A Comprehensive Expressed Sequence Tag Linkage Map for Tiger Salamander and Mexican Axolotl: Enabling Gene Mapping and Comparative Genomics in Ambystoma

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ABSTRACT

Expressed sequence tag (EST) markers were developed for *Ambystoma tigrinum tigrinum* (Eastern tiger salamander) and for *A. mexicanum* (Mexican axolotl) to generate the first comprehensive linkage map for these model amphibians. We identified 14 large linkage groups (125.5–836.7 cM) that presumably correspond to the 14 haploid chromosomes in the Ambystoma genome. The extent of genome coverage for these linkage groups is apparently high because the total map size (5251 cM) falls within the range of theoretical estimates and is consistent with independent empirical estimates. Unlike most vertebrate species, linkage map size in Ambystoma is not strongly correlated with chromosome arm number. Presumably, the large physical genome size (~30 Gbp) is a major determinant of map size in Ambystoma. To demonstrate the utility of this resource, we mapped the position of two historically significant *A. mexicanum* mutants, *white* and *melanoid*, and also *met*, a quantitative trait locus (QTL) that contributes to variation in metamorphic timing. This new collection of EST-based PCR markers will better enable the Ambystoma system by facilitating development of new molecular probes, and the linkage map will allow comparative studies of this important vertebrate group.

THE tiger salamander (*Ambystoma tigrinum*) species complex consists of several closely related and phenotypically diverse taxa that range from central Mexico to southern Canada (SHAFFER and McKNIGHT 1996). The complex as a whole is an important, naturalistic model system because taxa are characterized by extensive interspecific and intraspecific variation for a number of ecologically important traits, including expression of metamorphic *vs.* nonmetamorphic (paedomorphic) life histories (GOULD 1977; SHAFFER and VOSS 1996), timing of metamorphosis (ROSE and ARMENTROUT 1976; VOSS and SMITH 2005), cannibal *vs.* normal larval morphologies (POWERS 1907; HOFFMAN and PFENNIG 1999), infectious disease (COLLINS *et al.* 2004), variation in adult coloration and pigment patterning (REESE 1969; PARICHY 1996, 1998), and variation in general morphology (SHAFFER 1984; IRSCHICK and SHAFFER 1997). In addition, these salamanders are important laboratory models for olfaction (MARCHAND *et al.* 2004; PARK *et al.* 2004), vision (THORESON *et al.* 2004; CHICHILNISKY and REIKE 2005), cardiogenesis (DENZ *et al.* 2004; ZHANG *et al.* 2004), embryogenesis (BACHVAROVA *et al.* 2004; ERICSSON *et al.* 2004), and postembryonic development (PARICHY 1998; VOSS and SMITH 2005), including organ and tissue

regeneration (CHRISTENSEN *et al.* 2002; SCHNAPP and TANAKA 2005). Both natural and laboratory-based research areas are in need of a comprehensive genome map that can be used to identify the position and effect of loci that contribute to phenotypic variation and that can be used to compare features of the salamander genome to other vertebrates. Moreover, molecular markers that are used to develop linkage maps provide the material for generating nucleotide probes and sequences that can be used in a variety of ways, including *in situ* hybridization, population genetics, systematics, molecular evolution, and functional genomics.

Several lines of evidence suggest that meiotic or recombinational map size is large in tiger salamanders. The earliest mapping studies in *A. mexicanum* employed tetrad analysis and gynogenic salamanders for estimating genetic distance between a few phenotypic markers and their centromeres (*e.g.*, three in LINDSLEY *et al.* 1955 and seven in ARMSTRONG 1984). Most of these phenotypes were mapped to positions distant from centromeres (unlinked); this suggests a large recombinational map size for Ambystoma relative to other vertebrates where the majority of loci typically show linkage with the centromere (BROWN *et al.* 1998; STEEN *et al.* 1999; GROENEN *et al.* 2000; KELLY *et al.* 2000; KONG *et al.* 2002). Using a more direct method, CALLAN (1966) reported chiasmata counts from *A. mexicanum* oocyte nuclei ranging from 101 to 126, averaging 113 chiasmata per

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TABLE 1

Relationships between genome size and map size in vertebrates

Organism	Genome size (Gbp)[a]	N^b	Chromosomal arms[b]	Map size (cM)[c]	Obligatory map ($N \times 50$ cM)	Proportion obligatory[d]	cM/arm
Mouse	2.7	20	20	1361	1000	0.73 (0.73)	68.1
Rat	2.6	21	33	1749	1050	0.60 (0.94)	53.0
Human	3.0	23	46	3615	1150	0.32 (0.64)	78.6
Chicken	1.2	38	44	3800	1900	0.50 (0.58)	86.4
Zebrafish	1.8	25	50	3011	1250	0.42 (0.83)	60.2
Ambystoma (map)[e]	30.0	14	28	5152	700	0.14 (0.27)	184.0
Ambystoma (χ)[f]	30.0	14	28	5650	700	0.12 (0.25)	201.8

[a] References: mouse, Mouse Genome Sequencing Consortium (2002); rat, Rat Genome Sequencing Project Consortium (2004); human, International Human Genome Sequencing Consortium (2000); chicken, International Chicken Genome Sequencing Consortium (2005); zebrafish, Kelly *et al.* (2000); Ambystoma, Licht and Lowcock (1991).

[b] The haploid number of chromosomes. Smaller chicken microchromosomes (10–38; Masabanda *et al.* 2004) are tabulated as one-armed chromosomes. References: mouse, Mouse Genome Sequencing Consortium (2002); rat, Rat Genome Sequencing Project Consortium (2004); human, International Human Genome Sequencing Consortium (2000); chicken, International Chicken Genome Sequencing Consortium (2005); zebrafish, Gornung *et al.* (2000); Ambystoma, Callan (1966).

[c] References: mouse, Steen *et al.* (1999); rat, Brown *et al.* (1998); human, Kong *et al.* (2002); chicken, Groenen *et al.* (2000); zebrafish, Kelly *et al.* (2000).

[d] Numbers in parentheses represent the obligatory proportion of the map assuming one obligate chiasma per arm.

