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Integration of chicken genomic resources to enable whole-genome sequencing

J. Aerts,[a] R. Crooijmans,[a] S. Cornelissen,[a] K. Hemmatian,[a] T. Veenendaal,[a] A. Jaadar,[a] J. van der Poel,[a] V. Fillon,[b] A. Vignal[b] and M. Groenen[a]

[a] Animal Breeding and Genetics Group, Wageningen University, Wageningen (The Netherlands);
[b] Laboratoire de Génétique Cellulaire, INRA, Toulouse (France)

Abstract. Different genomic resources in chicken were integrated through the Wageningen chicken BAC library. First, a BAC anchor map was created by screening this library with two sets of markers: microsatellite markers from the consensus linkage map and markers created from BAC end sequencing in chromosome walking experiments. Second, *Hin*dIII digestion fingerprints were created for all BACs of the Wageningen chicken BAC library. Third, cytogenetic positions of BACs were assigned by FISH. These integrated resources will facilitate further chromosome-walking experiments and whole-genome sequencing.

Chicken (*Gallus gallus*, GGA) has a long history as a model organism for developmental biology, immunology and microbiology in vertebrates (Brown et al., 2003). In recent years, much effort has been made to create different genome mapping resources in this animal. A standardized karyotype of chicken was published by the International System for Standardized Avian Karyotypes in 1999 (Ladjali-Mohammedi et al., 1999). The detailed consensus linkage map of the chicken genome provides a large set of markers (n = 2,012; Groenen and Crooijmans, 2003), approximately one every 2 cM, that can be used in QTL studies for either whole-genome or regional scans. Schmid et al. (2000) published a first rough outline of chicken-human and chicken-mouse comparative maps. The comparative map provides an efficient way to identify relevant genes in livestock, based on the mapping information of species with more detailed maps such as human and mouse. However, the resolution of these comparative maps in chicken remains low because of a high number of inter- and intrachromosomal rearrangements between chicken and mammals (Crooijmans et al., 2001). Recently, Morisson et al. (2002) have created ChickRH6, a chicken whole-genome radiation hybrid panel that consists of 90 hybrid clones. This panel makes it possible to map markers by simple PCR, avoiding development of polymorphic markers as is required for genetic mapping. Finally, a detailed physical map – based on overlapping large insert clones such as bacterial artificial chromosomes (BACs) – is necessary to facilitate whole-genome sequencing (Gregory et al., 2002). The physical map is also an important resource for fluorescent in situ hybridization (FISH) studies. Although chromosome-walking experiments have resulted in parts of the physical maps for chicken chromosomes 8, 10, 13, 15, 24 and 28 (Crooijmans et al., 2001; Buitenhuis et al., 2002; Jennen et al., 2002), most chromosomal regions in chicken lack identified BAC clones.

Integration of the above resources is necessary to facilitate whole-genome sequencing of chicken, planned in the second

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Request reprints from Jan Aerts, Animal Breeding and Genetics Group
Wageningen University, Wageningen (The Netherlands)
telephone: +31 317 483397; fax: +31 317 483929
e-mail: jan.aerts@wur.nl

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and third quarter of 2003. The availability of the chicken DNA sequence will not only boost research in birds, but will also aid in the further detailed annotation of the human genome sequence. Human-mouse sequence comparisons using for example PipMaker (Frazer et al. 2003) show high similarity not only for coding and regulatory regions, but also for large parts of the "junk"-DNA. In contrast, preliminary results show that the evolutionary distance between human and chicken offers a very good signal-to-noise ratio in large-scale sequence comparisons for confirmation or negation of hypothetical genes, to highlight novel genes and for the identification of regulatory elements.

The research presented in this paper lays the foundation for the integration of different genomic resources in chicken through the Wageningen BAC library. First, a BAC anchor map is created to link BACs to the genetic map. Second, the complete BAC library is fingerprinted by *Hin*dIII digestion to be included in contig building to create the complete genome physical map. Third, using FISH mapping, cytogenetic positions are assigned to specific BACs. Fourth, BAC end sequencing is in progress to allow anchoring of shotgun-sequencing contigs to the physical map of chicken.

