lipoprotein (HDL) cholesterol in rabbit (Van Haeringen *et al.* 2001) and subsequently to construct a genetic map based on 103 AFLP markers organized into 12 linkage groups (Van Haeringen *et al.* 2002).

In 2001, l'Institut National de la Recherche Agronomique (INRA, France) launched a rabbit genome mapping program for the future development of projects aimed at mapping QTL and identifying candidate genes for production traits. Our objective was to build an integrated cyto-genetic and genetic map based on microsatellites regularly distributed over the rabbit genome. First, a standardized rabbit R-banded karyotype was established for precise and consensus fluorescence *in situ* hybridization (FISH) mapping (Hayes *et al.* 2002), and then 257 genes were FISH mapped (Chantry-Darmon *et al.* 2003, 2005a) with rabbit bacterial artificial chromosome (BAC) clones from an INRA library (Rogel-Gaillard *et al.* 2001). These localizations confirmed and/or refined most of the human–rabbit comparative data based on reciprocal chromosome painting (Korstanje *et al.* 1999). In parallel, 305 microsatellite sequences were produced either from gene-containing BACs with a known cytogenetic location or from a randomly produced genomic plasmid library (Chantry-Darmon *et al.* 2005b). One hundred and eighty-three of these 305

microsatellites were cytogenetically anchored across all the rabbit chromosomes except chromosome 22. In the present work, we report the genetic characterization of these microsatellites, including their polymorphism level and linkage analysis in rabbit reference families produced especially for this project. Parental strains of rabbits carrying wild and mutant alleles for *angora* and *albino* were chosen to construct these families. This allowed us to validate the relevance of the resulting genetic map by mapping the *albino* and *angora* characters for which preliminary mapping information was available.

Materials and methods

Animal resources

Three rabbit INRA strains were used to build three-generation families (Fig. 1), namely INRA2066 (de Rochambeau 1998), Castor Orylag® (INRA, Paris, France) (Vrillon *et al.* 1998) and Laghmere® (INRA, Paris, France). Strain INRA2066 carries two copies of the wild-type allele at the *angora* locus (LL) and two copies of the recessive *himalayan* allele ($c^h c^h$) at the *albino* locus. The Orylag® strain is wild type both at the *angora* locus (LL) and at the *agouti* colour locus (CC), while the Laghmere® strain carries recessive alleles at these respective loci (ll) and (cc), where cc confers the *albino* colour (Fig. 1). Two crosses were carried out to produce the F1 generation: (i) eight INRA2066 males with 22 Laghmere® does and (ii) three Laghmere® males with 12 Castor Orylag® does. The 53 F1 does from the (i) cross were then mated with the 29 F1 males from the (ii) cross, which produced 853 F2 rabbits. The following parameters were registered for each F2 individual: sex, weight at 63 days, hair length, texture, diameter and colour. Four phenotypic traits segregated in the families: two hair texture
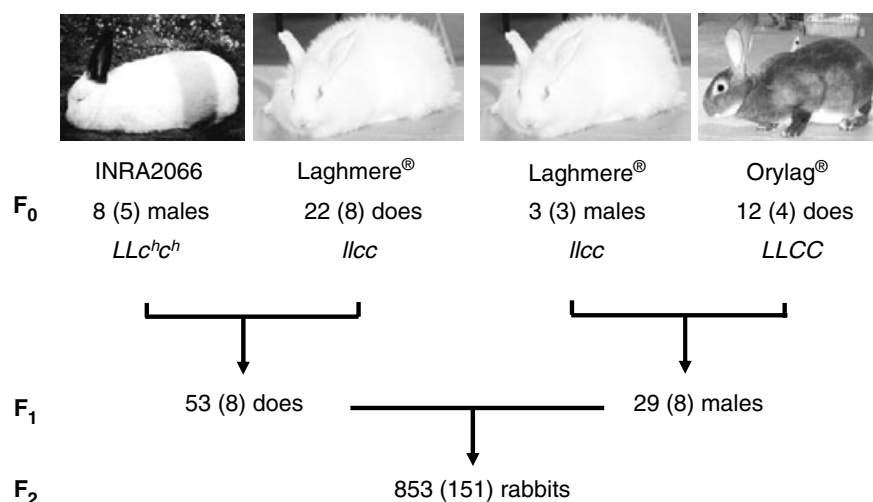

