学 位 論 文

Doctor's thesis

Genetic modifiers of otocephalic phenotypes in *Otx2* heterozygous mutant mice

(*Otx2* ヘテロ変異マウスにおける頭蓋奇形を修飾する遺伝子座の同定)

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関連論文

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CONTENTS

1. Summary

Mice heterozygous for the *Otx2* mutation display a craniofacial malformation, known as otocephaly or agnathia-holoprosencephaly complex (Matsuo et al., 1995). The severity of the phenotype is dependent on the genetic background of a C57BL/6 (B6) strain; most of the offspring of *Otx2* knock-out chimeras, which are equivalent to the F1 of CBA and B6 strains, backcrossed with B6 females display reduction or loss of mandible, whereas those backcrossed with CBA females do not show noticeable phenotype at birth. The availability of phenotypically disparate strains renders identification of *Otx2* modifier loci possible. In this study, a backcross of chimera with B6 was generated and genome-wide scans were conducted with polymorphic markers for non-mendelian distribution of alleles in *Otx2* heterozygous mutant mice displaying abnormalities in the lower jaw. One significant locus, *Otmf18*, between D18Mit68 and D18Mit120 on chromosomes 18, linked to the mandibular phenotype (LOD score 3.33) was identified. A similar replication experiment utilizing a second backcross (N3) mouse demonstrated the presence of another significant locus, *Otmf2* between D2Mit164 and D2Mit282 on chromosome 2, linked to the mandibular phenotype (LOD score 3.93). These two modifiers account for the distribution of the craniofacial malformations by the genetic effect between B6 and CBA strains. Moreover, *Otmf2* contain a candidate gene for several diseases in mice and humans. These genetic studies involving an otocephalic mouse model appear to provide new insights into mechanistic pathways of craniofacial development. Furthermore, these experiments

offer a powerful approach with respect to identification and characterization of candidate genes that may contribute to human agnathia-holoprosencephaly complex diseases.

要　旨

Otx2 ヘテロ変異マウスは、Otocephaly または agnathia-holoprosencephaly complex と呼ばれる頭蓋顔面奇形を呈することが報告されている。それらの頭蓋顔面奇形の種類や重症度は C57BL/6 (B6)の遺伝的背景に起因している（Matsuo et al. 1995）。

TT2 ES cell から作成された *Otx2* ノックアウトキメラマウスは、遺伝的には CBA と B6 の F1 である。この *Otx2* キメラマウスを B6 のメスに戻し交配した場合、生まれてくる仔の多くは、下顎の縮小や消失を示すが、CBA のメスに戻し交配した場合は明らかな外表奇形は認めない。そこで、この遺伝的背景による表現型の違いを利用し、*Otx2* 遺伝子に対する修飾遺伝子座を同定することを目的とした。

まず CBA と B6 間で多型を示すマーカーを約 20cM の間隔で全染色体にわたり設定した。次に *Otx2* キメラマウスを B6 のメスに戻し交配して得られるヘテロ変異マウス得た（N2）。各々の個体の外表奇形を観察後、出現頻度の最も高い下顎骨形成異常に注目した。*Otx2* ヘテロ変異マウス全個体の骨染色を行い、各個体の下顎の長さを測定した。下顎の長さにより normal mandible, small mandible, no mandible に分類し、各マーカーの遺伝子型（B6/CBA ヘテロ、B6/B6 ホモ）を決定した。それぞれのマーカーにおいてヘテロとホモの割合がメンデル率に従わず、どちらかに大きく偏っている場合は、その近隣部分は個体数、マーカー数を増やして精査した。統計処理においては QTL cartographer を利用し連鎖解析を行った（suggestive linkage は LOD score>1.9、significant linkage は LOD score>3.3 とした）。

その結果、*Otx2* キメラマウスを B6 に戻し交配し得られる *Otx2* ヘテロ変異マウス（N2）の解析では、18 番染色体の D18Mit68 から D18Mit120 の間に significant linkage（LOD score=3.33）を示す領域（*Otmf18*）を見出した。同様に N3（ある 1

匹の N2 のオスを B6 メスに戻し交配し得られたヘテロ変異マウス）の解析では、2 番染色体の D2Mit164 から D2Mit282 の間に significant linkage（LOD score=3.93）を示す領域（*Otmf2*）を見出した。

これら二つの領域には、B6 と CBA 間で何らかの遺伝子の違いが存在し、それが原因となり *Otx2* ヘテロ変異マウスに頭蓋顔面奇形などの表現型の違いを生じたと考えられる。*Otmf2* に関しては、その近傍に *Alx4* 遺伝子が存在すること、*Alx4* 遺伝子変異マウスの解析で頭蓋骨や下顎に異常を来たすこと、下顎に *Alx4* 遺伝子の発現を認め *Otx2* 遺伝子の発現領域と一致すること、さらに *Alx4* の DNA 結合配列が *Otx2* のプロモーター領域に認められることから、*Alx4* 遺伝子が *Otx2* 修飾遺伝子の候補遺伝子である可能性が示唆された。この様な otocephaly のマウスモデルを用いた今回の遺伝的研究から、頭蓋顔面の発生のメカニズムにおいて、*Otx2* 遺伝子が neural crest 形成のいくつかの異なった段階をそれぞれ特有の修飾遺伝子と相互作用することにより調節しているという新たな見解を得ることができた。さらに、これらの実験方法は、ヒト agnathia-holoprosencephaly complex に関係する候補遺伝子の同定と、その性質を明らかにするうえで、非常に有効な研究手段になることが示唆された。

2. Article

Genetic modifiers of otocephalic phenotypes in *Otx2* heterozygous mutant mice

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4. Abbreviations

Alx4 : Aristaless-like 4

A-P patterning : Anterior-Posterior patterning

B6 : C57BL/6

cM : Centi morgan

dpc : Date of post coitus

Emx1 : Empty spiracles homolog 1

Emx2 : Empty spiracles homolog 2

ES cell : Embryonic stem cell

EtBr : Ethidium bromide

LOD : Logarithm of odds

LRS : Likelihood ratio statistic

Otmf : *Otx2* modifier

Otx1 : Orthodenticle homolog 1

Otx2 : Orthodenticle homolog 2

PCR : Polimerase Chain Reaction

QTL : Quantitative trait loci

SHH : Sonic hedgehog

SSLP : Simple sequence length polymorphism

5. Introduction

Gene targeting is an important technology for analysis of gene function during embryogenesis; moreover, it offers the means for the generation of animal models for human congenital disease. Hundreds of mutant mice have been developed; furthermore, thousands will be available in the near future, providing researchers with an immense new resource and information regarding the developmental biology field. Additionally, numerous targeted loci show disparate phenotypes that depend on the genetic background of mouse strains, thus affording an even broader understanding of gene function (Horan et al., 1995; Matsuo et al., 1995; Proetzel et al., 1995; Rozmahel et al., 1996; LeCouter et al., 1998; Wojnowski et al., 1998; Wawersik et al., 1999). One powerful approach leading to identification of genes that are involved in a specific phenotype is the mapping of loci that modify the severity of the phenotype employing naturally occurring variations in existing inbred strains (Lander and Kruglyak, 1995). Because the genetic contributions to these traits are often caused by a combination of effects at multiple loci, these traits are termed complex traits. However, the identification of genetic loci which modify developmental malformations in knock-out mutant mice as an experimental model has not been attempted.

Mouse *Otx2* is a *paired*-like type homeobox gene functioning as a transcriptional activator (Simeone et al., 1992; 1993). It is sequentially expressed in the epiblast, anterior visceral endoderm, anterior definitive endoderm and anterior neuroectoderm prior to and during gastrulation; at the subsequent neurula stage, *Otx2* is expressed in the entire rostral brain region

(Simeone et al., 1992; Ang et al., 1993; Acampora et al., 1998; Kimura et al., 2000). Indeed, several knock-out and compound mutations of the *Otx2* gene suggest that it is involved in several steps for early A-P patterning and rostral brain development in cooperation with other regulatory genes (Matsuo et al., 1995; Acampora et al., 1995, 1997; 1998; Ang et al., 1996; Suda et al., 1996, 1997, 2001; Kimura et al., 2000, 2001; Tian et al., 2002).

In addition, *Otx2* is expressed in the cephalic mesenchyme, including the mesencephalic neural crest cells, which are distributed to the premandibular and distal portions of the mandibular regions (Kimura et al., 1997). Reflected by *Otx2* expression in the cephalic mesenchyme, *Otx2* also plays a crucial role in craniofacial development. *Otx2* single heterozygous mutant mice displayed craniofacial malformations that were strictly dependent on the genetic background of the murine strains (Matsuo et al., 1995). Previously, the *Otx2* knock-out chimera has been generated in TT2 ES cells, which are derived from F1 embryos obtained from crosses of inbred C57BL/6 (B6) and CBA strains (Yagi et al., 1993; Matsuo et al., 1995). Upon backcross of these chimeras with B6 females, the majority of heterozygous mutants are dead at birth, accompanied by severe craniofacial malformations, designated as otocephaly in many mammalian species and agnathia-holoprosencephaly complex in humans (Bixler et al., 1985; Juriloff et al., 1985; Winter, 1996; Wallis and Muenke, 2000). Notably, these mutants displayed reduction or absence of the lower jaw and/or eyes externally as well as holoprosencephaly by histological examination (Matsuo et al., 1995). On the other hand, when the chimeras were crossed with CBA females, most of the *Otx2* heterozygous mutant pups exhibited no noticeable

phenotype. This evidence explicitly suggests the presence of several genetic modifier genes exerting strong effects on the expressivity of the *Otx2* heterozygous mutant phenotype.

On the basis of facilitated recognition and quantitation of small changes in *Otx2* activity through lower jaw development, in particular, the length of the mandible, this phenotype provides a simple and sensitive assay for allelic differences at secondary loci interacting with the *Otx2* gene product. Changes in the gene products of secondary loci that lie upstream, downstream or interact directly with the *Otx2* protein would all impact the expression of the *Otx2* mutant mandible phenotype. With the advent of simple sequence length polymorphism (SSLP) markers, which are distributed throughout the entire genome (Dietrich et al., 1992), it is now possible to rapidly map the loci that contribute to such complex genetic traits. This situation provides an ideal opportunity for defining the genes that control the severity of otocephaly. Moreover, genetic analysis of well-defined experimental models of otocephaly offers the potential to markedly accelerate the genetic analysis of human agnathia-holoprosencephaly complex. In this study, two different mouse strains, B6 and CBA, were employed in order to identify and map modifier loci acting upon the expression of mandible abnormalities of *Otx2* heterozygous mutant mice. The modifier loci thus identified regulate a genetic pathway of craniofacial development interacting with *Otx2*; furthermore, these loci also may be possible genetic causes of human agnathia-holoprosencephaly complex diseases.

6. Materials and Methods

6-(1) Generation of *Otx2* mutant mice

Inbred B6 and CBA strains were purchased from Charles River. *Otx2* mutant chimeras were generated by homologous recombination in TT2 embryonic stem (ES) cells derived from F1 embryo from B6 × CBA mice. The gene was disrupted by inserting the neomycin resistance (neo^r) gene into the exon that encodes the homeobox sequence, interrupting the sequence between the first and second α-helix (Matsuo et al. 1995). Mice were housed in environmentally controlled rooms of the Center for Animal Resources and Development, Kumamoto University under the guidelines of Kumamoto University for animal and recombinant DNA experiments. The chimeras were obtained normally and mated with B6 females to generate N2 heterozygous mutant mice. A few N2 fertile males were obtained. Enough number of N3 embryos were got from only one of the N2 males. Backcross embryos (N2 and N3) were collected at 18.5 dpc.

6-(2) Genotyping of newborn mice

Genotypes of newborn mice and embryos were routinely assessed by PCR analyses and confirmed with genomic DNAs prepared from their tails. In PCR analysis, the mutant allele was detected as the 140bp product with primers below (Matsuo et al., 1995).

5'-TCGTGCTTTACGGTATCGCCGCTCCCGATT-3'

and

5'-GCACCCTGGATTCTGGCAAGTTGAT-3'

6-(3) External malformations of *Otx2* heterozygous mutant mice

External views of each embryo were photographically recorded. Those were classified into eight groups (no apparent abnormalities, reduction of low jaw, loss of low jaw, excencephaly, short nose cleft face, headless and others) according to their external morphology (Fig. 1). Frequency distribution of external malformations in consecutive mutant mice (n=200) was calculated in N2 and N3 respectively (Fig. 2).