Materials and methods

BAC library

This project used the Wageningen chicken BAC library, consisting of 50,208 BAC clones (Crooijmans et al., 2000). The clones have a reported average insert size of 134 kb, representing a 5.6× coverage of the chicken genome.

The BAC library is stored in 130 384-well plates. Row-, column- and platepools are created for each plate to enable PCR screening of the library.

BAC anchor map

The BAC library was screened with two sets of markers: STS (Sequence Tagged Site) markers located on the chicken linkage map and STSs created in chromosome walking experiments.

The first set was based on the extensive chicken consensus linkage map as published by Groenen et al. (2000) and updated by Schmid et al. (2000). The total number of microsatellite markers on this map is 1,255. For 37 of these, at least one positive BAC was already identified in previous work (Crooijmans et al., 2001; Buitenhuis et al., 2002; Jennen et al., 2002, in review). The remaining 1,218 microsatellite markers were used for an exhaustive screening of the Wageningen BAC library by PCR.

The second set of markers consisted of 853 STSs generated by BAC end sequencing in chromosome walking experiments. The genetic position of these markers is known indirectly, because walking experiments started with markers on the genetic map.

A two-dimensional screening was performed, as described by Crooijmans et al. (2000). In a first step, the plate pools were screened to identify the plates that are positive for the marker. In a second step, the row- and column-pools of these plates were screened to find the coordinates of the positive BAC clone. If this resulted in spurious or ambiguous results (i.e. weak signal or multiple positive rows/columns), the single BAC clone was tested. To obtain single BAC clone DNA, clones were grown overnight in LB medium with 12.5 mg/ml chloramphenicol at 37 °C. 5 µl cell suspension was diluted with 95 µl ddH_2O. DNA was obtained by lysis at 95 °C for 10 min, centrifugation at 1200 *g* for 3 min and discarding of the pellet.

Standard PCR techniques were used to find positive BACs for each marker. PCR volumes were 10 µl and reactions contained 5 pg/µl DNA, 0.195 µM of each primer, 0.14 U/µl Taq (Silverstar, Eurogentec, Belgium), 1.071 mM TMACl (tetramethylammoniumchloride), 0.186 mM dNTP's, 2.15% DMSO and 1× PCR buffer (1× PCR buffer contained 10 mM Tris-HCl pH9.0, 1.5 mM $MgCl_2 \cdot 6H_2O$, 50 mM KCl, 0.01% (*w/v*) gelatin and 0.1% Triton X-100). PCR conditions were 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 4 min. If necessary, the annealing temperature was increased to 50 °C or 55 °C to decrease aspecific binding of the PCR primers. Products were separated using standard agarose gel electrophoresis (1.5% multi-purpose agarose, 0.5× TBE buffer, 45 min, 120 V). In case of fragments smaller than 100 bp, a 4% nussieve agarose gel was used instead of the standard agarose gel.

For BACs identified in chromosome walking, BAC ends were sequenced and new PCR primers were developed on these sequences as described by Crooijmans et al. (2001).

BAC fingerprinting and contig building

Preparation of DNA, restriction endonuclease digestion with *Hin*dIII, agarose gel electrophoresis and data acquisition (using the Image program; http://www.sanger.ac.uk/Software/Image/) were adapted from Marra et al. (1997). DNA preparation was performed using polystyrene "Uni-Filter 800" receiver plates (Polyfiltronics). DNA was resolved in 20 µl of $T_5E_{0.1}$. Individual digestion brews contained 0.5 µl ddH_2O, 1 µl buffer "R+", 0.5 µl *Hin*dIII (40 U/µl) and 8 µl BAC DNA. The size standard marker for gel electrophoresis consisted of 46.6 µl Orange G, 6.0 µl Fermentas marker II, 0.8 µl Boehringer marker V and 223.2 µl TE buffer. Gels were scanned on a BioRad FX scanner.