	INRA2066	Laghmere®	Laghmere®	Orylag®
F₀	8 (5) males	22 (8) does	3 (3) males	12 (4) does
	LLc^hc^h	$llcc$	$llcc$	$LLCC$

F₁   53 (8) does ———————— 29 (8) males

F₂   853 (151) rabbits

Figure 1 Structure of the reference rabbit families. Two phenotypic traits segregate in the families: *angora*, with wild-type (*L*) and mutated (*l*) alleles, and *albino*, with wild-type (*C*), *albino* (*c*) and *himalayan* (c^h) alleles. The total number of animals is indicated, and the number of animals selected for genotyping and linkage analysis is given in parentheses.

characters (i.e. *angora* and normal) and two colour characters (i.e. *albino* and *agouti*). The segregation patterns of wild type (*L*) and recessive (*l*) alleles at the *angora* locus and of three alleles (wild type, *C*; *albino*, *c*; *himalayan*, c^h) at the *albino* locus were observed to determine the genetic positions of these loci.

Microsatellite resources

From 305 initial sequences (AJ874368–AJ874672), a set of 194 microsatellites, of which 183 were cytogenetically mapped (Chantry-Darmon *et al.* 2005b), was tested in this study. They are referred to as INRA microsatellites. A second set of seven microsatellite sequences *D1Utr4*, *D3Utr2*, *D5Utr3*, *D6Utr4*, *D7Utr4*, *D12Utr2* and *D19Utr2*, assigned to chromosomes 1, 3, 5, 6, 7, 12 and 19 respectively (Korstanje *et al.* 2001, 2003), were also included and are referred to as Utrecht microsatellites.

Animal genotyping

DNA was extracted from peripheral blood mononuclear cells recovered from 8 ml of blood sampled on ethylenediamine-tetraacetic acid (EDTA) (K3 EDTA, Vacutainer®B-D; Becton Dickinson, Rutherford, NJ, USA) by cardiac puncture, using a protocol described by Jeanpierre (1987). A low-cost technique described by Schuelke (2000) was applied for the genotyping of microsatellites. The polymerase chain reaction (PCR) reaction was carried out with three primers, including a primer specific for the locus to be amplified and extended by a universal sequence of 17 nucleotides (5′-GACCGGCAGCAAAATTG-3′), a reverse primer specific for the locus and a universal primer of 17 nucleotides that was 5′-labelled by 6-Fam, Hex or Tet (MWG AG Biotech, Ebersberg, Germany). The PCR protocol has been provided as Appendix S1. The results were analysed with the Genetic Profiler v1.1 software (ABI Prism 377A sequencer; Applied Biosystems, Foster City, CA, USA) or the Genotyper software (MegaBACE 1000 sequencer; Amersham Biosciences, New Haven, CT, USA). Genotyping data were checked using an in-house program (A. Neau, personal communication), which analysed the consistency of the allele distribution of each marker according to pedigrees.

Linkage analyses

The CRI-MAP 2.4 software (Green *et al.* 1990) was used to build the genetic map. The first step identified linked markers by a two-point analysis with the two-point option. In the second step, the linkage groups were examined by multipoint analyses using the build and flipsn options. Linkage data were merged with cytogenetic mapping data to confirm the order of markers or to identify linkage of isolated or weakly informative markers. These various analyses were performed with a LOD score of 3 (lowered to

1.8 in a few cases where the cytogenetic position of the markers made it possible to confirm the link). The size of the genetic map was calculated by adding up the genetic distances of all linkage groups plus 15 cM at both ends of each group and including 15 cM on both sides of each unlinked marker. The heterozygosity of each marker was calculated in the F_0 population using the formula $1 - \Sigma_{i=1}^{n}(f_i)^2$, in which n is the allele number and f_i equalled the frequency of allele i (ranging from 1 to n).