[e] Map size is estimated as the sum of LG1–14.

[f] Map size is estimated as 50 cM × the average number of chiasmata (Callan 1966).

nucleus. Assuming that one crossover is equivalent to a 50-cM map distance (Sturtevant 1913), this chiasmata frequency converts to an estimated map distance of 5650 cM, which is ~4.2× larger than that of the mouse genome and 1.6× larger than that of the human genome (Table 1). Finally, Voss *et al.* (2001) estimated map size from a linkage analysis in which 347 molecular genotypes were obtained for offspring from backcrosses between *A. mexicanum* and *A. mexicanum/A. t. tigrinum* hybrids. That analysis yielded one of the largest partial genetic linkage maps ever constructed (~3400 cM) and an overall map size estimate of 7291 cM based on the method of Hulbert *et al.* (1988). Thus, all studies to date suggest that recombinational map size is relatively larger in Ambystoma than in other vertebrates. Although recombinational map size in Ambystoma may simply be a consequence of a large physical genome size (Table 1), the contribution of additional factors that are known to strongly influence map length in other vertebrates (*e.g.*, chromosome number and morphology) have not been considered.

Recently completed expressed sequence tag (EST) sequencing projects for two tiger salamander species, *A. mexicanum* (Haberman *et al.* 2004; Putta *et al.* 2004) and *A. tigrinum tigrinum* (Putta *et al.* 2004), allowed us to rapidly develop a sufficient number of EST-based markers to generate a comprehensive genetic map for Ambystoma, and the first such map for any amphibian. Below we describe this new resource and show its utility in two areas: (1) mapping phenotypic mutants and (2) investigating the relative effects of genome size and chromosome number on recombination-based estimates of genome size. We also note the importance of these EST-based PCR markers as a general resource for tiger salamander research.

MATERIALS AND METHODS

Study species: *A. tigrinum tigrinum:* The Eastern tiger salamander is the archetype of the tiger salamander species complex. This relatively large salamander species breeds in ponds and temporary pools in the eastern and southern parts of the United States; the individual that was used in this study was purchased from Charles D. Sullivan, which collects and distributes amphibians from breeding ponds in the vicinity of Nashville, Tennessee. These salamanders undergo a typical amphibian life cycle, wherein they hatch from the egg in an aquatic-larval form and eventually undergo a metamorphosis through which they achieve a terrestrial adult form.

A. mexicanum: The Mexican axolotl is native to a single lake in central Mexico (Lake Xochimilco, Mexico D.F., Mexico). It is commonly used in biological research and strains have been maintained in laboratory culture for nearly a century and a half (Dumeril 1870; see also review by Smith 1989). The most notable feature of this salamander is that it exhibits a phenotype, known as paedomorphosis, wherein it forgoes metamorphic changes typical of most ambystomatid salamanders and retains a largely larval morphology throughout adult life. Several developmental mutants have been described for *A. mexicanum* (Malacinski 1989) and are readily available (http://www.ambystoma.org). We estimated the genomic position of two recessive pigment mutants: *white* (Häcker 1907) and *melanoid* (Humphrey and Bagnara 1967). In comparison to wild type, white is characterized by a general lack of epidermal pigment cells, which is caused by failure of pigment cell precursors to migrate or survive in epidermal tissues (reviewed by Parichy *et al.* 1999). In comparison to wild type, melanoid is characterized by a lack of iridophores in the skin and iris, relatively fewer xanthophores and greater numbers of melanophores (Humphrey and Bagnara 1967).

Molecular markers: A total of 1095 molecular markers were genotyped using a previously described AxTg mapping family that was generated by backcrossing *A. mexicanum/A. t. tigrinum* F_1 hybrids to a single *A. mexicanum* (Voss 1995). In a previous study using AxTg, Voss *et al.* (2001) reported linkage analyses of 323 "anonymous" molecular markers (315 AFLPs and 8 RAPDs) and 24 markers for nuclear protein-coding sequences. Here we report an additional 202 AFLP markers (scored for 44 backcross offspring) and 546 EST-based markers (scored for 91 backcross offspring). Marker fragments were amplified using standard PCR conditions (150 ng DNA, 50 ng each primer, 1.2 mM $MgCl_2$, 0.3 units Taq polymerase, 1× PCR buffer, 200 mM each of dATP, dCTP, dGTP, dTTP; thermal cycling at 94° for 4 min; 33 cycles of 94° for 45 sec, 60° for 45 sec, 72° for 30 sec; and 72° for 7 min). Polymorphisms were diagnosed by primer extension [Perkin-Elmer (Norwalk, CT) AcycloPrime-FP chemistry and Wallac (Gaithersburg, MD) Victor3 plate reader] (*e.g.*, CHEN *et al.* 1999; HSU *et al.* 2001; GARDINER and JACK 2002), thermal-gradient capillary gel electrophoresis (SpectruMedix, Reveal), allele-specific amplification, PCR fragment size polymorphism, and restriction digestion. Primer sequences, diagnostic polymorphisms, polymorphism detection assays, numbers of individuals genotyped, and NCBI GI numbers for source sequences for all 570 protein-coding loci are summarized in supplementary Table S1 at http://www.genetics.org/supplemental/. The design of AFLP markers, EST-based markers, and primer extension probes is described in detail below.

Generation of AFLP and RAPD markers: The methods used to generate AFLPs and RAPDs were reported previously (Voss and SHAFFER 1997; Voss *et al.* 2001). An additional 202 AFLPs were generated for this study using the same methods and backcross progeny. Primer sequences for all AFLPs are provided in supplementary Table S2 at http://www.genetics.org/supplemental/.

EST-based marker design: The majority of EST-based markers were designed to target 3′-unconserved regions of assembled EST sequences that were derived from *A. mexicanum* and *A. t. tigrinum* (PUTTA *et al.* 2004). These sequences were identified by first aligning (BLASTx) assembled sequences to a custom database of human protein sequences (Ref Seq Release 3, NCBI). The 3′-most 70 bp of aligning sequence and all remaining nonaligning 3′ sequence were targeted for primer design. These regions presumably correspond to sequence encoding carboxy-terminal regions and 3′-untranslated regions. Primers were designed within these regions using Primer3 software (http://www.mit.wi.edu/Primer3.html). When presumably orthologous sequences (>90% identity) were available for both *A. mexicanum* and *A. t. tigrinum*, marker design was additionally constrained to flank at least one polymorphism between the two sequences.