Contig building based on the fingerprints was performed using the FPC program (http://www.sanger.co.uk/Software/fpc/v6; Soderlund et al., 2000). All marker and BAC data from the BAC anchor map were loaded into the FPC database. Based on preliminary experiments, tolerance was set to 4 and flagged as variable. FPC was run three times with cutoff values $10e^{-10}$, $10e^{-12}$ and $10e^{-14}$. The DQ-er was run for contigs with two or more Q-clones. Manual editing was not performed.

Perl objects were developed to facilitate descriptive analysis of the FPC results and comparison of the outcomes using the three different cutoff values.

Full-length BAC sequences for length comparison in Table 2 were downloaded from the Comparative Vertebrate Sequencing website from the NIH Intramural Sequencing Centre website (http://www.nisc.nih.gov) on April 29, 2003.

Fluorescent in situ hybridization

Metaphase spreads were obtained from 9-day old embryo fibroblast cultures, synchronized with 0.06 µg/ml colcemid (Gibco BRL) and fixed by standard procedures.

Two-colour FISH for six labeled probes (see Table 4) was performed according to Morisson et al. (1998).

Results

BAC anchor map

Genetic positions of BACs were determined by PCR screening of the BAC library for two sets of DNA markers. The first set consisted of microsatellite markers with known location on the consensus linkage map. The second set consisted of sequence tagged sites (STSs) generated by BAC end sequencing in chromosome walking experiments (Crooijmans et al. 2001; Buitenhuis et al. 2002; Jennen et al. 2002, in review). The genetic position of set 2-markers is known indirectly, because walking experiments initiated at markers mapped to the genetic map.

Figure 1 provides an overview of the genetic positions of the BAC anchors; i.e. markers that directly link one or more BACs to the linkage map.

BAC anchors could be identified for 34 linkage groups. These linkage groups represent all of the macrochromosomes (GGA1–GGA8 and GGAZ), several microchromosomes (GGA9–GGA20, GGA23, GGA24 and GGA26–GGA28) and ten smaller linkage groups. The exact number of microchro-