Results and discussion

The objective was to construct a first-generation genetic map of the rabbit using markers at 10- to 20-cM intervals across the genome. Eight families with 10–25 offspring per family were selected among the three-generation rabbit families that were produced for the project (Fig. 1). From a total of 980 rabbits, 187 animals were genotyped, comprising 20 F_0, 16 F_1 and 151 F_2 individuals (Fig. 1). Among the 305 INRA microsatellites that were previously isolated in the laboratory, 194 microsatellites were successfully amplified by PCR with the primers already reported (Chantry-Darmon *et al.* 2005b). From this subset, 158 (81%) of the microsatellites were informative in the F_1 animals from the eight reference families (Table S1) with two to seven alleles (3.3 ± 1.0 on average). Among the seven Utrecht microsatellites reported by Korstanje *et al.* (2001, 2003), five (*D1Utr4*, *D3Utr2*, *D5Utr3*, *D6Utr4* and *D12Utr2*), mapping to OCU 1, 3, 5, 6 and 12 respectively were polymorphic with two to five alleles (Table S1). As already reported in rabbit by Queney *et al.* (2001), a small number of alleles was observed, but this may be due in part to the use of inbred rabbit strains for building the reference families. Heterozygosity was calculated for all the polymorphic markers using 20 F_0 animals, except for marker *INRACCDDV326*, which maps to OCUY (Table S2). This rate ranges from 0.17 (*INRACCDDV0101*, *INRACCDDV0202*, *INRACCDDV0292*) to 0.78 (*INRACCDDV0213*), with an average value close to 0.50. This heterozygosity rate agrees with the data reported for other mammals: 50% in cattle (Bishop *et al.* 1994), 55–80% in pig (Rohrer *et al.* 1994) and 63% in human (Hudson *et al.* 1992).

The 163 polymorphic microsatellite markers (158 INRA markers and five Utrecht markers) were genotyped in 187 animals of the reference population. Genotyping data were efficiently analysed for 109 microsatellite makers (Table S2). The genotyping data obtained for the remaining 54 polymorphic markers were difficult to interpret, probably due to the genotyping methodology used and to the standard cycling conditions applied to all the microsatellites. Polymorphic markers excluded from the map are listed in Table S1. These microsatellites might have been rescued either by changing PCR cycling and annealing temperature conditions with the three-primer technique or by using classical PCR conditions with one locus-specific

fluorescent primer. The resulting genetic map comprised 111 markers, including 109 microsatellites (104 INRA and five Utrecht markers) and the two phenotypic markers *angora* and *albino* (Table S2 and Fig. 2). The number of informative meioses varied from 24 to 260 (157 ± 63 on average) and from 0 to 250 for markers with known phase (94 ± 63 on average). Eighty-eight microsatellites and the two phenotypic markers were integrated into 20 linkage groups anchored to 17 chromosomes. The linkage groups were referred to as LG followed by the chromosome number and a lower case letter (Fig. 2 and Table S2). The size of the linkage groups ranged from 0 cM (2 markers) to 243.3 cM (11 markers) with an average of 24.9 cM. Nineteen polymorphic microsatellites with a cytogenetic localization were not included in any linkage group (Table S2 and Fig. 2). The two microsatellites *INRACCDDV0100* and *INRACCDDV0202* were not sufficiently informative to be mapped with certainty. Their most likely position is represented by a line drawn parallel to the linkage groups LG4b and LG13a respectively (Fig. 2). For each of the three chromosomes OCU4, OCU6 and OCU18, two overlapping linkage groups were unlinked (Fig. 2). Genotyping data analysis revealed that this was

due to microsatellites that were not informative in the same families in each linkage group.