Primer extension probe design: Primer extension probes were designed to target polymorphisms that were observed between presumptively orthologous EST sequences or between EST sequences and sequenced marker fragments that were amplified from *A. mexicanum* and *A. t. tigrinum*. Design of primer extension probes was constrained such that probes were complementary to sequence immediately 3′ of targeted SNP positions. The software package Array Designer 2.1 (PREMIER Biosoft International) was used to identify probes with T_m 60° ± 8° and high Δg for primer hairpin and primer dimer formation.

Construction of the linkage map: A linkage map was constructed for the tiger salamander using genotypes for 570 protein-coding markers (EST and gene based) and 525 AFLP markers. Linkage analyses were performed using Mapmaker/EXP 3.0b and linkage groups (LG) were visualized using MapManagerQXTb19 (MEER *et al.* 2004) and MapChart 2.1

(VOORIPS 2002). This linkage map was constructed by first identifying sets of markers that formed linear linkage groups supported by a log of odds (LOD) ≥5.0 and then by recursively adding markers to these initial groups at lower LOD thresholds (4.0, 3.5, and 3.0). At each step linkage groups were visually inspected to verify the linear arrangement among markers. At each LOD threshold, the addition of new markers to an existing linkage group or merger of two existing linkage groups was constrained such that addition or merger did not disrupt arrangements that were previously set at a higher LOD threshold. A few markers could not be assigned to precise positions within a linkage group, but could be assigned to a single linkage group at LOD >4.0 (supplementary Table S2 at http://www.genetics.org/supplemental/). Linkage distances among markers in the final map were estimated using the KOSAMBI (1944) mapping function in MapManagerQXTb19 (MEER *et al.* 2004).

QTL analysis: Interval analysis was used to identify the positions of two mutant phenotypes that segregated within the AxTg mapping family: *white*, which is specified by the recessive locus *d*, and *melanoid*, which is specified by the recessive locus *m*. Interval analysis was also used to identify the position of a major-effect QTL that contributes to segregation of metamorphosis *vs.* paedomorphosis and developmental timing. Segregation patterns of *white*, *melanoid*, and metamorphic phenotypes within the AxTg family have been previously described (Voss 1995). Likelihood-ratio statistics (LRS) for association of phenotypic variation with genotypic inheritance were estimated using the interval mapping (*e.g.*, HALEY and KNOTT 1992; MARTINEZ and CURNOW 1992; ZENG 1993, 1994) function of MapManagerQTXb19 (MEER *et al.* 2004). Significance thresholds for interval mapping were obtained through 10,000 permutations of trait values among backcross progeny. Confidence intervals for QTL positions were estimated by the method of DARVASI and SOLLER (1997).

RESULTS

Segregation of molecular markers: The segregation of alternate genotypes for a majority of the molecular markers was consistent with the Mendelian expectation of 1:1. Only 7% of protein-coding markers had genotypic ratios exceeding the 95% tail of the χ^2 distribution (Figure 1A). On average, ratios of anonymous markers were more deviant than those of protein-coding markers with 17% of markers exceeding the 95% tail of the χ^2 distribution (Figure 1B). Plotting χ^2 values for both marker classes against genomic position showed that the few markers with significantly deviant ratios were not strongly clustered at any position within the genome (Figure 1C).

Linkage analysis: Linkage analysis revealed 14 large linkage groups that ranged in size from 125.5 to 836.7 cM and consisted of 14–123 markers (Figure 2, Table 2). A total of 790 markers were assigned to these 14 linkage groups, with 486 protein-coding loci and 169 anonymous loci placed at precise positions. The total linkage distance spanned by these markers was 5251.3 cM and the average intermarker distance was 7.96 cM.

Thirty-six smaller linkage groups that ranged in size from 0 to 79 cM and consisted of 2–10 markers were identified. Thirteen of these linkage groups contained
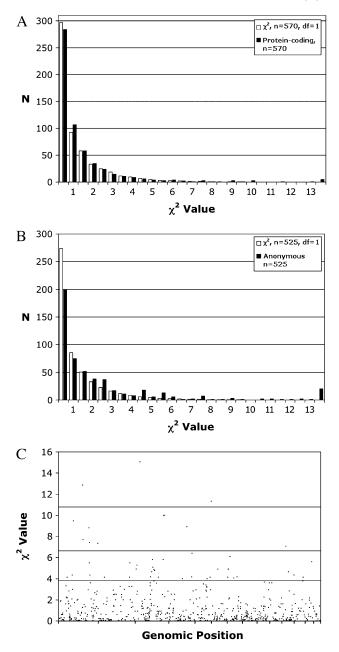
FIGURE 1.—(A) Plot of the distribution of the χ^2 statistic for 1:1 segregation of marker genotypes *vs.* standard χ^2 distributions for gene and EST-based markers. (B) Plot of the distribution of the χ^2 statistic for 1:1 segregation of marker genotypes *vs.* standard χ^2 distributions for anonymous markers (AFLPs and RAPDs). (C) Plot of χ^2 statistics for 1:1 segregation of marker genotypes *vs.* map position. The *x*-axis represents LG1–14 concatenated head to tail, and vertical bars demark boundaries between linkage groups.

at least one EST or protein-coding marker and spanned a total of 479.7 cM; 23 additional linkage groups that consisted of only AFLP markers spanned a total of 630.5 cM. A total of 165 markers (134 AFLP and 31 protein coding) did not show linkage to any other marker and were not assigned to any of the linkage groups.