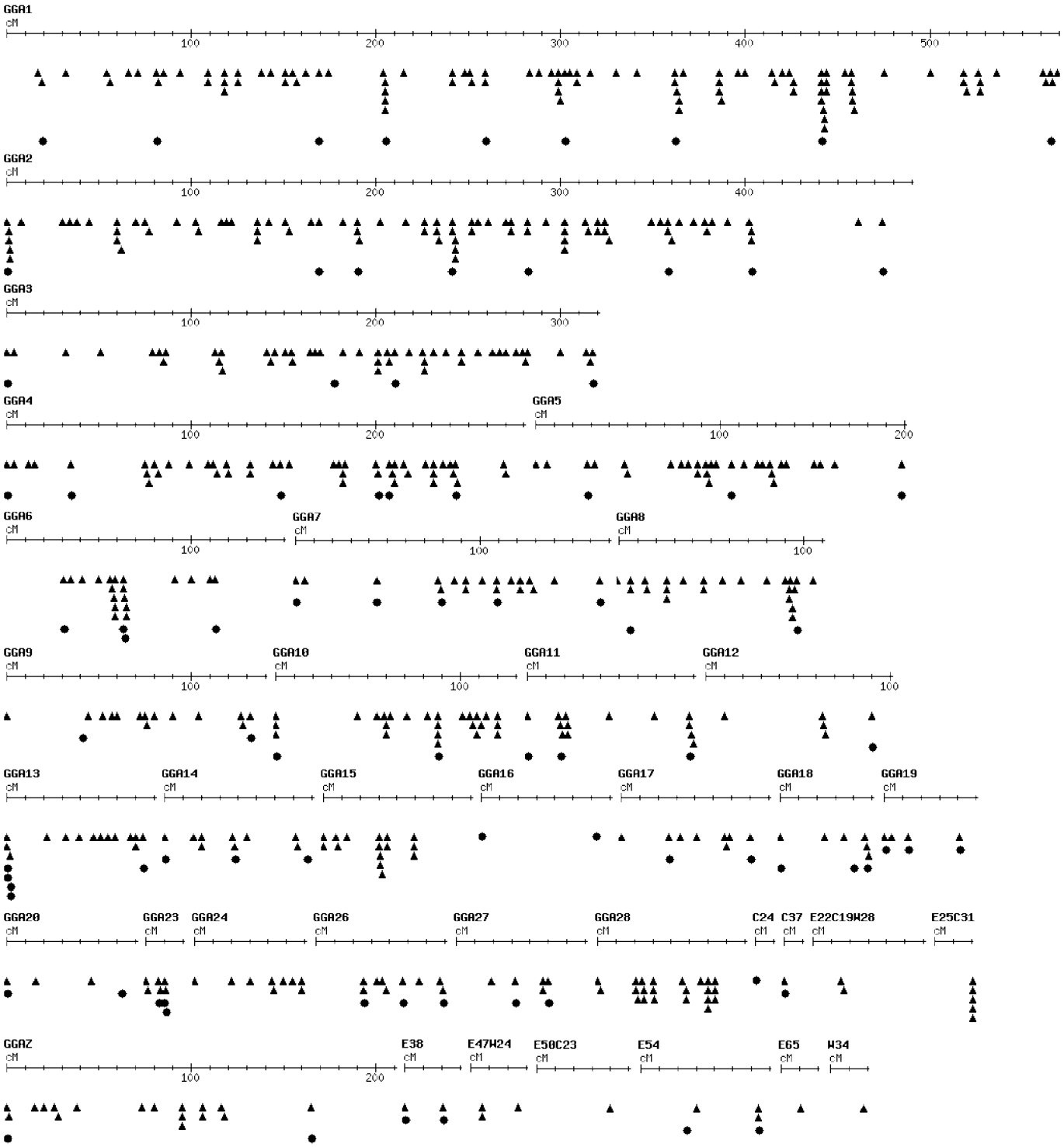

Fig. 1. Overview of the genetic positions of the BAC anchors for the first set (i.e. markers with a known location on the genetic map; triangles). Dots represent markers for which the cytogenetic position has been assessed. The ticks on the axis for each chromosome/linkage group represent 10 cM-steps.

mosomes covered is not yet known exactly because some of the microchromosomes might be represented by more than one linkage group.

An overview of the anchoring results is summarized in Table 1. In total, 1,522 markers identified 2,983 distinct BACs. The average amount of positive BACs per marker was 3.8. For set 1, we were unable to identify a BAC for 205 markers. Eighty-two percent of these could be attributed to markers that were not optimized for PCR screening (e.g. negative genomic control or amplification of aspecific bands; data not shown).

Table 1. General overview of mapping results. A distinction is made between markers from set 1 and set 2 (i.e. markers from the genetic map and markers from BAC end sequencing, respectively).

	No. of markers	No. of marker-BAC pairs	No. of distinct BACs
set 1	652	1,791	1,569
set 2	870	3,960	1,857
total	1,522	5,751	2,983

Table 2. Comparison of BAC lengths, as calculated by fingerprinting and sequencing

BAC	Length by fingerprinting (bp)	Length by sequencing (bp)	Difference (bp)
WAG-38H9	71,996	89,724	-17,728
WAG-55C14	87,671	55,589	32,082
WAG-65N20	94,593	109,569	-14,976
WAG-68G2	131,268	110,417	20,851
WAG-69H2	116,545	135,273	-18,728
WAG-71G10	104,076	120,464	-16,388
WAG-77D19	108,195	144,092	-35,897
WAG-93J15	115,703	136,822	-21,119
WAG-100N11	129,663	144,369	-14,706
WAG-105M15	81,541	98,793	-17,252
WAG-126P17	103,956	129,285	-25,329

Table 3. Summarized results of contig building using FPC for three different stringencies (with $10e^{-14}$ most stringent). Anchored contigs are contigs that contain at least one BAC from the BAC anchor map.