6-(4) Variation of mandible length in *Otx2* heterozygous mutant mice

Subsequently cartilage and bones were stained with alcian blue and alizarin red based on the method of Kelly et al (1983) and stored in 80% glycerol. After finishing that, the lengths of left and right mandibles of embryos were measured in millimeters and an average of both lengths was obtained (Fig. 3, 4). Similarly the lengths of mandibles in N2 wild-type embryos (n=30) were measured. According to the lengths of each mandible, the mutant embryos were qualitatively classified into three phenotypes, normal mandible (longer than 5.0 mm), small mandible (0.5 to 4.9 mm) or no mandible (0 mm). Those embryos with apparent facial and skull anomalies, for example cleft face, short nose and exencephaly, were excluded. Anomalies of microphthalmia and anophthalmia appeared frequently but were

ignored in this investigations due to the difficulty associated with judging defects from an external perspective.

6-(5) Genotypic analysis in the *Otx2* heterozygous mutant mice

Genomic DNA was prepared by standard procedures (Sambrook et al., 1989). Genotypes were determined by PCR amplification of polymorphic DNA fragments containing simple sequence repeats (Dietrich et al., 1994).

SSLP markers were selected so as to provide a spacing interval of approximately 20 cM. Sequences of the PCR primers are described in http://www.informatics.jax.org. Primers for the majority of these markers were purchased from Research Genetics (Huntsville, AL).

For initial genome-wide linkage analysis in N2 mutant mice, used SSLP markers were 92. On the other hand, in N3 mutant mice, only 51 markers were able to use to genotype in animals displaying three phenotypes, normal mandible, small mandible or no mandible. SSLP markers that we tried to use were summarized (Table 1). Finally for subsequent fine mapping in the concerned chromosomes, 35 and 15 additional markers were used to genotype these N2 and N3 mice, respectively.

PCR reactions were prepared in the followings.

10×Taq polymerase buffer (Promega)	1.5 μl
25mM MgCl	1.2 μl
2mM dNTP	1.5 μl
Taq polymerase (TOYOBO)	0.3 μl
Forward primer (6.6 μM)	1.5 μl

Reverse primer (6.6 μ M)	1.5 μ l
Mouse genome DNA (20pg/ μ l)	1.5 μ l
H_2O	6.0 μ l

Samples were amplified under the following conditions:

95℃ 20sec.

56℃ 20sec.

72℃ 40sec.

×36 cycles

PCR products were separated on 3% agarose gels (generally clear resolution by greater than 5-bp difference was achieved with Agarose-1000, Gibco). Staining was effected with EtBr for genotype determination.

Linkage of indivisual SSLP marker was initially evaluated by a χ^2 test which was compared with frequencies of homozygous B6/B6 and heterozygous B6/CBA in the phenotypes respectively.

At first the number of mutant embryos classified into three phenotypes that were used for analyses were 63 in normal mandible, 70 in small mandible and 66 in no mandible in N2 mutant mice (Table 2). While the number of mutant embryos in N3 were 56, 22 and 64, respectively. Those markers showed $p>0.1$ in χ^2 test were not utilized for further anaryses. At other markers used to genotyping were repeated whenever several heterozygous mutant embryos were collected (Table 2, 3, 4). At neighbors of these markers indicated statistical significance ($p<0.05$, χ^2 test), we searched additional markers for subsequent fine mapping.

6-(6) Linkage analysis

Statistical analyses were performed by composite interval mapping (Zeng, 1994; Jiang and Zeng, 1995) across the genome in 2-cM intervals by QTL cartographer (Basten et al., 2001). LRS scores were converted to logarithm of odds ratios (LOD scores) via division by 4.6. Suggestive and significant linkages were defined in accordance with the guidelines of Lander and Kruglyak (1995) as Lod score thresholds 1.9 and 3.3, respectively. Statistical evidence of suggestive and significant linkages would be expected to occur one time and 0.05 times at random in a genome scan, respectively (Lander and Kruglyak, 1995). A transmission distortion was not detected in the ratio of alleles at five modifier loci identified in this study for 100 random heterozygous N2 or N3 backcross mutant embryos (data not shown).

Table 1- (1) Variant alleles between B6 and CBA

~ ; no detected variant allele

> and < ; difference in size can be resoluted clearly

	marker	cM	B6 bp	B6	CBA		marker	cM	B6 bp	B6	CBA		marker	cM	B6 bp	B6	CBA
Chr1	D1Mit64	5.0	119	>		Chr2	D2Mit1	1.0	124	~		Chr3	D3Mit164	2.4	135	>	
	D1Mit295	8.3	190	~			D2Mit175	2.0	109	~			D3Mit130	3.9	149	~	
	D1Mit294	8.3	150	<			D2Mit464	9.0	126	~			D3Mit62	4.6	128	>	
	D1Mit296	8.3	150	~			D2Mit6	10.0	132	~			D3Mit265	4.6	142	<	
	D1Mit430	10.0	114	<			D2Mit80	10.0	192	~			D3Mit304	5.6	100	~	
	D1Mit231	12.0	267	>			D2Mit465	10.0	128	~			D3Mit178	13.8	191	~	
	D1Mit211	15.0	139	<			D2Mit521	15.3	123	~			D3Mit21	19.2	236	>	
	D1Mit70	17.8	176	~			D2Mit293	11.0	180	~			D3Mit306	22.0	125	~	
	D1Mit411	18.5	112	>			D2Mit416	10.0	124	~			D3Mit6	23.3	147	>	
	D1Mit171	20.2	158	~			D2Mit82	14.5	198	~			D3Mit241	33.0	116	<	
	D1Mit322	23.6	316	>			D2Mit365	17.0	102	<			D3Mit40	39.7	140	>	
	D1Mit75	32.1	194	~			D2Mit296	18.0	154	~			D3Mit29	45.2	150	~	
	D1Mit480	32.8	170	>			D2Mit367	26.2	149	~			D3Mit14	64.1	142	<	
	D1Mit178	34.8	167	~			D2Mit369	27.3	129	>			D3Mit200	77.3	131	>	
	D1Mit380	36.9	112	~			D2Mit241	30.0	135	~			D3Mit19	87.6	160	<	
	D1Mit7	41.0	105	~			D2Mit458	31.7	122	>							
	D1Mit253	48.8	149	>			D2Mit244	33.0	130	~		Chr4	D4Mit149	0.0	114	<	
	D1Mit84	58.4	250	~			D2Mit91	37.0	180	<			D4Mit55	19.8	184	>	
	D1Mit135	59.7	177	~			D2Mit472	38.3	100	~			D4Mit152	40.0	143	~	
	D1Mit493	62.1	111	>			D2Mit11	42.6	226	~			D4Mit116	40.0	136	~	
	D1Mit191	63.1	189	<			D2Mit247	44.0	124	~			D4Mit185	43.0	108	~	
	D1Mit94	64.0	156	<			D2Mit329	44.8	126	~			D4Mit124	57.4	157	>	
	D1Mit218	67.0	147	~			D2Mit418	44.8	174	<			D4Mit54	66.0	150	<	
	D1Mit193	68.0	123	~			D2Mit37	45.0	174	<			D4Mit48	69.8	152	~	
	D1Mit102	73.0	120	<			D2Mit94	47.0	160	<			D4Mit64	69.8	190	~	
	D1Mit14	81.6	180	<			D2Mit14	49.0	142	>			D4Mit14	78.5	131	~	
	D1Mit106	85.0	120	~			D2Mit102	52.5	152	<			D4Mit226	78.5	123	>	
	D1Mit399	85.0	138	<			D2Mit17	69.0	205	<							
	D1Mit150	100.0	138	>			D2Mit259	80.0	147	~		Chr5	D5Mit11	26.0	206	>	
	D1Mit407	101.5	120	<			D2Mit282	83.0	144	>			D5Mit7	45.0	160	>	
	D1Mit221	102.0	132	<			D2Mit51	95.5	128	<			D5Mit24	60.0	174	<	
	D1Mit222	106.2	129	~			D2Mit229	99.0	142	<			D5Mit95	68.0	116	<	
	D1Mit462	107.2	121	~			D2Mit148	105.0	117	<			D5Mit168	80.0	152	>	
	D1Mit155	112.0	252	>									D5Mit223	89.0	105	<	

Table 1- (2) Variant alleles between B6 and CBA

~ ; no detected variant allele

> and < ; difference in size can be resoluted clearly

	marker	cM	B6 bp	B6	CBA		marker	cM	B6 bp	B6	CBA		marker	cM	B6 bp	B6	CBA
Chr6	D6Mit86	0.5	130	>		Chr9	D9Mit219	5.0	119	~		Chr11	D11Mit78	2.0	106	>	
	D6Mit138	0.7	111	~			D9Mit297	15.0	104	>			D11Mit53	16.0	232	<	
	D6Mit264	3.2	124	>			D9Mit67	17.0	124	<			D11Mit51	18.0	140	>	
	D6Mit159	7.0	116	<			D9Mit227	23.0	83	<			D11Mit20	20.0	116	<	
	D6Mit74	20.5	150	>			D9Mit130	27.0	121	<			D11Mit240	28.0	150	~	
	D6Mit354	25.5	172	~			D9Mit22	28.0	218	~			D11Mit349	32.0	118	>	
	D6Mit384	28.0	126	<			D9Mit162	30.0	140	~			D11Mit208	33.0	129	~	
	D6Mit243	30.5	118	~			D9Mit174	33.0	206	~			D11Mit177	36.0	124	>	
	D6Mit188	32.5	130	<			D9Mit302	35.0	106	~			D11Mit5	37.0	219	>	
	D6Mit213	37.0	150	>			D9Mit74	41.0	136	<			D11Mit60	40.0	316	~	
	D6Mit54	48.2	188	~			D9Mit75	41.0	196	~			D11Mit90	42.0	150	~	
	D6Mit14	74.0	160	~			D9Mit50	49.0	160	>			D11Mit194	44.0	134	>	
	D6Mit201	74.0	148	>			D9Mit355	53.0	121	<			D11Mit195	47.0	136	~	
							D9Mit346	55.0	124	>			D11Mit36	47.7	234	~	
Chr7	D7Mit178	0.5	201	>			D9Mit53	57.0	212	>			D11Mit38	49.0	76	<	
	D7Mit340	1.2	112	<			D9Mit51	61.0	124	~			D11Mit58	65.0	234	>	
	D7Mit267	11.0	196	~			D9Mit243	61.0	95	<			D11Mit203	74.5	141	>	
	D7Mit78	16.0	202	~			D9Mit18	71.0	180	<							
	D7Mit270	18.0	146	>								Chr12	D12Mit1	2.0	230	~	
	D7Mit27	23.0	248	~		Chr10	D10Mit166	4.0	112	>			D12Mit182	2.0	132	~	
	D7Mit229	23.0	123	~			D10Mit51	9.0	150	<			D12Mit105	6.0	140	>	
	D7Mit145	26.5	189	>			D10Mit194	29.0	70	<			D12Mit153	15.0	142	<	
	D7Mit84	28.4	170	<			D10Mit115	38.4	121	<			D12Mit46	16.0	136	<	
	D7Mit146	37.0	152	>			D10Mit186	40.0	134	~			D12Mit36	28.0	122	<	
	D7Mit31	44.0	246	>			D10Mit42	44.0	184	<			D12Mit5	37.0	176	>	
	D7Mit301	46.5	115	<			D10Mit117	48.0	142	~			D12Mit7	50.0	108	<	
	D7Mit222	52.6	147	>			D10Mit10	51.0	180	>			D12Mit280	55.0	126	<	
	D7Mit66	57.5	164	>			D10Mit14	65.0	192	~			D12Mit8	58.0	168	~	
	D7Mit109	66.0	112	>			D10Mit237	67.5	87	~			D12Mit263	58.0	114	<	