QTL analyses: *white:* Interval analysis identified two LRS peaks for *white* on LG1 (*D1*, LRS 37.2, 95% C.I. =

17 cM; *D2*, LRS 12.4, 95% C.I. = 44 cM) (Figure 3). Although this suggests the possibility that *white* may depend upon more than a single locus, the lesser LRS peak is only suggestive of a significant QTL and an equally supportive LRS is expected to be observed once at random within the genome. In the mapping cross, color phenotypes segregated as F_2-type markers because F_1 hybrids and the recurrent *A. mexicanum* carried a single recessive allele (*d*) at *white* (D^{tig}/d^{mex} and D^{mex}/d^{mex} genotypes, respectively). In contrast, molecular genotypes segregated as backcross-type markers. As a result, only the *A. t. tigrinum* marker alleles were fully informative for association testing; that is, we expected that at the position of *white,* all individuals inheriting *A. t. tigrinum* molecular marker alleles would exhibit wild-type coloration, but never *white* coloration. Segregation ratios for two protein-coding markers (*E16A12* and *E23C5*) near the maximum LRS peak on LG1 are consistent with this expectation (Table 3). In contrast, incomplete association was observed for markers from the region with the suggestive LRS. These results indicate that *d* is most likely located within the 14.2-cM region that includes markers *E16A12* and *E23C5*.

melanoid: Interval analysis revealed a single region of LG14 that was strongly associated with segregation of the *melanoid* mutant phenotype (*M1*, LRS 17.5, 95% C.I. = 32 cM) (Figure 3). As was the case with *white,* the founding and recurrent *A. mexicanum* parents that were used to generate the AxTg family were heterozygous for the *melanoid* mutant (M_{mex}/m_{mex}). The expressed sequence marker *E17A7* is closest to the maximum inflection point of the LRS profile for *melanoid*. Genotypic ratios for this locus are consistent with tight linkage to *melanoid,* given the same expectations described above for segregation of molecular markers and the *white* phenotype (Table 4).

Metamorphosis *vs.* paedomorphosis: Interval analysis revealed a single region of LG3 that was strongly associated with segregation of metamorphosis *vs.* paedomorphosis in the AxTg family (LRS 101.7, 95% C.I. = 9 cM) (Figure 4). The marker *ctg325* lies closest to the maximum inflection point of the LRS profile. This QTL is located near the middle of LG3 and corresponds to the previously described *met* QTL (Voss and Shaffer 1997; Voss and Smith 2005). A region of LG4 also showed a suggestive association with segregation of metamorphosis *vs.* paedomorphosis, although it is much weaker relative to *met*.

BLAST alignments to the human RefSeq database: Our BLASTx analysis of 570 EST/gene-based markers yielded alignments with multiple human protein-coding genes. In total, 390 presumptive protein-coding markers had best BLASTx alignments with 350 nonredundant human RefSeq proteins. Best BLASTx matches and corresponding bitscore and *E*-values are provided in supplementary Table S3 at http://www.genetics. org/supplemental/. Analyses of conserved synteny and

LG1

cM	Marker
0.0	E20A12
21.2	A56.7
37.1	A32.19
45.0	G2M17
51.1	A16.9
63.1	Gapdh
66.7	E24C6
71.9	E5G11
80.1	A36.8
87.8	E17C11
94.7	E16A12
95.9	E23C5
102.0	A38.3
107.8	cntg26
115.3	A20.2 UBC549
126.3	E24C10
153.8	E20C6
160.6	E6G8 E22E10
172.3	A40.5
182.9	A11.8
185.7	A9.9
188.5	E7G8
212.7	E20G12
215.2	A21.8
217.7	A19.9
222.9	E8C10
224.0	E21G9
235.2	E20A6
241.3	E18E8
	E10C9 E10C9
242.6	E19C4 E8C1
244.8	E11G12
247.1	E13C8
248.3	E16E3 G1C5
260.5	E19C1
276.1	A10.3
278.9	A38.2
286.7	A55.8
292.1	A53.10
297.8	E22C12
313.1	E13C10
326.4	E15E5
342.4	E7A6
352.4	E20G3
357.8	A21.11
380.3	A52.16
388.1	A20.11
396.0	E13E11
401.0	E18C5
409.6	E12A5 E14G1
414.4	E12C9
419.0	E16C5
421.6	A16.1 A18.6
429.6	A53.2
445.5	A33.4
461.4	E19E4
477.0	Hoxc10
487.1	E18A10 E18A12
489.7	E23G3
507.9	E9E7
510.7	A24.2
516.1	Wnt1
534.2	E21G8
544.6	cntg116
561.9	A40.4
564.7	E5C5
569.3	E16C6
570.5	E12C6
576.4	E20C7
597.8	E9C4
609.5	A55.10
614.8	A34.6
627.2	A6.1
647.8	A12.9
652.7	A37.3
657.9	A38.4
666.0	A32.20
679.5	A6.8
692.6	A52.4
700.4	A4.7
708.3	A55.9
717.3	A8.10
729.6	ETG990
734.6	E13E4 NT001CB10
735.7	E24E9
742.4	E24G7
747.2	E13G1
755.4	E6C6
766.5	E22A7
772.9	E13A5
780.5	E12C8
798.9	E24E3
803.4	E11C1 E7C12
806.9	E10A4
815.5	G2I16
828.1	E14C7
836.7	E19C7

LG2

cM	Marker
0.0	E9E9
19.4	A21.4
25.8	E10C6
28.2	E23A7
35.3	E11E7
47.0	G1L7
52.2	E11G7 g1-e12
55.4	A8.12
58.3	E16G12 A6.7
87.6	Cspg2
92.4	E24E10
97.0	G1-J20
102.3	A6.9
117.8	A12.11
135.7	E11G8
146.1	E14E6
151.6	E14C5
167.5	E7G11
168.6	E6C12
173.1	E9G2
193.0	A29.10
208.5	A9.3 UBC608.1
226.4	E20A10
232.1	A52.11
235.3	Slc4a5
244.4	A26.7
260.3	E19A9
264.4	E26G7
265.7	E7C6
293.2	E20G10
301.1	E10C5 E26G3
310.8	E25C12
314.4	E23A12
333.7	A2.2
352.0	A31.3
364.8	A28.5
383.0	A29.2
401.8	E20A2
404.1	E23E3
413.8	E20A5
419.8	E15A11
453.6	E17E6
463.3	E9E1
466.9	E17A10
472.6	E25E1
473.7	E17E10
479.6	E21G10
487.1	E15C4
498.9	E12C5
516.7	A29.4 A36.2
539.2	E20A9
553.9	E8E9
558.8	E13A1
579.1	E20G6
595.2	E23E12
622.6	A31.1
638.5	E19E7
651.6	A15.3
654.1	A12.5
661.5	A32.13
675.7	G2I21
684.6	A39.12
695.3	E15A2
704.1	E24C11
720.3	E14G11
738.5	E9E6
749.5	A5.9
757.2	A21.3
767.6	A45.2