	Cutoff		
	$10e^{-10}$	$10e^{-12}$	$10e^{-14}$
No. of contigs			
anchored	609	597	521
not anchored	4,598	6,012	6,457
No. of contigs with > 10 BACs			
anchored	286	145	68
not anchored	845	639	334
No. of BACs			
in anchored contigs	9,256	4,968	2,991
in non-anchored contigs	32,615	31,357	26,387
as singeltons	7,419	12,965	19,912

A comprehensive list of marker-BAC pairs can be found as supplemental material at the journal's website (Supplemental Table 1, www.karger.com/doi/10.1159/000075766).

BAC fingerprinting and contig building

*Hin*dIII digestion of all 50,208 BACs of the Wageningen BAC library resulted in 49,290 high-quality fingerprints (98.2%). The average number of bands per BAC was 21 ± 5; average size of a band was 4241 ± 2873 bp. In consequence, the average BAC size was 89 ± 20 kb. The BAC sizes for 11 BACs were compared to BAC full-length sequences that are already available. On average, the BAC length based on the sequence was 12 ± 20 kb larger than the length as calculated from the BAC fingerprints. Length data are presented in Table 2.

The FPC program was used to build contigs based on these fingerprints. Manual editing was not performed. Results of the automated contig assembly are summarized in Table 3.

A full list of BACs and their assigned contigs is available at the journal's website (Supplemental Table 2). For each BAC, this list gives the contig names with FPC cutoff values $10e^{-10}$, $10e^{-12}$ and $10e^{-14}$. The less stringent cutoff value $10e^{-10}$ results in large contigs with a higher chance for misassembly. These large contigs can be split with higher cutoff stringency.

Using an in-house developed Perl script, the accuracy of each automatically assembled contig could be visualized. Figure 2 shows an overview of a contig of the $10e^{-10}$ FPC assembly. For each BAC on the x-axis, the y-axis shows the most stringent threshold (of the FPC cutoff values $10e^{-10}$, $10e^{-12}$ and $10e^{-14}$) at which that BAC is likely to overlap with the BAC at its left.

Fluorescent in situ hybridization

The BAC anchor map serves as a starting point for cytogenetic mapping. Fillon et al. (in preparation) used these BACs to map genetic positions to cytogenetic positions. In Fig. 1, these BACs are indicated by dots. A complete list is provided as supplemental material.

The cytogenetic position of six microsatellite markers on the genetic map of chromosome 4 was verified using positive BACs as probes for two-colour "caterpillar" FISH, i.e. WAG-112C24, WAG-125P16, WAG-118M14, WAG-33G16, WAG-12C6 and WAG-37E19 (see Fig. 3). All six probes could be clearly identified on the chicken chromosome spread and were located in the same order as on the genetic map.

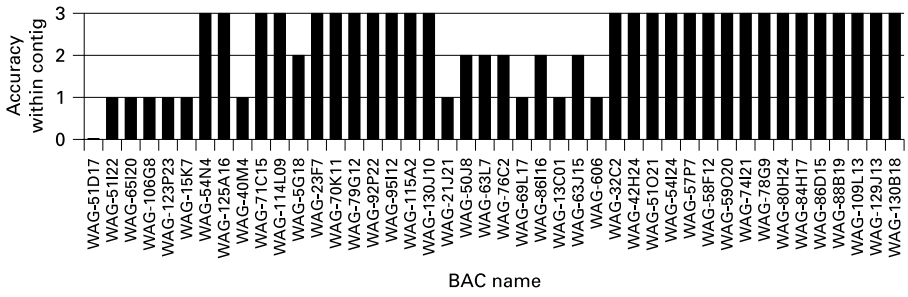

Fig. 2. Accuracy of a contig of the $10e^{-10}$ cutoff FPC assembly. For each BAC clone on the x-axis, the highest FPC cutoff stringency is given at which that clone is calculated to overlap with the clone at its left (1 = $10e^{-10}$, 2 = $10e^{-12}$, 3 = $10e^{-14}$). This figure does not reflect actual clone order within the contig.