Table 1- (3) Variant alleles between B6 and CBA

~ ; no detected variant allele

> and < ; difference in size can be resoluted clearly

	marker	cM	B6 bp	B6	CBA		marker	cM	B6 bp	B6	CBA		marker	cM	B6 bp	B6	CBA
Chr13	D13Mit3	10.0	159	<		Chr16	D16Mit182	3.4	215	>			D18Mit185	43.0	100	>	
	D13Mit94	31.0	155	<			D16Mit154	3.4	146	~			D18Mit186	45.0	126	>	
	D13Mit139	32.0	139	<			D16Mit129	3.4	148	~			D18Mit141	45.0	132	>	
	D13Mit13	35.0	148	>			D16Mit122	3.8	112	>			D18Mit188	47.0	100	<	
	D13Mit107	48.0	150	>			D16Mit9	4.0	146	>			D18Mit49	49.0	152	~	
	D13Mit76	61.0	106	>			D16Mit130	4.0	146	>			D18Mit162	50.0	96	~	
	D13Mit262	68.0	126	>			D16Mit131	4.3	144	<			D18Mit106	50.0	115	~	
	D13Mit151	71.0	123	>			D16Mit81	8.0	117	>			D18Mit3	54.0	184	~	
	D13Mit77	73.0	280	~			D16Mit146	16.9	116	~			D18Mit213	55.0	126	>	
	D13Mit35	75.0	190	>			D16Mit103	21.5	103	~			D18Mit4	57.0	210	~	
							D16Mit58	23.3	159	>							
Chr14	D14Mit10	1.5	132	>			D16Mit4	27.3	132	>		Chr19	D19Mit43	0.5	160	~	
	D14Mit121	17.0	149	>			D16Mit125	29.0	150	~			D19Mit109	4.0	126	>	
	D14Mit102	28.5	124	<			D16Mit5	38.0	156	<			D19Mit41	16.0	160	<	
	D14Mit185	54.0	142	>			D16Mit189	55.2	199	>			D19Mit117	22.0	110	>	
							D16Mit106	71.5	148	>			D19Mit46	24.0	115	<	
Chr15	D15Mit53	12.3	140	<									D19Mit53	43.0	110	>	
	D15Mit100	21.0	111	~		Chr17	D17Mit133	10.4	195	>			D19Mit70	51.0	196	~	
	D15Mit141	22.2	127	~			D17Mit22	19.0	157	~			D19Mit1	52.0	121	<	
	D15Mit63	29.2	146	>			D17Mit66	24.5	132	>			D19Mit33	53.0	252	<	
	D15Mit156	39.1	145	>			D17Mit20	34.3	180	>			D19Mit6	55.0	112	~	
	D15Mit239	40.9	112	>			D17Mit142	47.4	147	>			D19Mit137	55.7	122	<	
	D15Mit105	42.0	125	<			D17Mit123	56.6	133	<							
	D15Mit29	42.8	152	~								ChrX	DXMit124	2.8	180	~	
	D15Mit188	44.1	163	~		Chr18	D18Mit66	2.0	140	~			DXMit89	3.0	149	~	
	D15Mit241	46.4	102	~			D18Mit68	11.0	113	>			DXMit54	3.8	192	~	
	D15Mit189	48.5	128	~			D18Mit94	17.0	149	~			DXMit81	9.3	199	~	
	D15Mit96	48.9	140	~			D18Mit14	18.0	105	>			DXMit166	15.5	114	>	
	D15Mit159	49.6	146	~			D18Mit17	20.0	213	>			DXMit1	29.0	97	<	
	D15Mit34	52.2	148	~			D18Mit58	24.0	186	<			DXMit172	47.0	148	>	
	D15Mit245	58.9	119	>			D18Mit53	27.0	144	>			DXMit173	49.2	125	~	
	D15Mit161	69.2	128	~			D18Mit123	31.0	116	<			DXMit130	58.0	168	~	
							D18Mit52	32.0	140	~			DXMit153	62.4	145	~	
							D18Mit51	37.0	198	>			DXMit189	65.6	120	~	
							D18Mit50	41.0	154	<			DXMit135	69.0	118	~	
							D18Mit9	42.0	170	>			DXMit186	69.0	128	>	

Table 2-(1) Linkage of marker loci to phenotype in N2 backcross mutant mice *

			no mandible					small mandible					normal mandible				
				Homo	Hetero		p<0.05		Homo	Hetero		p<0.05		Homo	Hetero		p<0.05
Chr	SSLP	cM	n	B6/B6	B6/CBA	χ2	p	n	B6/B6	B6/CBA	χ2	p	n	B6/B6	B6/CBA	χ2	p
1	D1Mit64	5.0	95	50	45	0.26		101	50	51	0.01		66	35	31	0.24	
	D1Mit211	15.0	63	34	29	0.40		101	52	49	0.09		66	33	33	0.00	
	D1Mit253	48.8	63	31	32	0.02		101	49	52	0.09		66	30	36	0.55	
	D1Mit191	63.1	63	32	31	0.02		101	53	48	0.25		66	31	35	0.24	
	D1Mit14	81.6	63	30	33	0.14		101	60	41	3.57		66	30	36	0.55	
	D1Mit150	100.0	92	49	43	0.39		117	68	49	3.09		66	32	34	0.06	
	D1Mit221	102.0	143	76	67	0.57		117	67	50	2.47		66	33	33	0.00	
	D1Mit155	112.0	143	80	63	2.02		117	71	46	5.34	0.02084	66	36	30	0.55	
2	D2Mit365	17.0	143	95	48	15.45	0.00009	181	82	99	1.60		114	55	59	0.14	
	D2Mit369	27.0	143	91	52	10.64	0.00113	182	85	97	0.79		114	53	61	0.56	
	D2Mit458	31.7	143	88	55	7.62	0.00577	182	85	97	0.79		114	53	61	0.56	
	D2Mit418	45.0	143	86	57	5.88	0.01531	165	87	78	0.49		112	53	59	0.14	
	D2Mit14	49.0	143	84	59	4.37	0.03658	182	96	86	0.55		114	54	60	0.32	
	D2Mit102	53.0	143	87	56	6.72	0.00953	181	101	80	2.44		114	55	59	0.14	
	D2Mit395	66.9	143	89	54	8.57	0.00342	181	101	80	2.44		114	53	61	0.56	
	D2Mit17	69.0	143	87	56	6.72	0.00953	182	103	79	3.16		112	53	59	0.32	
	D2Mit164	71.0	143	87	56	6.72	0.00953	181	103	78	3.45		112	53	59	0.32	
	D2Mit282	83.0	143	84	59	4.37	0.03658	181	105	76	4.65	0.03105	112	51	61	0.89	
	D2Mit263	92.0	143	80	63	2.02		181	102	79	2.92		112	53	59	0.32	
	D2Mit229	99.0	143	72	71	0.01		181	102	79	2.92		114	55	59	0.14	
	D2Mit148	105.0	143	67	76	0.57		182	93	89	0.09		114	56	58	0.04	
3	D3Mit164	2.4	143	84	59	4.37	0.03658	70	34	36	0.06		66	30	36	0.55	
	D3Mit62	4.6	143	85	58	5.10	0.02393	70	35	35	0.00		66	30	36	0.55	
	D3Mit21	19.2	143	87	56	6.72	0.00953	117	56	61	0.21		66	30	36	0.55	
	D3Mit6	23.3	143	86	57	5.88	0.01531	70	32	38	0.51		66	29	37	0.97	
	D3Mit40	39.7	143	80	63	2.02		70	32	38	0.51		66	29	37	0.97	
	D3Mit14	64.1	143	75	68	0.34		70	34	36	0.06		66	31	35	0.24	
	D3Mit200	77.3	63	35	28	0.78		70	39	31	0.91		66	34	32	0.06	
	D3Mit19	87.6	92	53	39	2.13		70	37	33	0.23		66	34	32	0.06	
4	D4Mit149	0.0	63	33	30	0.14		70	38	32	0.51		66	27	39	2.18	
	D4Mit55	19.8	63	33	30	0.14		70	38	32	0.51		66	25	41	3.88	0.04886
	D4Mit124	57.4	63	29	34	0.40		70	36	34	0.06		66	30	36	0.55	
	D4Mit54	66.0	63	27	36	1.29		70	36	34	0.06		66	32	34	0.06	
	D4Mit226	78.5	63	28	35	0.78		70	37	33	0.23		66	36	30	0.55	
5	D5Mit11	26.0	63	27	36	1.29		70	30	40	1.43		66	31	35	0.24	
	D5Mit7	45.0	63	30	33	0.14		70	38	32	0.51		66	29	37	0.97	
	D5Mit24	60.0	63	32	31	0.02		70	38	32	0.51		66	25	41	3.88	0.04886
	D5Mit95	68.0	63	33	30	0.14		70	39	31	0.91		66	26	40	2.97	
	D5Mit168	80.0	63	32	31	0.02		70	36	34	0.06		66	24	42	4.91	0.02670
	D5Mit223	89.0	63	34	29	0.40		70	37	33	0.23		66	24	42	4.91	0.02670
6	D6Mit86	0.5	63	30	33	0.14		101	44	57	1.67		66	28	38	1.52	
	D6Mit74	20.5	92	40	52	1.57		101	52	49	0.09		66	35	31	0.24	
	D6Mit188	32.5	63	28	35	0.78		117	67	50	2.47		66	37	29	0.97	
	D6Mit213	37.0	63	28	35	0.78		117	70	47	4.52	0.03350	66	36	30	0.55	
	D6Mit149	46.3						117	63	54	0.69						
	D6Mit201	74.0	63	34	29	0.40		117	63	54	0.69		66	29	37	0.97	

Table 2-(2) Linkage of marker loci to phenotype in N2 backcross mutant mice *

			no mandible					small mandible					normal mandible				
Chr	SSLP	cM	n	Homo B6/B6	Hetero B6/CBA	χ2	p<0.05 p	n	Homo B6/B6	Hetero B6/CBA	χ2	p<0.05 p	n	Homo B6/B6	Hetero B6/CBA	χ2	p<0.05 p
7	D7Mit178	0.5	143	80	63	2.02		117	70	47	4.52	0.03350	66	34	32	0.06	
	D7Mit270	18.0	143	75	68	0.34		117	72	45	6.23	0.01256	66	35	31	0.24	
	D7Mit145	26.5	92	47	45	0.04		117	71	46	5.34	0.02084	66	36	30	0.55	
	D7Mit222	52.6	92	44	48	0.17		117	68	49	3.09		66	31	35	0.24	
	D7Mit66	57.5	92	45	47	0.04		117	67	50	2.47		66	30	36	0.55	
	D7Mit109	66.0	92	53	39	2.13		101	55	46	0.80		66	29	37	0.97	
8	D8Mit3	10.0	63	35	28	0.78		70	37	33	0.23		66	29	37	0.97	
	D8Mit25	32.0	63	32	31	0.02		70	37	33	0.23		66	30	36	0.55	
	D8Mit33	45.0	63	33	30	0.14		70	34	36	0.06		66	28	38	1.52	
	D8Mit200	58.0	63	35	28	0.78		70	34	36	0.06		66	31	35	0.24	
	D8Mit156	73.0	63	35	28	0.78		70	34	36	0.06		66	31	35	0.24	
9	D9Mit64	7.0	143	67	76	0.57		117	75	42	9.31	0.00190	66	35	31	0.24	
	D9Mit90	10.0						117	74	43	8.21	0.00417					
	D9Mit297	15.0						117	74	43	8.21	0.00417					
	D9Mit67	17.0	143	66	77	0.85		117	74	43	8.21	0.00417	66	31	35	0.24	
	D9Mit227	22.0						117	72	45	6.23	0.01256					
	D9Mit130	27.0	63	30	33	0.14		117	73	44	7.19	0.00733	66	33	33	0.00	
	D9Mit53	57.0	63	31	32	0.02		117	65	52	1.44						
	D9Mit18	71.0	63	27	36	1.29		117	55	62	0.42		66	36	30	0.55	
10	D10Mit166	4.0	143	68	75	0.34		166	82	84	0.02		114	56	58	0.04	
	D10Mit194	29.0	143	79	64	1.57		165	88	77	0.73		114	52	62	0.88	
	D10Mit274	34.0	143	91	52	10.64	0.00111	165	88	77	0.73		112	53	59	0.32	
	D10Mit115	38.4	143	93	50	12.93	0.00032	182	96	86	0.55		114	55	59	0.14	
	D10Mit42	44.0	143	89	54	8.57	0.00342	182	100	82	1.78		114	55	59	0.14	
	D10Mit10	51.0	143	84	59	4.37		182	98	84	1.08		114	62	52	0.88	
11	D11Mit78	2.0	143	65	78	1.18		117	68	49	3.09		66	27	39	2.18	
	D11Mit295	11.0	143	64	79	1.57											
	D11Mit340	11.0	143	61	82	3.08											
	D11Mit185	12.0	143	61	82	3.08											
	D11Mit53	16.0	143	57	86	5.88	0.01531	117	61	56	0.21		66	28	38	1.52	
	D11Mit231	17.0	143	56	87	6.72	0.00953										
	D11Mit51	18.0	143	55	88	7.62	0.00577	117	55	62	0.42		66	28	38	1.52	
	D11Mit20	20.0	143	59	84	4.37	0.03658	117	58	59	0.01		66	30	36	0.55	
	D11Mit349	32.0	143	58	85	5.10	0.02393	117	58	59	0.01						
	D11Mit5	37.0	143	62	81	2.52		117	54	63	0.69		66	23	43	6.06	0.01383
	D11Mit194	44.0	143	61	82	3.08							66	23	43	6.06	0.01383
	D11Mit38	49.0	143	58	85	5.10	0.02393	117	55	62	0.42		66	25	41	3.88	0.04886
	D11Mit58	65.0	143	67	76	0.57		70	38	32	0.51		66	27	39	2.18	
	D11Mit203	74.5	143	67	76	0.57		70	39	31	0.91		66	26	40	2.97	
12	D12Mit105	6.0	92	50	42	0.70		117	68	49	3.09		66	32	34	0.06	
	D12Mit46	16.0	124	69	55	1.58											
	D12Mit36	28.0	124	73	51	3.90	0.04829	117	65	52	1.44		66	36	30	0.55	
	D12Mit5	37.0	92	50	42	0.70		101	57	44	1.67		66	32	34	0.06	
	D12Mit7	50.0	63	33	30	0.14		101	56	45	1.20		66	36	30	0.55	