LG3

cM	Marker
0.0	A45.5
15.5	A45.3
22.0	E9E3
23.4	E21C8
33.4	E23G8
50.3	E21A4
84.4	E13A11
111.9	Rho
117.5	E12A3
126.9	E7G12
129.1	E10E9
130.3	E19A10
138.8	E15E6
149.2	A1.13
157.2	E10E2
164.1	E21A3
171.6	GNAT1
182.1	E20C5
189.9	E18C6
194.7	E18C7
197.0	E8C9
205.0	E28A6
206.1	E12E6
217.8	E17G3
223.4	E9G8
235.2	E16E8
238.0	A5.3
246.6	A8.8
255.8	A25.8 A40.7
258.4	Foxa3
262.2	E8A9 E24E4
264.6	E23C10
265.9	E18G2
267.1	E14C12
269.4	E16G10
275.6	E15G5 E6E2 / E9G12 E25C2 / E29A6 / E10G4 E12A8 / E6G4 Calm3 / E19A4
280.2	
281.4	E17E9 E21A5
293.6	E13G5 E7A8
294.7	E9G11
298.2	A11.7
306.4	A24.5
311.8	E6C5 E7G7
315.3	E18G5
321.4	E18C8
322.6	E7G2
333.2	ctg325 / E18E7 E5E4
343.2	E5E6
365.1	A32.17
383.6	A34.5
413.2	E10G12 E7C10
420.7	E6C2
427.0	E16G1
431.7	E19G10
438.9	E7C8
445.7	E17G8
450.1	E10A8
455.3	E22G5 E25G1
458.0	A5.8
473.4	A18.12
494.6	A15.8
497.1	A14.7
499.5	A9.11
501.9	A12.7
509.5	A18.8
527.9	E18G10
531.7	E10A12 E18A3
546.7	E15E2
557.3	HLP1
564.9	Hoxa4 Hoxa9
589.5	E6C8
604.2	A52.9
614.6	A4.10
643.0	E17E8 / E13E5 E19G3
646.5	E20E2
647.7	E12C2
661.4	betaTH
671.3	A56.8

LG4

cM	Marker
0.0	E9A2
1.2	E8A1
6.9	E5A8
12.7	E25E3
16.2	E23A1
24.2	A27.1
36.9	A4.6
65.3	E24A11
71.1	E14C8 E19G2
76.1	E13E7
77.3	E6C7
85.0	A32.9
102.8	A19.10
107.8	A20.18
119.1	E20C1
139.1	A38.9
146.4	A54.5
162.8	A24.6
176.2	UBC274
180.7	UBC134
188.1	E14A11 E19A2
190.5	E8C8
196.8	E25A8
204.3	E24A12
211.7	E14A7
223.7	CoxV1b
224.8	E12C7
229.6	E10G3
233.2	E18A4
235.5	ETG93
242.5	E13E2
243.7	E12E12 E8E1
250.6	E14G10
267.7	E16G9
287.8	E16A7
295.9	E23E9
301.8	E6E5 E8A10
306.6	E26G10
312.4	E23G7
330.5	E9A3
336.6	E7C5
345.4	E12E11
362.3	E11G6
392.9	bl005ba01
398.6	E5G10
405.7	E23C6
408.1	E8E5
413.9	E17C2
416.3	E5E7
435.4	A1.1
443.1	A25.9
445.7	A45.1
453.0	A37.1
469.4	E15A1
478.8	E14G12
495.3	E19C8
502.4	E12E2 E18C3
503.6	E10C1
519.2	E10C11
527.1	E16A9
531.8	cntg66
538.9	E16G8

LG5

cM	Marker
0.0	A11.9
13.1	A39.10
31.9	A57.3
39.7	E14E10
52.6	E12A6
63.3	E15E12
81.1	E12A11
86.2	E11G11
96.0	A32.6
101.4	E17E4
112.3	E16C7 E9C9
116.9	E22E3
119.5	E9A6
123.1	E5G5
133.5	A15.7
146.7	A56.9
155.6	E11G4
162.6	E17C12
181.8	E10C3
198.4	E20A8
203.0	E7A11
207.6	E12G1 E12G7
212.3	E22G12
213.5	E13E6 E14E8
217.5	g1-d5
227.0	Hoxd11 Hoxd8
230.4	E13A7
231.6	E23G2
245.6	E26A8
263.1	E14E2
281.3	E26C11
294.9	E24C3
300.5	A31.8
305.8	A30.16
323.1	A39.8

FIGURE 2.—The tiger salamander linkage map (LG1–14).

LG6

0.0	E12G2
10.3	E24G11
34.3	E7C1
45.2	E9A11
51.5	E19E5
57.6	E24C12
61.3	E14A1 G2-L21
83.4	E13C7
97.5	G1-I8
98.8	E7C4 E24G3
99.9	E24G9
107.5	E17C1
113.2	A39.5
119.1	E14A2
139.1	E13G4
155.3	E10G8 A52.8
158.0	G2F2
177.5	E14E3
186.7	E17G4
190.1	E15E9
195.4	A32.14
210.9	A50.4
221.3	A18.7
229.4	G2G14
241.0	E23E11
251.7	E25G7
258.6	E9E11 brachyury
268.7	E15A6
270.0	E12G12
292.1	E8A7
293.3	E16C8
294.4	E14C3
308.6	E13G11 G1D6