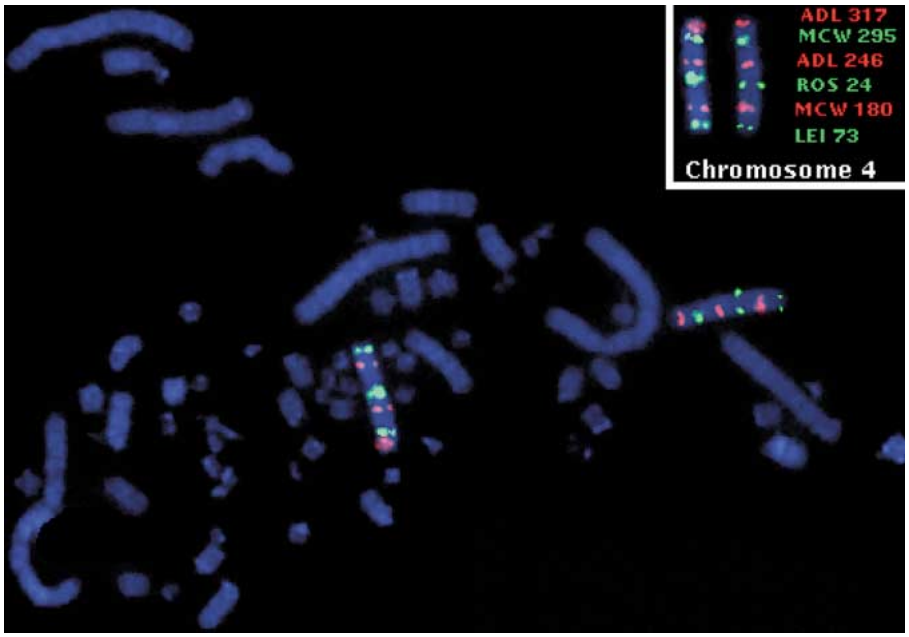

Fig. 3. Assessment of cytogenetic position of six microsatellite markers on chicken chromosome 4. Probes used were BACs positive for the markers shown in the upper right corner of the figure.

Table 4. BACs used as probes for two-colour FISH on GGA chromosome 4. For chromosomal position and genetic marker, the BAC that is used as a probe is specified.

Position (cM)	Marker	BAC
12	ADL0317	WAG-112C24
75	MCW0295	WAG-125P16
112	ADL0246	WAG-118M14
128	ROS0024	WAG-33G16
207	MCW0180	WAG-12C6
243	LEI0073	WAG-37E19

Discussion

BAC anchor map

Figure 1 shows that the BAC anchor map provides a broad coverage of the chicken genome. The BAC anchor map alone already covers about 8% of the chicken whole-genome physical map; markers of set 1 can account for 5.7%. On average, a BAC anchor is identified every 6.5 cM. The largest gap in the BAC anchor map is 58 cM and located on the q-arm of chromosome 2. The marker density of the genetic map of some of the microchromosomes is too low to enable building BAC anchor maps for these chromosomes (e.g. E57, E58, WAU31 and WAU32).