Table 2-(3) Linkage of marker loci to phenotype in N2 backcross mutant mice *

			no mandible					small mandible					normal mandible				
				Homo	Hetero		p<0.05		Homo	Hetero		p<0.05		Homo	Hetero		p<0.05
Chr	SSLP	cM	n	B6/B6	B6/CBA	χ2	p	n	B6/B6	B6/CBA	χ2	p	n	B6/B6	B6/CBA	χ2	p
13	D13Mit3	10.0	63	32	31	0.02		101	51	50	0.01		66	31	35	0.24	
	D13Mit13	35.0	63	32	31	0.02		101	59	42	2.86		66	35	31	0.24	
	D13Mit107	48.0	63	29	34	0.40		117	69	48	3.77		66	34	32	0.06	
	D13Mit287	57.0	92	49	43	0.39		117	70	47	4.52	0.03350					
	D13Mit76	61.0	63	29	34	0.40		117	69	48	3.77		66	36	30	0.55	
	D13Mit151	71.0	63	28	35	0.78		101	59	42	2.86		66	35	31	0.24	
15	D15Mit53	12.3	63	34	29	0.40		117	48	69	3.77		66	36	30	0.55	
	D15Mit63	29.2	63	34	29	0.40		70	35	35	0.00		66	37	29	0.97	
	D15Mit239	40.9	63	29	34	0.40		70	37	33	0.23		66	34	32	0.06	
	D15Mit245	58.9	63	29	34	0.40		70	36	34	0.06		66	34	32	0.06	
16	D16Mit9	4.0	92	40	52	1.57		70	40	30	1.43		66	28	38	1.52	
	D16Mit4	27.3	92	41	51	1.09		70	37	33	0.23		66	31	35	0.24	
	D16Mit5	38.0	92	42	50	0.70		70	36	34	0.06		66	33	33	0.00	
	D16Mit106	71.5	63	28	35	0.78		70	32	38	0.51		66	35	31	0.24	
17	D17Mit143	5.0	143	84	59	4.37	0.03658										
	D17Mit133	10.0	143	81	62	2.52		70	41	29	2.06		66	39	27	2.18	
	D17Mit148	20.3	143	81	62	2.52											
	D17Mit66	25.0	143	85	58	5.10	0.02393	70	39	31	0.91		66	36	30	0.55	
	D17Mit88	30.0	143	87	56	6.72	0.00953	117	69	48	3.77						
	D17Mit20	35.0	143	88	55	7.62	0.00577	117	69	48	3.77		66	38	28	1.52	
	D17Mit193	41.0	143	88	55	7.62	0.00577	117	66	51	1.92						
	D17Mit142	47.0	143	82	61	3.08		70	35	35	0.00		66	36	30	0.55	
	D17Mit123	57.0	143	84	59	4.37	0.03658	70	35	35	0.00		66	33	33	0.00	
18	D18Mit110	4.0	143	51	92	11.76	0.00061	181	76	105	4.65	0.03105	112	64	48	2.29	
	D18Mit68	11.0	143	51	92	11.76	0.00061	181	79	102	2.92		114	64	50	1.72	
	D18Mit120	16.0	143	57	86	5.88	0.01531	181	79	102	2.92		112	65	47	2.89	
	D18Mit14	18.0	143	58	85	5.10	0.02393	182	77	105	4.31	0.03789	114	65	49	2.25	
	D18Mit17	20.0	143	56	87	6.72	0.00953	182	78	104	3.71		114	64	50	1.72	
	D18Mit58	24.0	143	57	86	5.88	0.01531	181	77	104	4.03	0.04470	114	64	50	1.72	
	D18Mit53	27.0	143	60	83	3.70		181	77	104	4.03	0.04470	114	65	49	2.25	
	D18Mit51	37.0	143	66	77	0.85		182	78	104	3.71		114	64	50	1.72	
	D18Mit9	42.0	143	70	73	0.06		182	81	101	2.20		114	58	56	0.04	
	D18Mit213	55.0	143	78	65	1.18		182	89	93	0.09		114	57	57	0.00	
19	D19Mit109	4.0	63	31	32	0.02		70	35	35	0.00		66	35	31	0.24	
	D19Mit46	24.0	63	27	36	1.29		70	36	34	0.06		66	36	30	0.55	
	D19Mit1	52.0	63	30	33	0.14		70	36	34	0.06		66	29	37	0.97	
	D19Mit137	55.7	63	28	35	0.78		70	35	35	0.00		66	31	35	0.24	

* Listed are the SSLPs that were used (SSLP), the chromosomes are on which they are located (Chr), the distance frome the centromere in cM (cM), the number of mice that are homozygous (Homo B6/B6) and heterozygous (Hetero B6/CBA), the total number of N2 mice tested (n), the resulting χ^2 value (χ^2) and p value (p)

Table 3. Linkage of marker loci to phenotype in N3 backcross mutant mice *

			no mandible					small mandible					normal mandible				
				Homo	Hetero		p<0.05		Homo	Hetero		p<0.05		Homo	Hetero		p<0.05
Chr	SSLP	cM	n	B6/B6	B6/CBA	$\chi 2$	p	n	B6/B6	B6/CBA	$\chi 2$	p	n	B6/B6	B6/CBA	$\chi 2$	p
1	D1Mit64	5.0	72	48	24	8.00	0.00468	50	29	21	1.28		64	28	36	1.00	
	D1Mit211	15.0	72	46	26	5.56	0.01838	50	27	23	0.32						
	D1Mit480	32.8	72	44	28	3.56		50	23	27	0.32						
	D1Mit253	48.8	72	41	31	1.39		50	23	27	0.32						
	D1Mit191	63.1	72	37	35	0.06		50	21	29	1.28						
2	D2Mit365	17.0	72	46	26	5.56	0.01838	50	25	25	0.00		64	32	32	0.00	
	D2Mit369	27.0	72	47	25	6.72	0.00953	50	24	26	0.08		64	31	33	0.06	
	D2Mit458	31.7	72	48	24	8.00	0.00468	50	24	26	0.08		64	31	33	0.06	
	D2Mit418	45.0	72	47	25	6.72	0.00953	50	24	26	0.08						
	D2Mit14	49.0	72	49	23	9.39	0.00218	50	24	26	0.08		64	26	38	2.25	
	D2Mit102	53.0	72	51	21	12.50	0.00041	50	24	26	0.08		64	24	40	4.00	0.04550
	D2Mit395	66.9	72	53	19	16.06	0.00006	50	23	27	0.32		64	23	41	5.06	0.02448
	D2Mit17	69.0	72	53	19	16.06	0.00006	50	23	27	0.32		64	22	42	6.25	0.01242
	D2Mit164	71.0	72	53	19	16.06	0.00006	50	23	27	0.32		64	22	42	6.25	0.01242
	D2Mit282	83.0	72	49	23	9.39	0.00218	50	25	25	0.00		64	16	48	16.00	0.00006
	D2Mit263	92.0	72	44	28	3.56		50	29	21	1.28		64	15	49	18.06	0.00002
	D2Mit229	99.0	72	45	27	4.50	0.03389	50	30	20	2.00		64	15	49	18.06	0.00002
	D2Mit148	105.0	72	42	30	2.00		50	29	21	1.28		64	15	49	18.06	0.00002
4	D4Mit149	0.0	56	25	31	0.64		50	22	28	0.72						
	D4Mit55	19.8	56	28	28	0.00		22	10	12	0.18						
	D4Mit111	21.9	56	29	27	0.07		22	10	12	0.18						
5	D5Mit11	26.0	72	30	42	2.00		50	20	30	2.00						
	D5Mit7	45.0	72	33	39	0.50		50	22	28	0.72						
	D5Mit24	60.0	56	25	31	0.64		22	10	12	0.18						
	D5Mit95	68.0	56	27	29	0.07		22	11	11	0.00						
	D5Mit168	80.0	56	32	24	1.14		22	12	10	0.18						
	D5Mit223	89.0	56	30	26	0.29		22	11	11	0.00						
8	D8Mit3	10.0	72	35	37	0.06		50	18	32	3.92	0.04771	64	24	40	4.00	0.04550
	D8Mit25	32.0	56	29	27	0.07		50	21	29	1.28						
	D8Mit33	45.0	56	28	28	0.00		22	12	10	0.18						
	D8Mit200	58.0	56	28	28	0.00		22	12	10	0.18						
9	D9Mit297	15.0						50	28	22	0.72						
	D9Mit67	17.0	72	41	31	1.39		50	28	22	0.72						
	D9Mit227	22.0						50	27	23	0.32						
	D9Mit130	27.0	72	38	34	0.22		22	10	12	0.18						
	D9Mit53	57.0	56	26	30	0.29		22	11	11	0.00						
	D9Mit18	71.0	56	29	27	0.07		22	13	9	0.73						
10	D10Mit166	4.0	56	23	33	1.79		50	28	22	0.72						
11	D11Mit78	2.0	72	27	45	4.50	0.03389	50	24	26	0.08						
	D11Mit185	12.0	72	28	44	3.56											
	D11Mit51	18.0	72	27	45	4.50	0.03389	50	24	26	0.08		64	35	29	0.56	
	D11Mit20	20.0	72	29	43	2.72		22	11	11	0.00						
	D11Mit5	37.0	72	32	40	0.89		22	12	10	0.18						
	D11Mit38	49.0	72	35	37	0.06		50	23	27	0.32						
	D11Mit58	65.0	72	46	26	5.56	0.01838	50	20	30	2.00						
	D11Mit203	74.5	72	47	25	6.72	0.00953	50	21	29	1.28		64	33	31	0.06	
13	D13Mit3	10.0	72	32	40	2.57		50	30	20	2.00						
	D13Mit13	35.0	56	29	27	0.07		50	31	19	2.88						
	D13Mit107	48.0	72	39	33	0.07		50	31	19	2.88						
	D13Mit287	57.0	72	44	28	3.56		50	31	19	2.88		64	23	41	5.06	0.02448
	D13Mit76	61.0	72	42	30	0.64		50	30	20	2.00						
	D13Mit151	71.0	56	31	25	0.64		50	29	21	1.28						
15	D15Mit53	12.3	72	31	41	1.39		50	19	31	2.88		64	43	21	7.56	0.00597
	D15Mit63	29.2	56	30	26	0.29		50	23	27	0.32						
	D15Mit239	40.9	56	29	27	0.07		50	24	26	0.08						
16	D16Mit9	4.0	56	24	32	1.14		50	28	22	0.72						
	D16Mit4	27.3	56	23	33	1.79		50	27	23	0.32						
	D16Mit5	38.0	56	25	31	0.64		50	23	27	0.32						
	D16Mit106	71.5	56	27	29	0.07		50	25	25	0.00						
18	D18Mit17	20.0	72	34	38	0.22		50	27	23	0.32						
	D18Mit58	24.0						50	25	25	0.00						
	D18Mit53	27.0	56	28	28	0.00		50	25	25	0.00						
	D18Mit51	37.0	56	31	25	0.64		50	25	25	0.00						
	D18Mit9	42.0	56	32	24	1.14		50	22	28	0.72						
	D18Mit213	55.0	56	32	24	1.14		50	21	29	1.28						
19	D19Mit137	55.7	72	33	39	0.50		22	10	12	0.1818						

* Listed are the SSLPs that were used (SSLP), the chromosomes are on which they are located (Chr), the distance frome the centromere in cM (cM), the number of mice that are homozygous (Homo B6/B6) and heterozygous (Hetero B6/CBA), the total number of N2 mice tested (n), the resulting χ^2 value (χ^2) and p value (p)

7. Results

7-(1) Variation and classification of *Otx2* heterozygous mutant phenotypes

In an effort to map loci responsible for modification of the severity of craniofacial defects in *Otx2* heterozygous mutant mice, two strains of mice, displaying disparate phenotypes of *Otx2* heterozygosity, were utilized (Fig. 1, 2). *Otx2* mutant chimeras employing a TT2 ES cell line derived from F1 embryos of B6 females and CBA males have been generated (Yagi et al., 1993; Matsuo et al., 1995). Upon backcross of chimeric males with wild type B6 females to generate heterozygous mutant mice, severe craniofacial malformations occurred in the majority of the *Otx2* heterozygous mutants at 18.5 dpc (Fig. 1, 2). External abnormalities were mainly characterized as the reduction or loss of the lower jaw and eyes (Fig. 1D, E and 2). Additionally, the severity of the phenotype varied greatly from a normal condition to the appearance of acephaly (Fig. 1C-I). In contrast, when chimeric males were backcrossed with wild type CBA females, craniofacial malformations were not observed (Fig. 1B).