LG7

0.0	A11.3
7.6	A17.6
12.6	A17.10
17.9	A39.7
25.8	E26G9
41.4	E13A3
61.9	A25.6
75.4	A26.3
91.3	A20.13
96.7	E12C4
102.8	E22C3
113.4	E11E11 E12C1 E16E10
120.6	E12G10
129.0	A40.2
136.8	A9.4
142.1	A7.14
158.0	A7.10
160.5	A25.10
178.4	E12A7
186.3	E11G2 E17A2 E19G7
189.0	A9.1
191.7	E15G9
207.7	E21G12
212.8	E13C1
216.4	E15C8 E22A3
221.8	A20.8
239.7	E9E10
260.3	E18E11
261.5	v2p3a5
263.9	E16E2
273.8	E7G9
278.8	E6A11
284.9	A27.2
287.4	A54.6
294.9	A28.3

LG8

0.0	E14A8
13.9	E8A2
15.1	E7G10
19.7	E29C4
20.8	E14G3
25.7	E15G10
36.4	E12A1
39.3	A26.4
47.0	A1.6
60.5	E16C12
64.1	E20C8
65.3	E19A11
	E15E3 E17G2
66.5	E19A12 v2p3b1
	E24C7
79.6	E16A5
85.2	E16C3
86.5	E10A3
87.8	E10G10
89.2	E12E10
91.7	E13E8
96.3	E18C10
106.3	pcna
124.2	E21E4
128.0	E11A8
148.6	E7C9
166.2	E14C4 E14G7
174.3	A37.5
179.2	A38.8
181.9	A9.13
201.8	A14.5
216.9	A33.10
231.2	E27E7
255.4	E12A9 E7A1
265.2	E14E1
270.2	E7A3
290.1	A19.13

LG9

0.0	A19.11
5.1	E27A3
22.5	E24A1
30.9	ntf3
43.4	E17C9
58.1	Wnt5B
59.3	E8E2
61.7	E30G6
78.4	E6G10
86.6	E6E12
105.8	G2-K2
107.5	E19C9
112.6	E12A4
119.8	E9A7
121.0	E20G2 E8G1
125.7	E17C6
129.2	E17C5 E8G11
136.5	E23G6
151.9	E20G5
154.3	E10E3
155.5	E19E8
159.0	E23G1
	E10G2 E12E1
166.4	E15C7
170.1	UV
176.4	E13C12 E6A12
191.5	E18E3
206.5	E8C7
217.5	A19.17
220.1	A55.11
236.4	A50.1
244.4	A10.6

LG10

0.0	E23A10
6.4	E24C2
27.0	A28.6
48.2	A7.11
67.5	E20C4
75.0	E24A6
84.6	E20C2
97.0	E20C3
100.5	E7A7
105.3	E5G4
109.0	E22A41
115.0	E12C3
117.5	E5G1
118.7	E26G8
122.2	E17G11
124.7	E12G5
136.3	E9E8
156.9	E21C6
174.7	E7G1
191.0	E17A4
200.2	E20E4
222.9	E25A12
233.3	E17G7
241.0	A56.6

LG11

0.0	A45.6
5.0	A27.3
10.3	GNAT2 A16.3
21.1	E21C7
41.8	E9C11
60.4	G2G21
63.0	E11C3
71.5	E20G11
72.6	E17C3 E9C2
76.2	E9G1
80.0	E15A9
94.3	E19E12
95.5	E13G8
107.8	E25E5
112.6	E17E411
122.8	E10A2 E15G4
132.9	E15E10
136.0	E6E11
137.2	E13E9 G2H18
139.7	E11C5
142.8	A10.4
145.7	Col1A
149.6	Dlx3
162.4	G1-L11
167.1	E18A9
168.2	E10C4 ETG1506 Krt17 Krt24
171.1	E13A8
176.9	E9A10
178.0	Hoxb13
180.2	E22G4
190.4	E6E7
192.8	G2G13
194.0	THRAP4
203.4	Thra
218.5	Rpl19
229.1	A18.5
234.4	A21.13

LG12

0.0	E5A9
8.7	E9E4
26.0	A20.19
42.8	E6G1
51.2	E12E3
61.6	UBC722.1
74.1	A33.15
95.3	E14A6
117.0	E11E8
130.6	E5A5
147.4	A33.11
166.7	A30.7
177.4	A6.6
185.0	A4.4
202.3	E12G3
211.4	E7E3

LG13

0.0	A28.7
12.8	A31.5
26.2	E10C8
31.5	A12.4
50.3	A10.1
64.2	E20E3 A56.1
74.1	E8G8
78.8	E23A2
97.6	A40.6
111.4	A8.1
120.3	E24E1
138.0	E13A6
155.6	E24A5
163.3	A11.2

LG14

0.0	E7A5
31.3	E20E5 E7E9 ctg1459 ETG926 E26A9
32.6	ctg1381
47.2	A25.11
52.5	A26.9
69.9	UBC144
98.0	E17A7
105.0	E12C11
118.1	A34.2
125.5	A31.9

FIGURE 2.—*Continued.*

segmental homology between Ambystoma and other vertebrate species are presented elsewhere.

DISCUSSION

We have generated the first comprehensive linkage map for Ambystoma by mapping >1000 anonymous markers and EST loci in an interspecific mapping panel. Fourteen large (>125–836 cM) and 35 small (0–75 cM) linkage groups were identified. Because the number of large linkage groups equaled the haploid chromosome number in *A. t. tigrinum* and *A. mexicanum* (PARAMENTER 1919; FANKHAUSER and HUMPHREY 1942), and because

TABLE 2

Distribution of markers on Ambystoma LG1–14

LG	EST based[a]	Anonymous markers[a]	All markers	cM	% map	Average intermarker distance
1	73 (70)	50 (37)	123 (107)	836.7	15.9	7.8
2	56 (51)	47 (24)	130 (75)	767.6	14.6	10.2
3	80 (78)	34 (21)	114 (99)	671.3	12.8	6.8
4	55 (54)	21 (14)	76 (68)	538.9	10.3	7.9
5	32 (30)	18 (9)	50 (39)	323.1	6.2	8.3
6	34 (34)	15 (5)	49 (39)	308.6	5.9	7.9
7	27 (25)	24 (17)	51 (42)	294.9	5.6	7.0
8	34 (34)	17 (8)	51 (42)	290.1	5.5	6.9
9	32 (31)	9 (5)	41 (36)	244.4	4.7	6.8
10	21 (21)	13 (3)	34 (24)	241.0	4.6	10.0
11	39 (38)	10 (6)	49 (44)	234.4	4.5	5.3
12	9 (9)	12 (7)	21 (16)	211.4	4.0	13.2
13	7 (7)	12 (8)	19 (15)	163.3	3.1	10.9
14	9 (9)	5 (5)	14 (14)	125.5	2.4	9.0
Total	508 (491)	287 (169)	795 (660)	5251.3	100.0	8.0
% mapped	87.4	54.7	71.9			