Multiple reasons exist for the fact that several markers could not identify a positive BAC. First, coverage of 5.6 means that approximately 99.2% of the genome is represented (Crooijmans et al., 2000). Therefore, markers in the remaining 0.8% will not identify any clones. Second, the markers were developed for fluorescent genotyping of large populations for linkage and QTL studies. Using fluorescent dyes, a small quantity of amplified DNA is sufficient to be detected. In several cases, the quantity of the complete amplification product of a marker is still too low to be visible in an agarose gel screening system. In addition, many markers amplified too many aspecific bands, which made detection of the right PCR band impossible, or amplified products smaller than 100 bp. These small fragments are difficult to call on a standard 1.5% agarose gel because of interference with primer-dimer bands. In case of spurious (e.g. many aspecific bands) or ambiguous results, the BAC was considered negative.

The average number of BACs per marker in set 2 is higher than in set 1, because markers of set 2 were developed specifically for testing on agarose gels and not for fluorescent genotyping. Furthermore, spurious results after agarose gel interpretation for set 1 were discarded by definition, while those for set 2 could often be resolved by incorporating other data from chromosome walking experiments.

The BAC anchor map allows for integration and quality control of the genetic and physical maps. In total, 184 of the 1,569 distinct BACs of set 1 in Table 1 were identified by more than one marker. Using this information, it is possible to assess the quality of the consensus genetic map. We identified six locations on the genetic map where markers that are several cM apart map to the same clone (i.e. WAG-11C21, WAG-32A2, WAG-41H14, WAG-54F10, WAG-83J16 and WAG-90M16). The BAC WAG-90M16, for example, is positive for markers ALD0105, MCW0271, ROS0075 and MCW0351, even though markers ROS0075 and MCW0351 are 9 cM apart (94 cM and 105 cM on GGA8, respectively). This can be caused either by a recombination hotspot between 94 cM and 105 cM on this chromosome, or by an error in the genetic map. First, genotyping errors result in erroneous location of a genetic marker. Second, some markers have a low number of informative meioses

or do not segregate in all 12 families used to construct the chicken consensus linkage map.

BAC fingerprinting and contig building

Based on the fingerprinting results, we found an average BAC insert size of 89 kb, which is smaller than the estimated 134 kb as published by Crooijmans et al. (2000) by pulsed-field gel electrophoresis (PFGE). Several reasons exist for this difference. First, the size as calculated by fingerprinting is an underestimation. Small digestion products are difficult to identify in the Image program and were not called. Second, it is often difficult to detect co-migration of two or more digestion fragments. A third important reason is the inherently rough nature of fragment size estimation by PFGE. This can be attributed to the large difference in concentration between BAC and marker DNA, the fact that each band appears as a smear and, most important, the non-linear dependency of migration speed on fragment size.

To further investigate the difference in average BAC size, we compared BACs that have already been sequenced with our fingerprinting data. A trifold comparison between sequence, fingerprints and PFGE data for the same BACs was not possible, because the PFGE length assignments by Crooijmans et al. (2000) were performed on anonymous BACs before picking the BAC clones eventually making up the BAC library. On average, the sequenced lengths were larger by a factor of 1.11 than the lengths as calculated after fingerprinting. This confirms the systematic underestimation also found by Le Hellard et al. (2001). However, for clones WAG-55C14 and WAG-68G2, the sizes as calculated based on restriction digestion fragment sizes are larger than the sizes as calculated by sequencing. For WAG-68G2, the fingerprint pattern is questionable, as it does not show the characteristic decrease in intensity with smaller fragment size. Interestingly, for WAG-55C14, in silico *Hin*dIII digestion of the nucleotide sequence showed that fragment sizes were all smaller with a factor 1.10 to 1.25 compared to the fragments read from the agarose gel. Re-evaluation of the gel pattern of this clone and its adjacent markers confirmed consistent gel migration and correct band calling.

Based on the fingerprints and the average underestimation by a factor 1.1 compared to the sequenced clones, our estimate for average insert size of the Wageningen BAC library is 100 kb.