Initially, in order to investigate the variation of severity of craniofacial malformations, the chimeras were backcrossed with wild type B6 females, resulting in N2 heterozygous offspring. Subsequently, the external abnormalities of these offspring were examined at 18.5 dpc (Fig. 1, 2A). Descriptions of eye and holoprosencephaly malformations were excluded in this investigation due to the difficulty associated with judging defects from an

external perspective; moreover, further histological analysis is required for the precise description of these abnormalities (Matsuo et al., 1995). 37% of heterozygous pups (N2) did not exhibit prominent abnormalities in jaw, nose or head externally (Fig. 1C, 2A). 19% and 21.5% of these offspring displayed reduction and absence of the lower jaw, respectively (Fig. 1D, E and 2A). A small percentage of mutants exhibited excencephaly (7.0%; Figs. 1F and 2A), short nose (3.0%; Fig. 1G, 2A), cleft face (2.0%; Fig. 1H, 2A) and acephaly, showing loss of the entire head (5.5%; Fig. 1I, 2A). The remaining small percentage of mutants revealed additional phenotypes, including ethmocephaly (5.0 %; Fig. 2A; and data not shown). Consequently, the distribution of these craniofacial abnormalities is characteristic of a monogenic trait that is caused by modifier loci (Fig. 1, 2; Lander and Schork, 1994).

The most frequently observed phenotype was lower jaw abnormality; consequently, we focused on the jaw anomalies. In order to investigate the phenotype of lower jaws more precisely, the morphology of the mandibular skull following bone and cartilage staining by alcian blue and alizarin red was further examined (Fig. 3). It was found that even in reduced lower jaws, mandible formation was affected to varying extents, ranging from simple fusion of the anterior tips of the incisors to involution of the entire mandible in a small single median bone (Fig. 3F,H,J). Furthermore, to determine the severity of the anomalous mandibles, the length of each was measured (Fig. 3, 4). Normally, lengths of wild type mandibles of B6 and CBA strains were consistently longer than 5.0 mm at 18.5 dpc (Fig. 3B, 4A and data not shown). Similarly, lengths of *Otx2* heterozygous mutant mandibles on CBA genetic

background were also longer than 5.0 mm (data not shown). In contrast, mandibles of heterozygous mutants backcrossed to B6 females exhibited varying lengths (Fig. 3, 4B). Mutant mandibles demonstrated lengths in excess of 5.0 mm as well as in the range of 0.5 to 4.9 mm; additionally, the mandible was absent in several samples (Fig. 3C-K, 4B).

In order to define the genetics underlying this dramatic variation in mandible phenotype, a whole genome search for modifier loci involved in the modulation of mandible abnormalities was conducted. Thus, all mutant individuals exhibiting no apparent abnormalities, reduction of lower jaw and loss of lower jaw (Fig. 2), were genotyped; however, mutant embryos displaying other external phenotypes, such as excencephaly, short nose, cleft face, acephaly, etc., were not investigated with respect to further genotyping experiments (Fig. 1, 2).

7-(2) Linkage analysis using N2 offspring

It is hypothesized that the variable severity in the *Otx2* heterozygous mutant mandible of the B6 strain was due to the variation in genetic background, particularly involving modifier loci, the alleles of which differed between B6 and CBA. With the discovery of the highly polymorphic and simple genotyping protocols of simple sequence length polymorphisms (SSLPs) (Love et al., 1990), these markers are very applicable to the mapping of the location of genetic loci involved in genetic background-dependent phenotypic differences. However, the utility of CBA for genetic mapping studies has been limited by the lack of information regarding DNA variants

alleles (Dietrich et al., 1992, 1994; http://www.informatics.jax.org/). In order to map locations of modifiers of *Otx2* mutant mice, we first surveyed variant SSLP markers between CBA and B6 strains for the entire genome scan (Fig. 5). Of the 293 markers tested, 180 were variant based on agarose gel electrophoresis (Table 1). This rate of variant alleles is comparable to that observed in other inbred laboratory mouse strains (Dietrich et al. 1994). Given the high frequency of variant alleles and large litter size, the CBA strain could be useful in mapping studies of genetic modifiers in transgenic or knock-out mice that are widely generated in the CBA and B6 genetic background.

In order to map the regions of the genome containing modifying loci, 199 mutant pups displaying no apparent abnormalities, reduction of lower jaw and loss of lower jaw (Fig. 1, 2) were initially selected from the first generation of B6 backcrossed animals (N2). These were subjected to further skull staining and the lengths of each mandible were measured. Then, the severity of the lower jaws phenotypes was designated as normal mandible (the mandible length is longer than 5.0 mm), small mandible (the mandible length corresponds to 0.5 to 4.9 mm) and no mandible (the length is 0 mm). Subsequently, 92 SSLP markers were chosen, covering approximately 20-cM intervals throughout the entire genome with the exception of two chromosomes: chromosome 14, on which the *Otx2* gene is located, and chromosome X, which is derived solely from the B6 strain. These 199 offspring were then genotyped using 92 PCR markers for the initial genome scan (Fig. 5). Markers (e.g., chromosomes 2, 10 and 18) showing trends for the potential linkage ($p<0.05$) were subjected to extended genotyping so as to

include a total of 439 mutant embryos displaying the phenotype of normal mandible, small mandible or no mandible together with 35 additional microsatellite DNA markers surrounding potential loci (Fig. 6, Table 1, 2).

Thus, linkage analysis was conducted with the composite interval mapping of QTL-cartographer program (Fig. 6; Basten et al., 2001); in addition, to investigate whether the genetic loci can modify the phenotype for small mandible (the mandible length corresponds to 0.5 to 4.9 mm) or no mandible (0 mm) qualitatively, genetic analysis was also performed with mutant individuals displaying normal mandible (the mandible length is longer than 5.0 mm) and no mandible or those displaying normal mandible and small mandible, respectively (Fig. 6). Consequently, one significant linkage on chromosome 18, which was defined as *Otx2* modifier (*Otmf*) *18*, was obtained exhibiting a peak LOD score of 3.33 at 11.1 cM (Fig. 6C, Table 2). One suggestive linkage was found on chromosome 10, with a peak LOD score of 2.56 at 38.1 cM (Fig. 6B, Table 2). These two loci exert effects on both the no mandible and small mandible phenotypes (Fig. 6B, C, Table 2). Unexpectedly, *Otmf18* was derived from the CBA strain (Table 2), suggesting epistatic interactions between modifiers. Additionally, two weak linkages were also detected on chromosome 2; these linkages exhibited peak LOD scores of 1.59 at 17 cM and 1.8 at 66.9 cM, respectively (Fig. 6A). Thus, these findings acquired via the survey of N2 offspring indicate that at least one modifier locus *Otmf18* is significantly involved in the severity of mandible phenotypes in *Otx2* mutant embryos.

7-(3) linkage analysis using N3 offspring

As described previously, small numbers of N2 backcross heterozygous mutant mice survived to weaning, followed by fertility that afforded further progeny (Fig. 1, 2; Matsuo et al., 1995). In order to confirm and refine modifier location, a single N2 male was selected; subsequently, allele distribution between B6 and CBA was genotyped employing the 92 polymorphic markers from the N2 initial genome scan (Fig. 5). Chromosomes 3, 6, 7, 12 and 17 were found to be homozygous for B6 in this male mouse; chromosomes 2, 5, 9, 11, 13 and 16 were heterozygous for CBA, whereas chromosomes 1, 4, 8, 10, 15, 18 and 19 contained both B6 homozygous and B6 and CBA heterozygous regions, respectively (Fig. 5; marked in gray or black color). Consequently, among the chromosomes on which the modifier candidates by N2 linkage analysis were located, chromosome 2 was heterozygous for B6 and CBA and chromosomes 10 and 18 were homozygous for B6. Thus, the latter two chromosomes were excluded from further N3 analysis.

Next, this N2 male was backcrossed with wild type B6 females, resulting in heterozygous N3 animals. External phenotypes were classified as described above (Fig. 1). The frequency of external phenotype in these N3 mutant embryos is summarized in Fig. 2B. 29% of heterozygous pups did not display prominent abnormalities in jaw, nose or head (Fig. 2B). 28.5 and 31.5% of mutant progeny exhibited reduction of and loss of the lower jaw, respectively (Fig. 2B). All mutant animals exhibiting no apparent abnormalities, reduction of lower jaw, and loss of lower jaw (Fig. 2), were

subjected to skeletal staining and the lengths of each mandible were measured (Fig. 4C). Then, the severity of the mandibular phenotype was designated as normal mandible (the mandible length is longer than 5.0 mm), small mandible (the mandible length corresponds to 0.5 to 4.9 mm) and no mandible (the length is 0 mm). For the modifier mapping, these 202 mutant N3 pups were genotyped initially with 51 microsatellite markers which were not homozygous for B6 strain allele in this male (Fig. 5, marked in gray color). Thus, genetic analysis was conducted with the composite interval mapping of QTL-cartographer program as described (Fig. 7; Basten et al., 2001). For markers (e.g., chromosome 2) with potential linkage ($p<0.05$), extended genotyping was performed along with 16 additional SSLP markers (Fig. 7, Table 1). Additionally, to investigate whether the loci can modify the phenotype for small mandible or no mandible qualitatively, genetic analysis was also performed with mutant individuals displaying normal mandible and no mandible or those displaying normal mandible and small mandible, respectively (Fig. 7). Consequently, we found that one significant locus was mapped on chromosome 2. *Otmf2*, which was also linked weakly in the N2 linkage analysis (Fig. 6A), regulates the phenotype displaying no mandible with a peak LOD score of 3.93 at 77 cM (Fig. 7). Furthermore, one suggestive locus, which was characterized by a peak LOD score of 3.13 at 96 cM on chromosome 2, regulates the phenotype of the small mandible (Fig. 7). The above results, in conjunction with N2 linkage data, indicated that at least two distinct modifier loci, *Otmf2* and *Otm18*, regulate the severity of the otocephalic phenotypes in *Otx2* heterozygous mutant mice.

8. Discussion

In the present investigation, genetic linkage analysis (Lander and Botstein, 1989; Lander and Kruglyak, 1995; Darvasi, 1998) was employed to identify genetic loci modifying the otocephalic phenotype in *Otx2* heterozygous mutant mice. A genome-wide screen comparing the pattern of strain means to the severity with SSLP markers detected two significant modifier loci, *Otmf2* and *Otmf18* (Fig. 6, 7; Table 4). This data offers the first evidence that these genetic loci regulate the severity of the otocephalic phenotype. Furthermore, the findings indicate that these loci are genetically associated with *Otx2* locus. In addition, these modifier may interact with other unidentified modifier loci epistatically. One locus, *Otmf18*, was mapped on the CBA allele (Table 2, 4). Since the otocephalic phenotype is not evident on the CBA genetic background (Fig. 1), the *Otmf18* locus on the CBA strain alone appears to be insufficient to induce mandible abnormalities. Thus, a second undetermined modifier, probably located on the B6 strain, may be required for expression of mandible abnormalities.

Therefore, these findings, in conjunction, suggest that the genetic mechanism of the otocephalic phenotype is substantially more complex than originally expected. Nevertheless, the modifier loci account for the genetic effect between B6 and CBA strains and can, in part, explain the distribution of craniofacial malformations brought about by haploinsufficiency of the *Otx2* gene. Indeed, identification and characterization of these genetic loci provide new insights into mechanistic pathways of mandible development derived from mesencephalic neural crest. Furthermore, the otocephalic

mouse model may afford a powerful approach with respect to identification and characterization of candidate genes that may contribute to human agnathia-holoprosencephaly complex diseases.