[a] Numbers in parentheses represent the number of markers that were placed at precise positions within the map.

these linkage groups yielded a map size estimate that agrees with a theoretical and empirical (see below) estimate of total map size, we propose this set as a framework for the 14 chromosomes in the tiger salamander genome. It is unlikely that the collection of smaller linkage groups represent any additional chromosomes within the Ambystoma genome because the haploid chromosome number is indisputably 14 and microchromosomes are not known in this group.

Map coverage: The combined map distance of our 14 largest linkage groups (5251 cM) is consistent with previous studies that indicated a large genetic map for Ambystoma. By comparison, the combined map length of LG1 and LG2 is greater than the total map length of

the mouse genome! Marker-based estimates of genome size vary greatly for Ambystoma (*e.g.*, 2600–6276 cM in ARMSTRONG 1984 and 7291 cM in VOSS *et al.* 2001). This variation may be attributed to the large genome size of Ambystoma and the nonrobust nature of estimators; genotyping errors and missing data cause upward bias in size estimates and nonrandom distribution of markers with respect to recombinational distances causes downward bias (CHAKRAVARTI *et al.* 1991). We estimated genome size using linkage data from this study and the method of HULBERT *et al.* (1988). Analyses were performed separately for protein coding and anonymous marker classes and for the combined data set using several linkage thresholds (10, 15, 20, 25, and 30 cM; data not shown). Estimates of map size varied greatly depending on data set and linkage threshold: estimates based on protein-coding markers ranged from 3741 to 4320 cM (15 and 30 cM thresholds, respectively), estimates from anonymous markers ranged from 6925 to
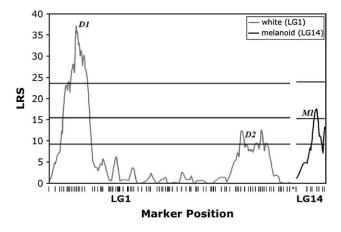
FIGURE 3.—LRS plot for association between segregation of pigment phenotypes and marker genotypes in LG1 and LG14. Horizontal lines represent LRS thresholds for suggestive (37th percentile), significant (95th percentile), and highly significant (99.9th percentile) associations (LANDER and KRUGLYAK 1995) estimated using MapMaker QTXb19.

TABLE 3

Segregation of markers associated with *white*

QTL	Genotype	white	+
		Phenotype	
D1	$E16A12^{Am}/E16A12^{Am}$	18	20
D1	$E16A12^{Am}/E16A12^{Att}$	0	46
D1	$E23C5^{Am}/E23C5^{Am}$	18	18
D1	$E23C5^{Am}/E23C5^{Att}$	0	43
D2	$A32.20^{Am}/A32.20^{Am}$	16	31
D2	$A32.20^{Am}/A32.20^{Att}$	2	38

QTL	Genotype	Phenotype	
		melanoid	+
M1	$E24E9^{Am}/E24E9^{Am}$	10	30
M1	$E24E9^{Am}/E24E9^{Att}$	6	41
M1	$E17A7^{Am}/E17A7^{Am}$	14	23
M1	$E17A7^{Am}/E17A7^{Att}$	2	44

10,782 cM (25 and 10 cM thresholds, respectively), and estimates based on the entire data set ranged from 7624 to 8824 cM (25 and 10 cM thresholds, respectively). Presumably, higher intrinsic error rates of AFLP markers (*e.g.*, ISIDORE *et al.* 2003) (Figure 1, A and B) yield inflated estimates of map size in the anonymous and combined data sets.

Fortunately, a more direct and independent estimate of recombination frequency is available for *A. mexicanum*. CALLAN's (1966) counts of chiasmata in *A. mexicanum* oocyte nuclei convert to an estimated map size of 5650 cM. This estimate is only slightly higher than the sum of our 14 largest linkage groups (5251 cM). Several factors likely contribute to the relatively small difference between these two estimates, including incomplete coverage of the linkage map (especially near the telomeres), the degree to which the KOSAMBI (1944) mapping function models recombination in Ambystoma, variation in average recombination rate between the male F_1's used in this study and the female *A. mexicanum* used by CALLAN (1966), and possibly small-scale differences in gene order between *A. t. tigrinum* and *A. mexicanum* (SYBENGIA 1992; TRUE *et al.* 1996). Close agree-

ment between the CALLAN (1966)-derived estimate and our recombination-based estimates of genome size suggest that the 14 large linkage groups that we identified frame the majority of the Ambystoma genome.

Some of the smaller linkage groups may represent distal portions of chromosomes that are represented by larger linkage groups. Recombination rates are known to increase toward the ends of chromosomal arms in a wide array of organisms, including mammals (*e.g.*, JENSEN-SEMEN *et al.* 2004), chickens (INTERNATIONAL CHICKEN GENOME SEQUENCING CONSORTIUM 2005), and grasses (KING *et al.* 2002; ANDERSON *et al.* 2004). In agreement with this pattern, CALLAN (1966) reported a higher-than-average recombination rate between the nucleolar organizing region (NOR) and the adjacent telomere in *A. mexicanum*. Alternately, some of these linkage groups may consist completely or partly of markers with genotyping error rates that prevent assignment to larger linkage groups at the LOD 4.0 threshold. Presumably as more loci are mapped, linkage groups that represent distal regions will be integrated into the framework map and spurious linkage groups will be excluded from the map.