As the coverage of the BAC library is too low to create large contigs, manual editing of the automated contig assembly by FPC was not performed. To enable contig assembly by FPC, either the resolution of the BAC library has to be increased by using additional enzymes (Tao et al., 2001), or the genome coverage (i.e. the number of BACs that are fingerprinted) has to be increased. Therefore, fingerprints are merged with fingerprint data of other chicken BAC libraries derived from Red Jungle Fowl that have been created by Washington University. The library collection consists of the Michigan State University TAM31 *Bam*HI, TAM32 *Eco*RI and TAM33 *Hin*dIII libraries and the CH261 *Eco*RI library (Children's Hospital Oakland Research Institute). Contig building and manual editing of the combined fingerprinting data is currently in progress at Washington University. Combining chromosome-walking data with the fingerprint contigs acts as a quality check for the Wageningen fingerprints. Preliminary results show that clones that overlap based on chromosome walking experiments also overlap based on fingerprints.

The fingerprint data and contigs built by FPC on the Wageningen data speed up ongoing chromosome walking in two ways. First, because fingerprint fragment sizes are known for each BAC, the largest BAC can be selected for BAC end sequencing from a list of overlapping BACs. Second, finding a positive BAC for a BAC-end marker generally involves three steps: identifying a positive plate pool, identifying the positive row- and column-pools, and checking the individual BAC clone. Using results from FPC, and representations as in Fig. 2, it is often possible to skip the second step and check the individual BAC directly (data not shown). Marra et al. (1999) showed that the FPC program does a good job in putting overlapping BACs in the same contig, but their order within the contig has to be corrected manually. Therefore, as no manual editing was performed, Fig. 2 reflects the possible relationships between clones, but not the clone order within the contig. The example in Fig. 2 clearly shows that at a stringency level of $10e^{-10}$ the 44 BACs in the figure are within a single contig. Increasing the stringency to $10e^{-12}$ results in breaking up the contig into six smaller contigs and five unlinked BACs. When stringency is increased even more to $10e^{-14}$, 31 BACs remain grouped in four contigs and 13 BACs become singletons. A program (named coral) is developed at the University of Vancouver to recalculate the actual clone order. This program was not yet available at the time of our analysis.

Fluorescent in situ hybridization

The BAC resource described in this paper has already proven to be of high importance for the integration of the linkage and cytogenetic maps (Schmid et al., 2000; Fillon et al. 2003). As a further illustration of the strength and possible applications for this BAC resource, we performed a "caterpillar" FISH as shown in Fig. 3. The FISH experiment shows clearly that the data presented in this paper is an interesting resource for FISH mapping. Using this technology, both chromosomal location and orientation of the integrated genetic, physical and cytogenetic maps can be identified. By comparing the position of the hybridization signal of probes at the end of the genetic map, relative to the telomeric ends, the distance between the ends of the genetic map and the physical map can be assessed. Chromosome numbers and rearrangements can be accurately defined. Furthermore, although most chicken genetic markers cannot be used in other bird species, the chicken BAC clones are excellent probes in cross-species hybridization in other birds, for example FISH in golden pheasant, quail and turkey (Schmid et al., 2000).

In conclusion, our research allows for integration of multiple genomic resources in chicken, i.e. genetic, physical, cytogenetic and sequence maps. This integration is a prerequisite for whole-genome sequencing, which is currently in progress. The BAC anchor map and *Hin*dIII digestion fingerprints allow for chromosomal positioning of clone contigs in the construction of the whole-genome physical map. FISH mapping using BACs of the BAC anchor map integrates the physical and cyto-

genetic maps. The BAC-end sequences will align shotgun-sequencing contigs to the physical map. These integrated resources will be valuable tools in genomic research before and after publication of the full chicken DNA sequence.

The availability of the chicken DNA sequence will not only boost research in birds, but will also aid in the further annotation of the genomes of other species, in particular those of man and mouse. The easy access of the chicken embryo in combination with the availability of a full set of molecular resources (ESTs, BACs, genome sequence) and a way of switching easily between different maps (i.e. the BAC anchor map) will also boost the use of chicken as a model species in developmental biology.

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