8-(1) *Otx2* modifier loci may control several distinct steps for the formation of neural crest cells

The modifier loci identified in this study are considered to regulate the developmental processes of mandible, which originates from mesencephalic neural crest. Fate mapping experiments in chicks have suggested that skull bones of the premandibular and the distal portions of the mandibular regions originated from cephalic neural crest mainly at the level of mesencephalon (Couly et al., 1993; Koentges and Lumsden, 1996). Similarly in mouse, mesencephalic neural crest cells contribute to the mesenchyme of premandibular and mandibular regions (Osumi-Yamashita et al., 1994; Imai et al., 1996). Notably, endogenous *Otx2* is expressed in neural plate, neural crest and neural crest cells at the level of mesencephalon; moreover, distal elements of mandibular arch skeletons are lacking or severely affected in *Otx2* heterozygous mutants (Matsuo et al., 1995; Kimura et al., 1997; this study). Thus, the *Otx2* heterozygous mutant defects relate primarily to *Otx2* function in the formation of mesencephalic neural crest (Kimura et al., 1997).

Since the genetic modifier loci were crucial for development of neural crest-derived structures, it is likely that they play an important role in the induction, guidance, migration or differentiation of mesencephalic neural crest in the identical genetic pathway of the *Otx2* gene. Neural crest is

induced at the dorsolateral edge of the neural plate; from that point, neural crest cells delaminate and migrate along specific routes to many destinations in the vertebrate embryo (Le Douarin, 1982). Grafting experiments in the chick have shown that interactions between embryonic non-neural ectoderm (presumptive epidermis) and neural plate induce the formation of neural crest cells at their interface, and that each of these tissues contributes to the neural crest (Selleck and Bronner-Fraser, 1995; Liem et al., 1995). Following induction, neural crest delaminates from neural tube; that is, neural crest undergoes an epithelial to mesenchymal conversion and begins to migrate along specific pathways, differentiating into several structures. An important link exists between the guidance and differentiation of neural crest cells. In some cases, specified cells are targeted to the correct destinations, whereas in other instances, cells migrate to sites where they encounter inductive signals. These crest cells finally differentiate into a wide variety of cell types, including neurons and glial cells of the peripheral nervous system, melanocytes and smooth muscle cells, and cartilaginous and skeletal elements in the head (Le Douarin, 1982).

Two genetic loci which significantly modify the severity of mandible phenotypes of *Otx2* heterozygous mutants have been identified. Notably, the *Otmf18* locus appeared to be linked to phenotypes of no mandible and small mandible (Fig. 6C; Table 4). This finding suggests that *Otmf18* may direct the formation of mesencephalic neural crest cells fated to the entire mandible. The *Otmf2* locus was linked solely with the phenotype of no mandible (Fig. 7, Table 4), indicating that this locus may regulate earlier processes in neural crest formation, i.e., induction or delamination of neural

crest. In contrast, one suggestive locus at 96.0 cM on chromosomes 2, was linked with the small mandible phenotype but not with the no mandible phenotype (Fig. 7), suggesting that this locus may regulate later processes, such as the migration or differentiation of mesencephalic neural crest cells which exclusively contribute to the most distal portion of the mandible. Thus, *Otx2* may regulate several distinct steps of neural crest formation at that stage, interacting with distinct modifier genes. Further precise mechanisms of mandible development by modifiers await the identification of modifier genes.

8-(2) Candidate genes and mechanism of interaction with *Otx2*

Two modifier have been identified; however, mapping resolution is not sufficiently fine to determine the single gene that is responsible for modification of the mandible phenotype. Nevertheless, from this survey, many genes that are believed to interact with *Otx2*, such as the *Emx1*, *Emx2*, *Otx1*, *Cripto* and *Lim1* genes (Matsuo et al., 1995; Suda et al., 1996, 1997, 2001; Acampora et al., 1997, 1998; Kimura et al., 2001; Zoltewicz et al., 1999), were excluded as a genetic modifier of *Otx2* in craniofacial development. A potential *Otmf2* candidate is *Alx4*. The modifier, *Otmf2*, identified on the middle of chromosome 2, was located near the *Alx4* gene which is located at 65.0 cM of chromosome 2 (Table 1; Qu et al., 1998). *Alx4* is a closely related member of the family of *paired*-related homeobox genes named as *prx* family (Qu et al., 1998). The *prx* family consists of *prx1*, (previously referred to as *Mhox*), *prx-2*, *Cart-1*, *Alx3* and *Alx4*. All of

these genes are expressed in the cranial mesenchyme of the mandibular arch (Zhao et al., 1994, 1996; Martin et al., 1995; Qu et al., 1997; Berge et al., 1998a, 1998b; Lu et al, 1999). Indeed, *Alx4*$^{-/-}$ mutation in mouse and haploinsufficiency of human *ALX4* cause ossification defects of the skull (Qu et al., 1997; Wu et al., 2000, Wuyts et al., 2000; Mavrogiannis et al., 2001). Furthermore, the *Alx4* heterozygous mutant phenotype is subject to strain-specific genetic modifying loci in mouse (Forsthoefel, 1962, 1968; Qu et al., 1999). Moreover, in *Alx4*$^{-/-}$; *Cart1*$^{-/-}$ double mutant mice, the distal portion of the mandible was severely truncated (Qu et al., 1999). Indeed, based on our N2 analysis, no mandible and small mandible phenotypes were suggestively associated in chromosome 10, on which *Cart1* is located (Fig. 6B; Zhao et al., 1994). Furthermore, expressions of *prx* family and *Otx2* genes were consistently co-localized in the mesenchyme of the mandibular arch (data not shown). These results support our hypothesis that *Alx4* may genetically interact with *Otx2* in skull development.

One possible interaction between *Otx2* and *Alx4* involves direct transactivation by these transcriptional factors of *Otx2* expression in cephalic mesenchyme. Consistent with this hypothesis, we previously found that DNA sequences, termed motif B (TAATTA), were highly conserved in *cis*-regulatory elements between mouse and pufferfish *Otx2*; additionally, these sequences were essential for *Otx2* expression in cephalic mesenchyme (Kimura et al., 1997). Motif B is a suitable candidate for the *prx* family homeodomain binding sites (Cserjesi et al., 1992; Kimura et al., 1997; Cai, 1998; Qu et al., 1999). These *prx* family proteins exhibit similar DNA-binding activity; moreover, these proteins also form heterodimers and activate

transcription in a similar fashion (Qu et al., 1999). Furthermore, they are dosage-sensitive genes and function in a partially redundant manner in mandible development (see above). These lines of evidences supports our hypothesis that *prx* family transcription factors directly transactivate the level of *Otx2* expression in cephalic mesenchyme.

Differences in the amino acid sequences of these candidate genes between B6 and CBA could underlie subtle changes in the function of these proteins, affecting the mechanisms by which interaction occurs with downstream target genes or transcription factor complex. Alternatively, slight differences may exist between the B6 and CBA alleles in the temporal or spatial patterns and level of expression of these genes. Therefore, assessment of the aforementioned candidates as modifiers of *Otx2* will require high resolution mapping studies employing congenic strains to obtain a more precise localization of these loci. Moreover, sequence comparisons and analysis of relative timing and expression levels in the B6 and CBA alleles are necessary.

8-(3) Human agnathia-holoprosencephaly complex

Otocephaly, also referred to as agnathia-holoprosencephaly, is a lethal developmental field complex characterized by extreme hypoplasia or absence of the mandible, microstomia, aglossia and synotia (Bixler et al., 1985). Agnathia-holoprosencephaly, defined a complex of agnathia and holoprosencephaly has been reported more than 80 cases. Agnathia-microstomia-synotia defined a complex of agnathia, microstomia and synotia

without holoprosencephaly is a similar congenital anomaly and has been published more than 90 cases. Significant advances in the study of this disease have revealed the genetic and gene-environment bases of numerous common and rare craniofacial disorders (Winter, 1996; Wallis and Muenke, 2000). In humans, this condition can occur alone or in association with various other anomalies, including cyclopia, holoprosencephaly, cerebellar hypoplasia and other visceral anomalies (Opitz, 1980; Pauli et al., 1983). Moreover, the otocephalic phenotype has been observed in many animal species, including mouse (Juriloff et al., 1985), sheep (Willson, 1966; Smith, 1968), guinea pig (Wright and Wagner, 1934) and rabbit (Faller and Rossier, 1969). In mouse, the *otocephaly* (*oto*) mutation was identified in a screen for lethal mutations on chromosome 1 (Juriloff et al., 1985). This locus has been defined as internal between D1Mit79 and D1Mit134 in a region of synteny with human 2q35-36 (Zoltewicz et al., 1999). Strong linkage with the *oto* locus for mandible phenotypes of *Otx2* heterozygous mutants was not detected in the current investigation; however, further consomic or congenic analysis is required in order to finally determine whether the *oto* locus is associated with the *Otx2* mutant phenotype.

In addition to mandible abnormalities, most $Otx2^{+/-}$ mutant mice also displayed holoprosencephaly (Matsuo et al., 1995). In humans, holoprosencephaly is the most common developmental defect of the forebrain (Wallis and Muenke, 1999). It exhibits an incidence as high as 1:250 during early embryogenesis (Matsunaga and Shiota, 1977). The phenotype of holoprosencephaly is quite variable and proceeds in a continuous spectrum from severe manifestations with major brain and face anomalies to clinically

normal individuals (Wallis and Muenke, 1999). Several distinct human genes for holoprosencephaly have been identified recently, including *SHH*, *ZIC2*, *SIX3*, *TGIF* and *HESX1* (Roessler et al., 1996; Brown et al., 1998; Wallis et al., 1999; Gripp et al., 2000; Dattani et al., 1998). Intrafamilial variability of clinical findings exists in kindreds carrying specific mutations in either *SHH* or *SIX3* (Nanni et al., 1999; Brown et al 1998). Indeed, heterozygous carriers for mutations in either *SHH* or *SIX3* can appear phenotypically normal; in contrast, other heterozygous mutation carriers within the same family may be severely affected. This observation suggests the possibility of the occurrence of an undetermined second mutation in the same gene. Alternatively, other gene products or environmental factors may act in these pathways and alterations in the identical or additional genes or factors could be required for severe holoprosencephaly manifestations (Nanni et al., 1999; Brown et al 1998). It is not known as to whether *Otx2* is involved in human holoprosencephaly. The modifier loci identified in this study might be suitable candidates for genetic causes of human craniofacial congenital diseases. Identification of human mutations of *Otx2* modifier genes and evaluation of interaction between these genes and environmental causes awaits molecular identification of these modifier genes.

9. Conclusion

In this study, two significant loci, *Otmf18* and *Otmf2*, which modify *Otx2* mutant phenotypes have been identified. These two modifiers account for the distribution of craniofacial malformations by the genetic effect between B6 and CBA. *Otmf18* between D18Mit68 and D18Mit120 on chromosome 18 linked to the small mandible and no mandible phenotype (LOD score=3.33). It has been demonstrated that *Otmf18* is mapped on the CBA allele and functions with a dominant manner. *Otmf2* between D2Mit164 and D2Mit 282 on chromosome 2 linked to the no mandible phenotype (LOD score=3.93). It is indicated that *Otmf2* is mapped on the B6 allele and functions with a recessive manner. These modifier loci are considered to regulate the developmental processes of mandible, which originates from mesencephalic neural crest. Thus, *Otx2* may control several distinct steps of neural crest formation interacting with distinct modifier genes. Moreover, *Otmf2* contains a candidate gene for several diseases in mice and humans. These genetic studies involving an otocephalic mouse model appear to provide new insights into mechanistic pathways of craniofacial development. Furthermore, these experiments offer a powerful approach with respect to identification and characterization of candidate genes that may contribute to human agnathia-holoprosencephaly complex disease.