Size of the tiger salamander genetic map: The salamander genome is one of the largest genomes and our linkage map is the largest yet reported for any vertebrate. The tiger salamander genome is estimated to be 10–25× larger than other sequenced vertebrate genomes, yet the genetic map is only 1.5–5× larger (Table 1). Thus, in terms of genome size, the salamander linkage map appears at to be ∼5× smaller than expected. However, because physical and linkage distances do not scale linearly among organisms, variables in addition to physical size must be considered. Chromosome number contributes directly to the size of the genetic map because at least one chiasma must be formed per bivalent to ensure normal segregation of chromatids during meiosis in most species (EGEL 1995; ROEDER 1997; MOORE and ORR-WEAVER 1998), although achiasmate meiosis has evolved in several plant and invertebrate species (reviewed by JOHN 1990). Therefore, the minimum map size of any eukaryotic genome is 50 cM × the haploid number of chromosomes. Furthermore, there is a strong correlation between chromosome arm number and genetic map size among mammals, suggesting that formation of at least one chiasma per chromosomal arm may be necessary in mammals (DURTILLEAUX 1986; PARDO-MANUEL DE VILLENA and SAPIENZA 2001). Map sizes for human, mouse, rat, chicken, and zebrafish are much closer to their theoretical obligatory minima, on the basis of chromosome arm number, than is the Ambystoma map (Table 1). Analyses that include multiple vertebrate taxa, especially those with large and intermediate genetic map lengths, are needed to fully elucidate the effect of chromosome arm number and other variables on genetic map size. However, it is apparent from the comparisons made here that linkage map size in Ambystoma is
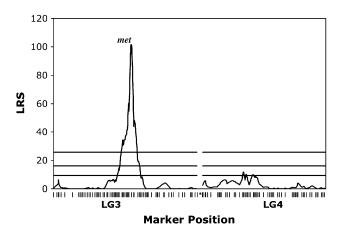

FIGURE 4.—LRS plot for association between segregation of metamorphosis *vs.* paedomorphosis and marker genotypes in LG3 and LG4. Horizontal lines represent LRS thresholds for suggestive (37th percentile), significant (95th percentile), and highly significant (99.9th percentile) associations (LANDER and KRUGLYAK 1995) estimated using MapMaker QTXb19.

influenced by factors in addition to chromosome arm number, the most likely factor being the large physical genome size of Ambystoma.

Refining the positional estimate of *white* and tentative assignment of LG1 to the chromosome harboring the NOR: The *white* phenotype has been a subject of several linkage analyses. Early tetrad mapping studies located *d* in the distal region of an unknown chromosome (Lindsley *et al.* 1955; Armstrong 1984) and several studies have observed an association between segregation of *white* and segregation of NOR variants (reviewed by Sinclair *et al.* 1978). The NOR is located subterminally on one of the four largest chromosomes (Paramenter 1919; Dearing 1934; Haschuka and Brunst 1965; Callan 1966; Cuny and Malacinski 1985). In our study we identified a single region of the genome that was significantly associated with *white*. The maximum inflection point of the LRS profile for association with *white* is located 95 cM from the end of the largest linkage group (LG1). Callan's (1966) chiasmata counts place the NOR at ~44 cM from the end of a chromosomal arm. Localization of *white* to the distal portion of a large linkage group is consistent with previous studies that indicated linkage between *white* and the NOR. We therefore conclude that LG1 likely represents the NOR-containing chromosome, with marker *E20A12* located near the distal portion of the arm harboring the NOR.

Identification of the genomic interval containing *melanoid*: We have identified a single region within the smallest of our 14 linkage groups that shows strong association with segregation of the *melanoid* phenotype. Armstrong's (1984) tetrad analyses yielded an estimated distance of 59.1 cM between *melanoid* and its centromere. Assuming that LG14 corresponds to one of the smaller chromosomes, the maximum inflection point of the LRS profile for *melanoid* is consistent with the earlier gene-centromere mapping study. The size of LG14 relative to the rest of the genetic map appears to support the assignment of LG14 to the smallest chromosome. Precise chromosomal measurements have been made for *A. tigrinum* (Paramenter 1919) and *A. mexicanum* (Callan 1966); the proportion of the genome occupied by the smallest chromosome was estimated to be 2.65% for *A. tigrinum* and 2.4% for *A. mexicanum*. Our LG14 represents 2.4% of the current genetic map (LG1–14). Although we do not expect a linear relationship between genetic and physical distance, our results are consistent with *melanoid* being located on one of the smaller tiger salamander chromosomes.

The genetic basis of metamorphic failure in lab *A. mexicanum*: It has been known for some time that expression of paedomorphosis in laboratory *A. mexicanum* is largely determined by a single genetic factor (Tompkins 1978; Voss 1995). A previous QTL analysis of the AxTg mapping panel using 234 AFLP markers identified a major-effect QTL (*met*) that is associated with expression of paedomorphosis *vs.* metamorphosis (Voss and Shaffer 1997); *met* was later shown to affect metamorphic timing in crosses using wild-caught *A. mexicanum* (Voss and Smith 2005). Our QTL analysis using all of the newly mapped markers did not identify statistically significant factors in addition to *met*; however, a second region of the genome yielded a statistically suggestive result. The relatively small number of individuals in the AxTg panel does not provide sufficient power to identify small-effect QTL. This second genomic region will be tested using the much larger WILD2 mapping panel (Voss and Smith 2005) in future work.

EST markers and Ambystoma research: The collection of EST markers that constitute our linkage map are significant new resources for Ambystoma research. Because these markers correspond to polymorphic expressed sequences with known linkage relationships, they are especially informative as probes for developmental, populational, systematic, molecular, comparative, and functional studies (Putta *et al.* 2004). Using the PCR primer information provided in supplementary Table S1 at http://www.genetics.org/supplemental/, it will now be possible to develop probes for many protein-coding genes for *A. mexicanum* and *A. t. tigrinum*, as well as for other ambystomatid species (Riley *et al.* 2003; Fitzpatrick and Shaffer 2004; Putta *et al.* 2004). Primer sequences for protein-coding markers, their corresponding assembled and curated EST sequences, and BLAST alignments can also be obtained at the Salamander Genome Project website (http://www.ambystoma.org).

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