Thirty-six microsatellites, which have been FISH mapped and are associated with an identified gene (Table S2 and Fig. 2; Chantry-Darmon *et al.* 2005b), provide a direct integration of genetic and cytogenetic data. The size of this genetic map was 2766.6 cM averaged across the male and female maps, which are 2942 and 2930 cM respectively. In order to integrate previously reported linkage groups (Fox 1994; Korstanje *et al.* 2001, 2003), we included seven Utrecht microsatellites assigned to OCU1 (*D1Utr4*), OCU3 (*D3Utr2* in LG XI), OCU5 (*D5Utr3*), OCU6 (*D6Utr4*), OCU7 (*D7Utr4*), OCU12 (*D12Utr2*) and OCU19 (*D19Utr2*) (Table S1 and Fig. 2). Only five were interpretable and polymorphic. The *D1Utr4* microsatellite allowed us to link LGI established by Fox (1994) and further mapped by Korstanje *et al.* (2001) on OCU1 (LG1a; Fig. 2). Similarly, *D3Utr2* links LGXI to LG3a on OCU3; *D5Utr3* links LGVI to LG5a on OCU5; *D6Utr4* links LG6a to a linkage group previously assigned to OCU6 (Korstanje *et al.* 2003) and *D12Utr2* anchors LGVII (Fox 1994) to LG12a on OCU12. *D6Utr4* was integrated in the linkage group LG6a, which is anchored at both ends on OCU6p and confirms the orientation
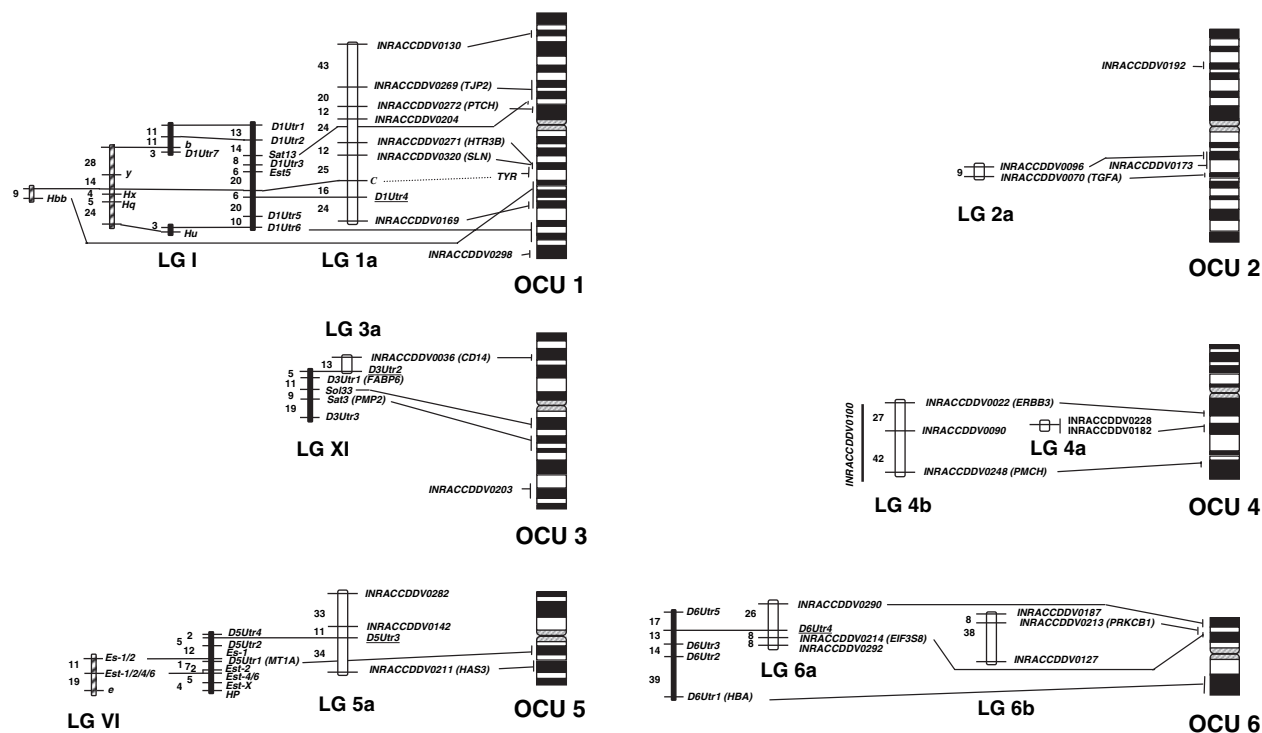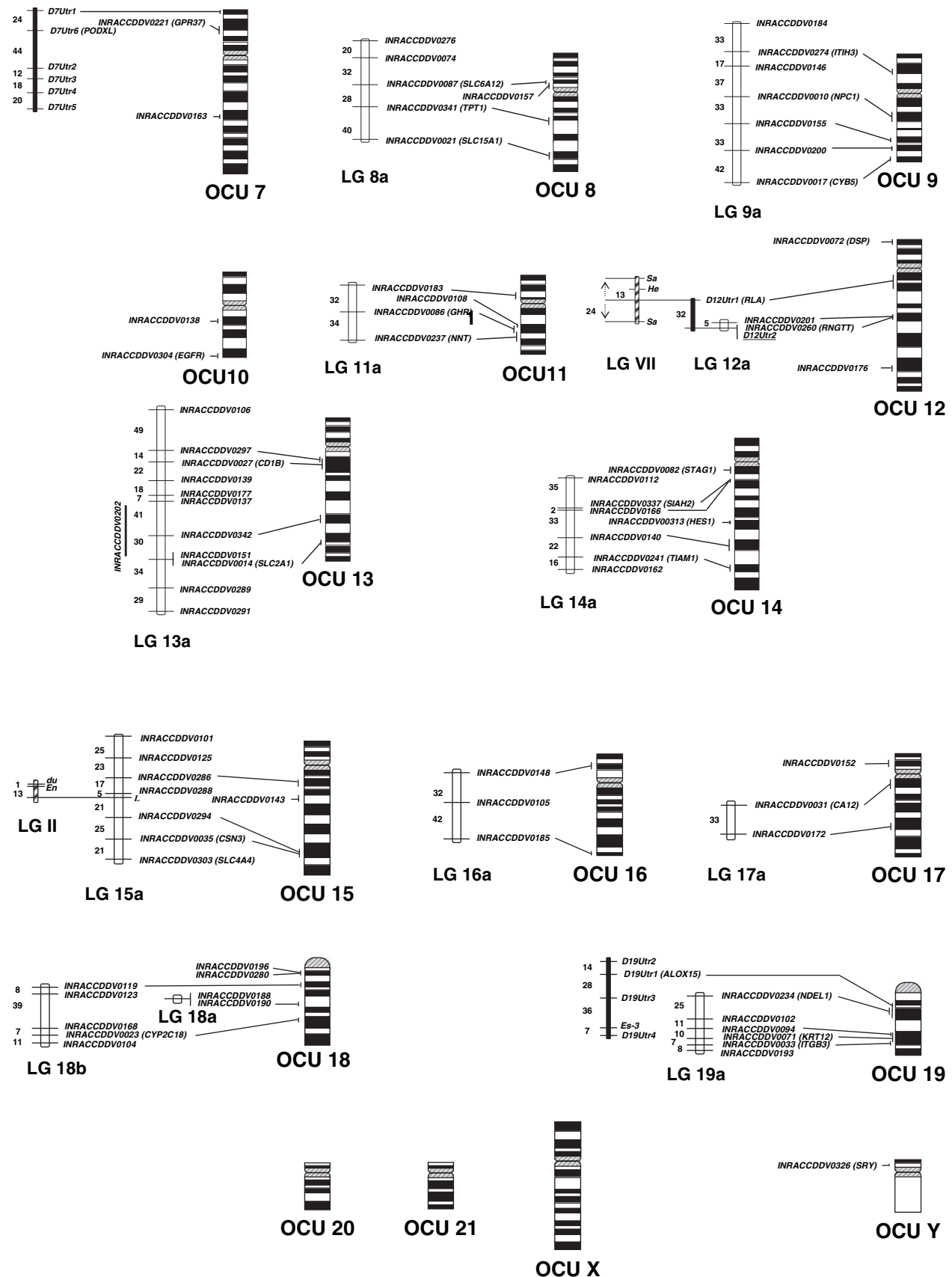