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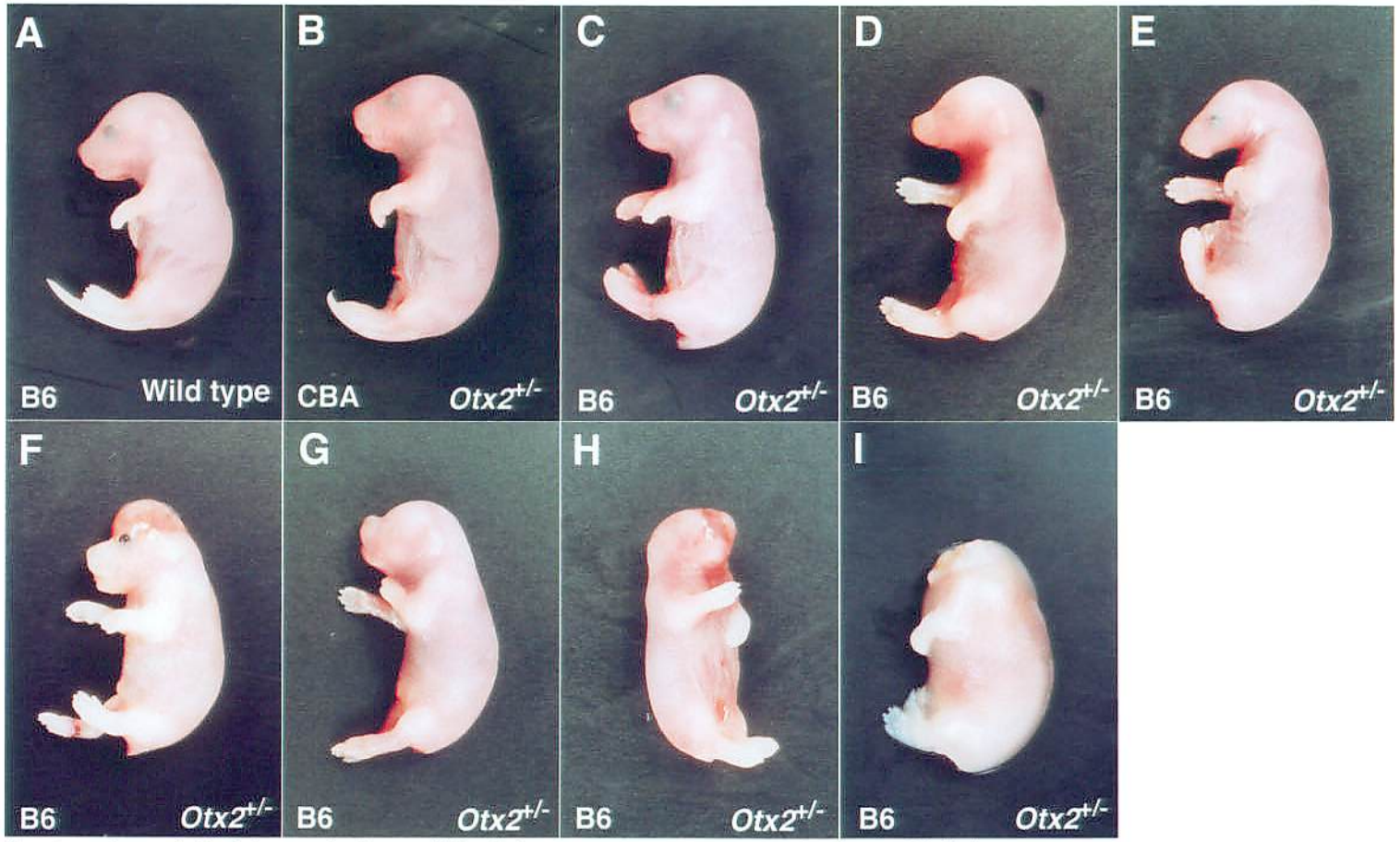

Fig. 1. Variation of external craniofacial morphology of Otx2 heterozygous mutant embryos at 18.5 dpc.
Wild type mouse backcrossed Otx2 knock-out chimeras with wild type B6 females (A). Otx2 heterozygous mutant mice (N2) backcrossed Otx2 mutant chimeras with wild type CBA females (B) and with wild type B6 females (C-I), respectively. No noticeable malformations are evident in the mutant mouse on the CBA strain genetic background (B). No apparent external abnormalities are observed in the mutant mouse on the B6 strain genetic background (C). The mutant mouse displays reduction of the lower jaw (D). The mutant mouse lacks an entire lower jaw (E). The mutant mouse displays excencephaly (F). The distal portion of the face is shortened in the mutant mouse (short nose) (G). The face is cleft in the mutant mouse (cleft face) (H). The entire head is lacking (acephaly) in the mutant mouse (I).

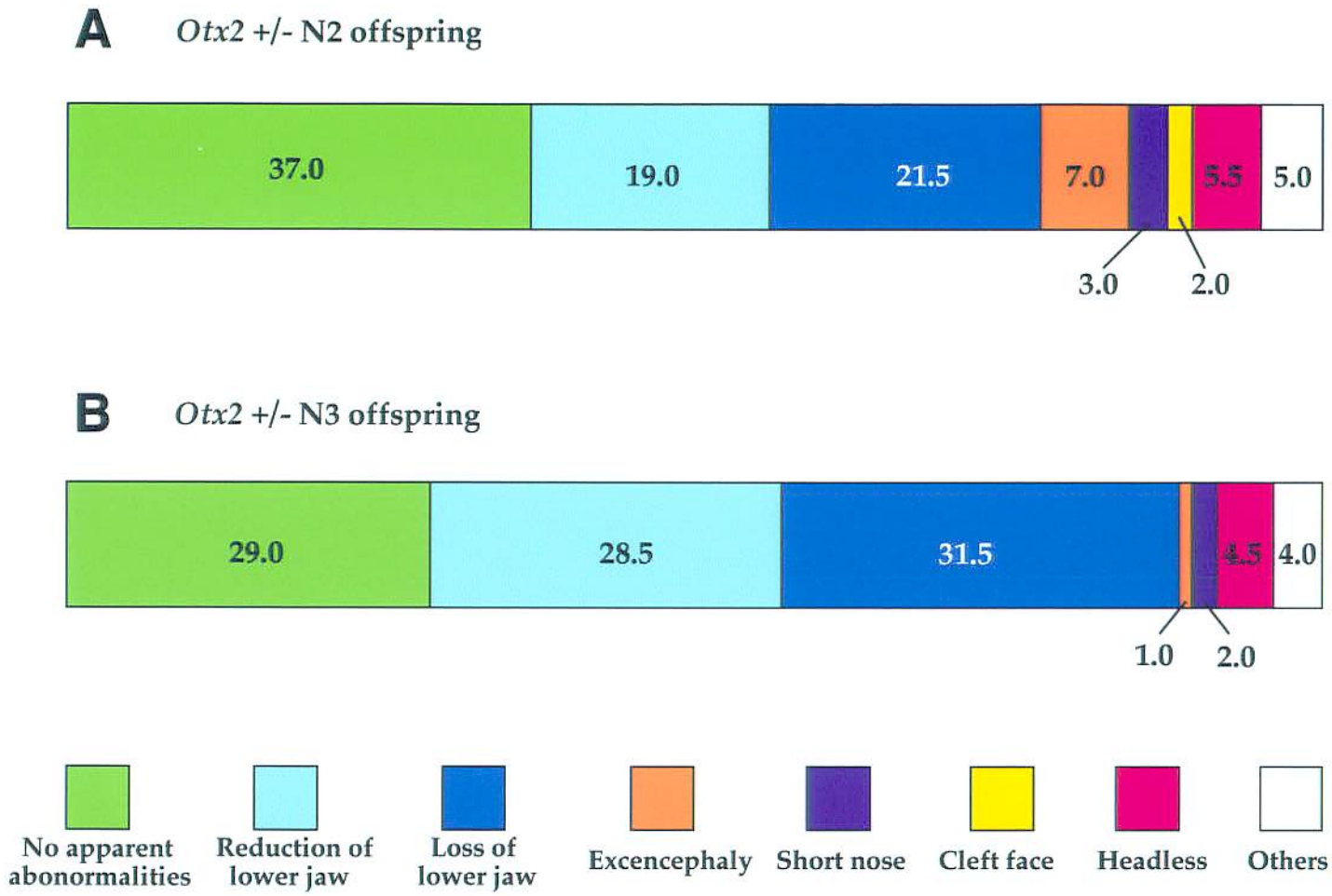

Fig. 2. Frequency distribution of external malformations in mutant mice with B6 background.
Otx2 heterozygous mutant N2 embryos (n=200) at 18.5 dpc obtained by backcrossing chimeras with wild type B6 females are phenotypically classified into eight groups according to their external morphology (A). *Otx2* heterozygous mutant N3 embryos (n=200) at 18.5 dpc obtained by backcrossing the N2 male with wild type B6 females are phenotypically classified into eight groups according to their external morphology (B).

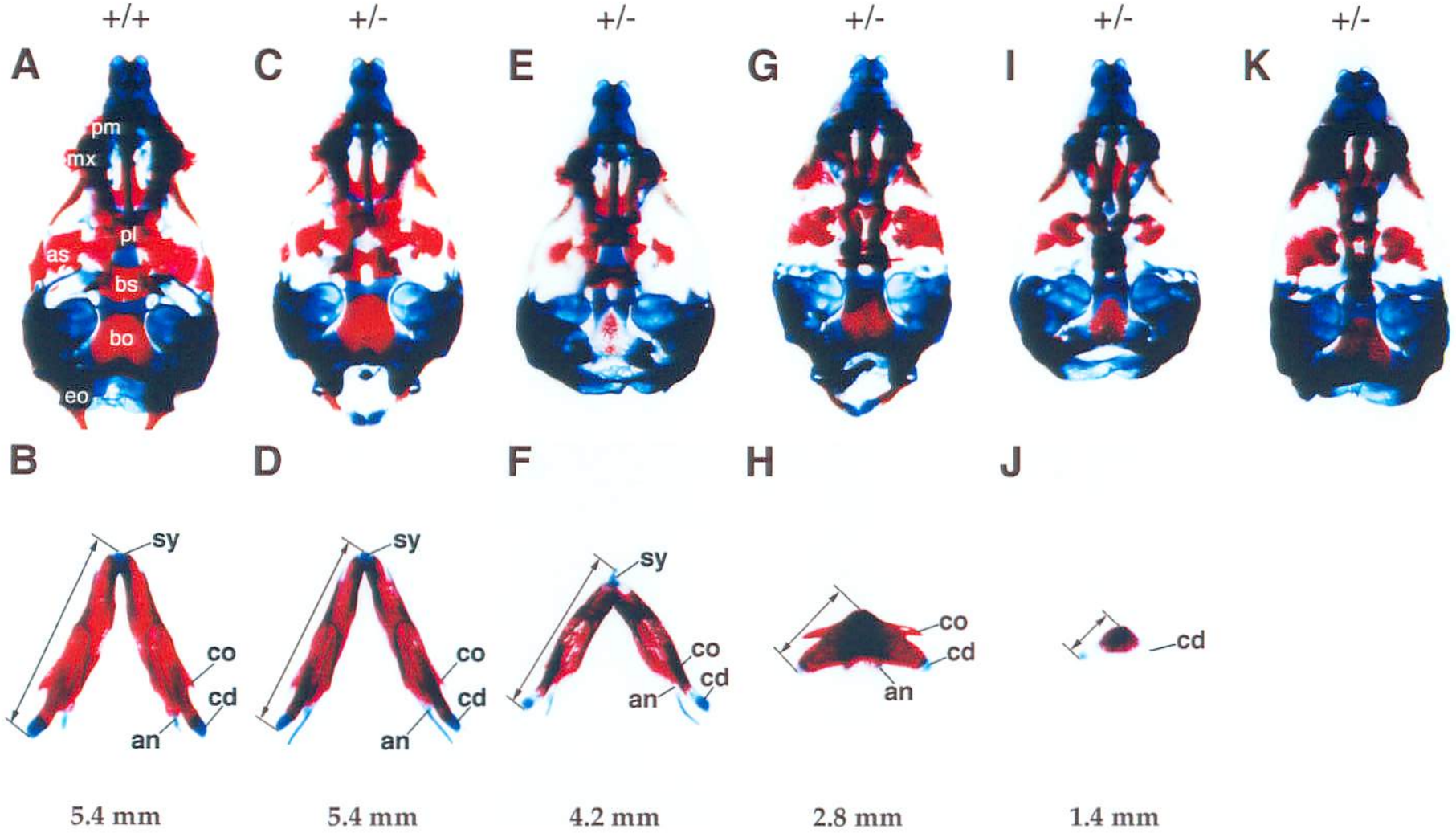

Fig. 3. Variation of mandible length in Otx2 heterozygous mutant mice.
Whole-mount views of skull morphology of wild type (A,B) and heterozygous mutant embryos (C-K) at 18.5 dpc following cartilage and bone staining. Mandibles have been separated in cases in which they were present (A-J). An embryo lacking a mandible (K). The length of each mandible from proximal to distal is 5.4 mm (A), 5.4 mm (C), 4.2 mm (E), 2.8 mm (G) and 1.4 mm (I), respectively. Abbreviations; an, angular process; as, alisphenoid; bs, basisphenoid; bo, basioccipital; cd, condyloid process; co, coronoid process; eo, exoccipital; mx, maxillar; pl, palatine; pm, premaxillar; sy, symphysis.