Figure 2 Rabbit integrated genetic and cytogenetic map, including anchorage of previously published linkage groups. The linkage groups are represented on the left side of each R-banded ideogram of rabbit chromosomes. Bars indicate the cytogenetic positions of microsatellites previously mapped by fluorescence *in situ* hybridization (Chantry-Darmon *et al.* 2005b). When an associated gene is known, it is given in italicized letters within parentheses. Genetic distances are estimated in Kosambi cM and mentioned on the left of the linkage groups. The new linkage groups have names referred to in Table S2 and are represented by open boxes. The linkage groups reviewed in Fox (1994) are represented as hatched boxes, and the linkage groups reported by Korstanje *et al.* (2001, 2003) are represented as large black bars. The two microsatellites *INRACCDDV0100* and *INRACCDDV0202*, which could not be precisely positioned in linkage groups, are written to the left of a bar representing the most likely position. The morphological characters *albino* (C) and *angora* (L) were mapped to linkage groups LG1a and LG15a respectively.

Figure 2 (continued)

of the linkage group reported by Korstanje *et al.* (2003). For chromosomes 7 and 19, the previously identified linkage groups could not be integrated in our genetic map but are represented in Fig. 2. The *angora* phenotypic marker made it possible to link LG15a to LGII (Fox 1994) on OCU15.

The *angora* character mapped 3 cM from IN-RACCDDV0288 by a two-point analysis (LOD = 7.63). Multipoint analysis revealed that the *angora* character belongs to the linkage group LG15a on OCU15 between microsatellites INRACCDDV0288 and INRACCDDV0294 at 4.8 and 20.7 cM respectively (Fig. 2 and Table S2). In mice, the *angora* phenotype is due to an autosomal recessive mutation in *FGF5* (Hebert *et al.* 1994). The *FGF5* gene is localized on human chromosome 4q21.21 (Ensembl Human Genome Browser 27.35a.1), which is conserved with rabbit chromosome 15q, according to the human–rabbit comparative map (Korstanje *et al.* 1999). Moreover, our results agree with those of Mulsant *et al.* (2004) revealing a tight genetic linkage between the *angora* character and the *FGF5* gene in rabbit, although no causative mutation has been identified.

The *albino* character mapped 14 cM from *D1Utr4* by two-point analysis (LOD = 18.62), and it belongs to the linkage group LG1a on OCU1, between microsatellites IN-RACCDDV0320 and *D1Utr4* at 25.2 and 16 cM respectively. The linkage analyses reported here provide a genetic linkage with LGI, which contains the *haemoglobin beta chain* (*Hbb*) locus (Fig. 2). Mutations of the *tyrosinase* (*TYR*) gene are responsible for the *albino* phenotype in mouse (Beermann *et al.* 1990), human, rat, cow, dog and chicken (OMIM #203100). In addition, the rabbit *TYR* gene has been mapped previously by FISH on OCU1q14–q15 (Chantry-Darmon *et al.* 2005a) and the *Hbb* locus has been mapped on OCU1q14–q21 (Xu & Hardison 1989). These cytogenetic data agree with the genetic linkage analyses (Fig. 2), and our results confirm a previous report demonstrating genetic linkage between *albino* and *Hbb* with an estimated map distance of 9 ± 6 cM (Sandberg & Andersson 1987). The *TYR* gene variants are probably involved in fur pigmentation of rabbit (Aigner *et al.* 1996, 2000), and indirect experiments strongly suggest that the *TYR* gene variants are responsible for the *albino* phenotype because albinism was rescued in transgenic rabbits by introducing a yeast artificial chromosome containing the mouse *tyrosinase* gene by pronuclear injection (Brem *et al.* 1996).

We report a first-generation cytogenetically anchored genetic map based on microsatellites for the rabbit. We have applied a technique using locus-specific primers and three fluorescently labelled universal primers (Schuelke 2000). This technique was successful on 64% of the markers, allowing us to test a large number of markers and to construct a low-cost genetic map. The reservoir of published microsatellites (Chantry-Darmon *et al.* 2005b), together with the rabbit whole-genome sequence corresponding to a threefold coverage, will help to increase map density and to provide markers for still-uncovered genomic segments. In addition, because many rabbit microsatellite sequences have hits on the rabbit sequence traces that are released in public databases (data not shown), it is anticipated that our work will help to anchor sequence traces onto the rabbit genome.

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Supplementary Material

The following supplementary material is available online at http://www.blackwell-synergy.com:

Table S1 Polymorphism of rabbit microsatellites.

Table S2 Summary of the data associated with microsatellites integrated in the genetic map.

Appendix S1 Genotyping PCR conditions.