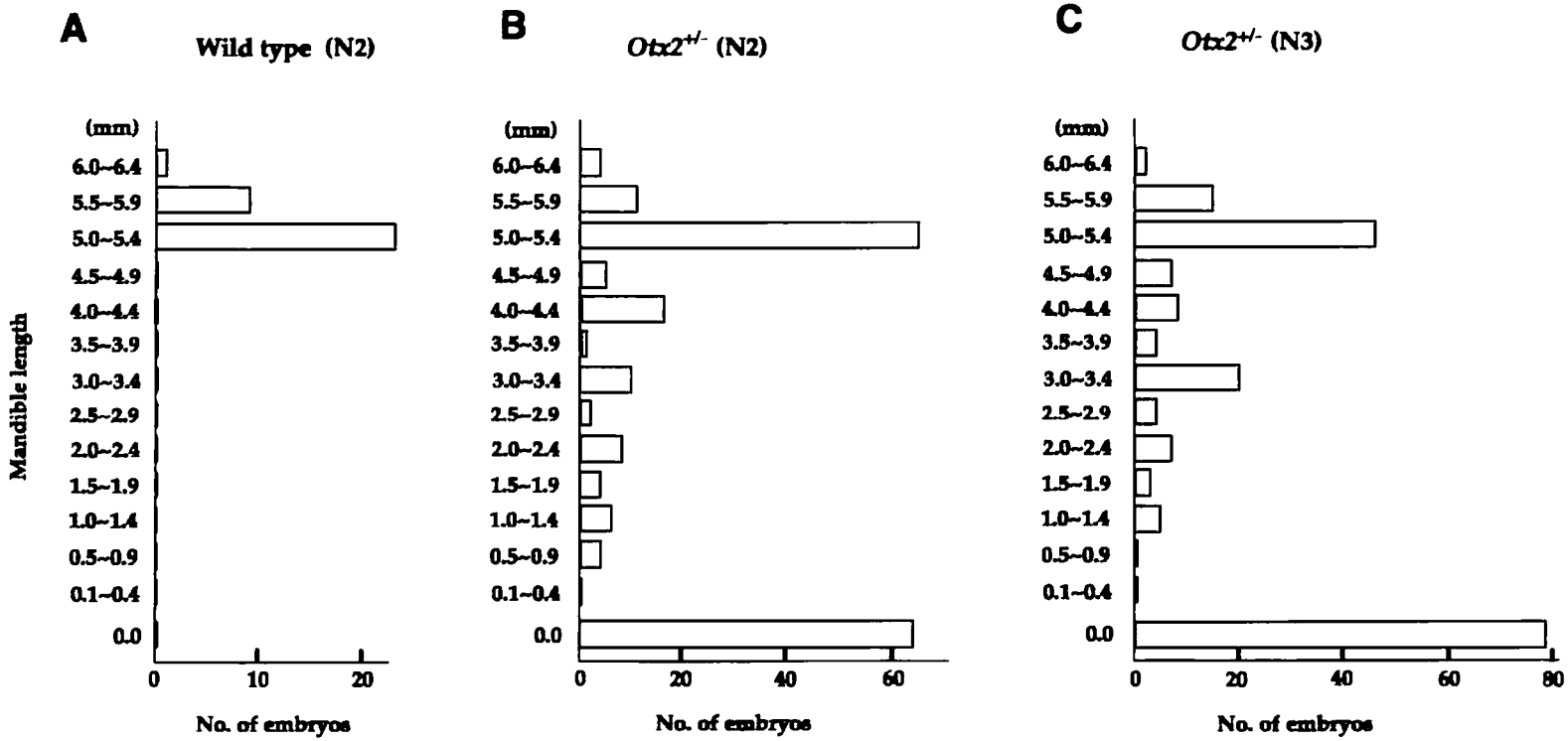

Fig. 4. Frequency distribution of mandible length in 18.5 dpc embryos. Distribution of mandible length in N2 wild-type embryos (n=30) chimeras crossed with wild type B6 females (A), heterozygous N2 mutants (n=200) crossed with wild-type B6 females (B) and heterozygous N3 mutants (n=200) backcrossed twice with wild-type B6 females (C).

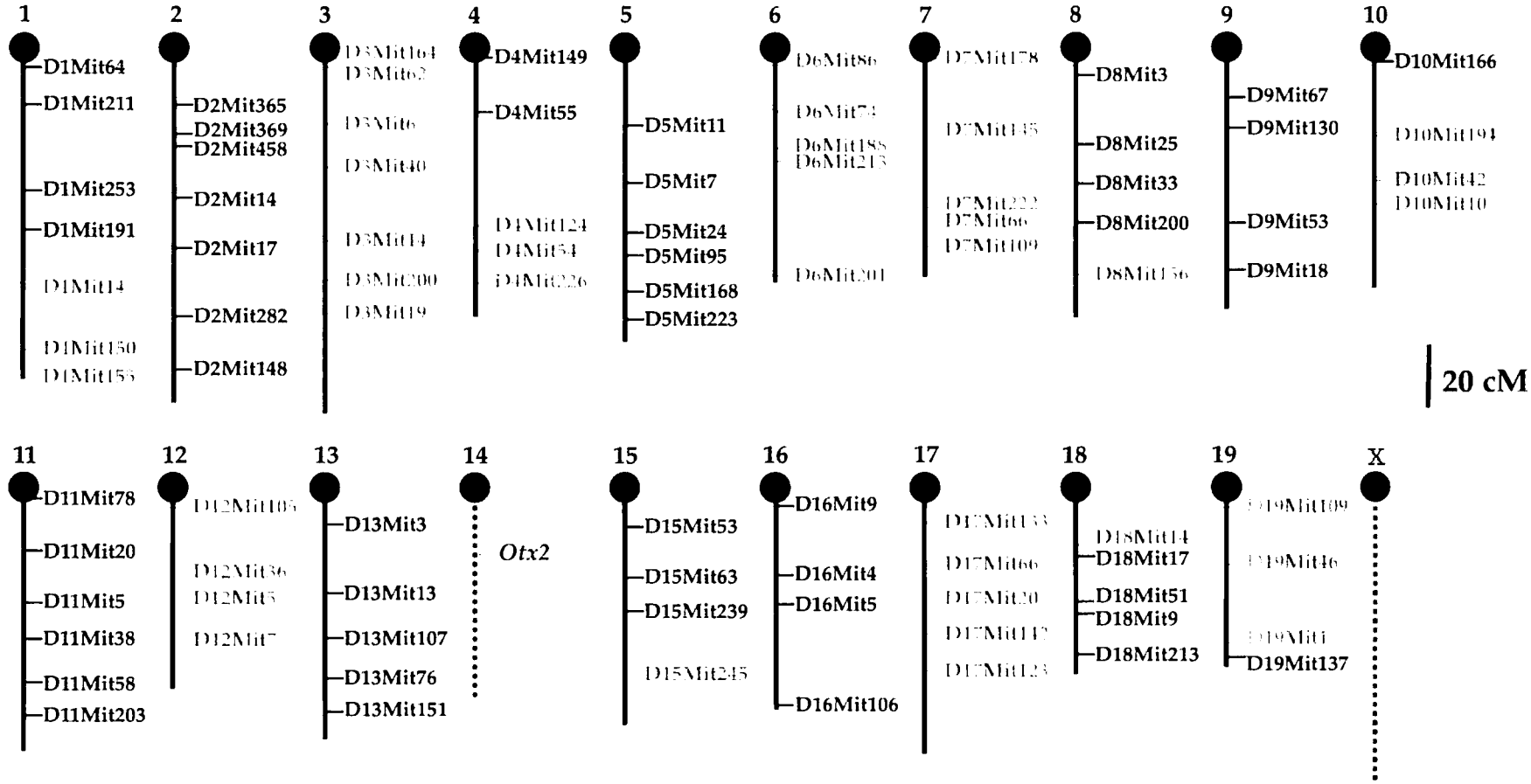

Fig. 5. Chromosomal location of microsatellite markers selected for analysis of phenotypic pools in the first level screen. Marker positions were obtained from the microsatellite map distributed by the MIT mouse genome database. All SSLP markers colored in both black and gray are employed in the N2 analysis. Markers colored in black represent a heterozygous (B6/CBA) and those colored in gray represent a homozygous (B6/B6) allele in the N2 male. SSLP markers colored in black representing heterozygous (B6/CBA) allele are used in the N3 mapping analysis.

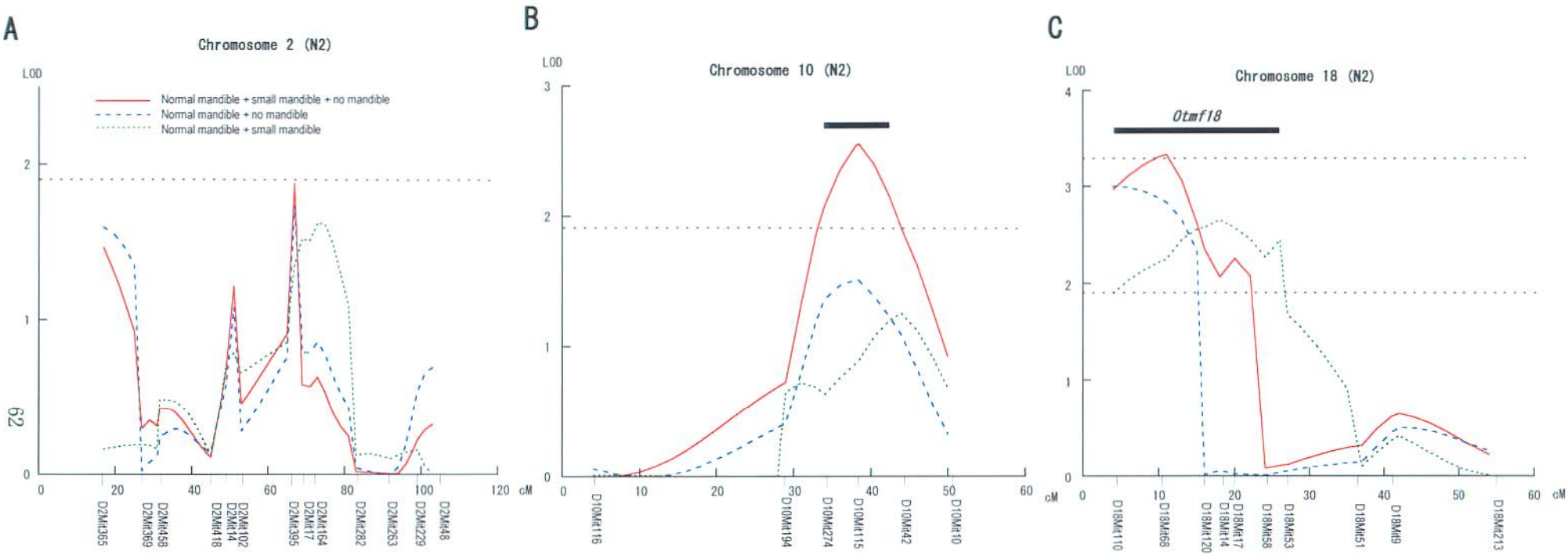

Fig. 6. Interval mapping of the modifier locus in the N2 analysis.

Chromosome 2 (A), chromosome 10 (B) and chromosome 18 (C), respectively.
The vertical and horizontal axes display LOD scores
and the relative positions of the markers along the chromosomes from centromere (Left) to telomere (Right) in cM, respectively (determined by QTL cartographer). Two dashed horizontal lines depict the LOD scores (1.9 and 3.3), which represent suggestive and significant linkages (Lander and Kruglyak, 1995), respectively. Red lines represent values obtained from mutant embryos displaying phenotypes of no mandible, small mandible and normal mandible. Blue dashed lines represent values obtained from mutants exhibiting no mandible and normal mandible phenotypes, and green dashed lines represent values obtained from those mutants characterized by small mandible and normal mandible phenotypes, respectively. The most likely position for each locus, determined by its two (B, C) LOD support interval, is indicated by the closed bar above the plot.

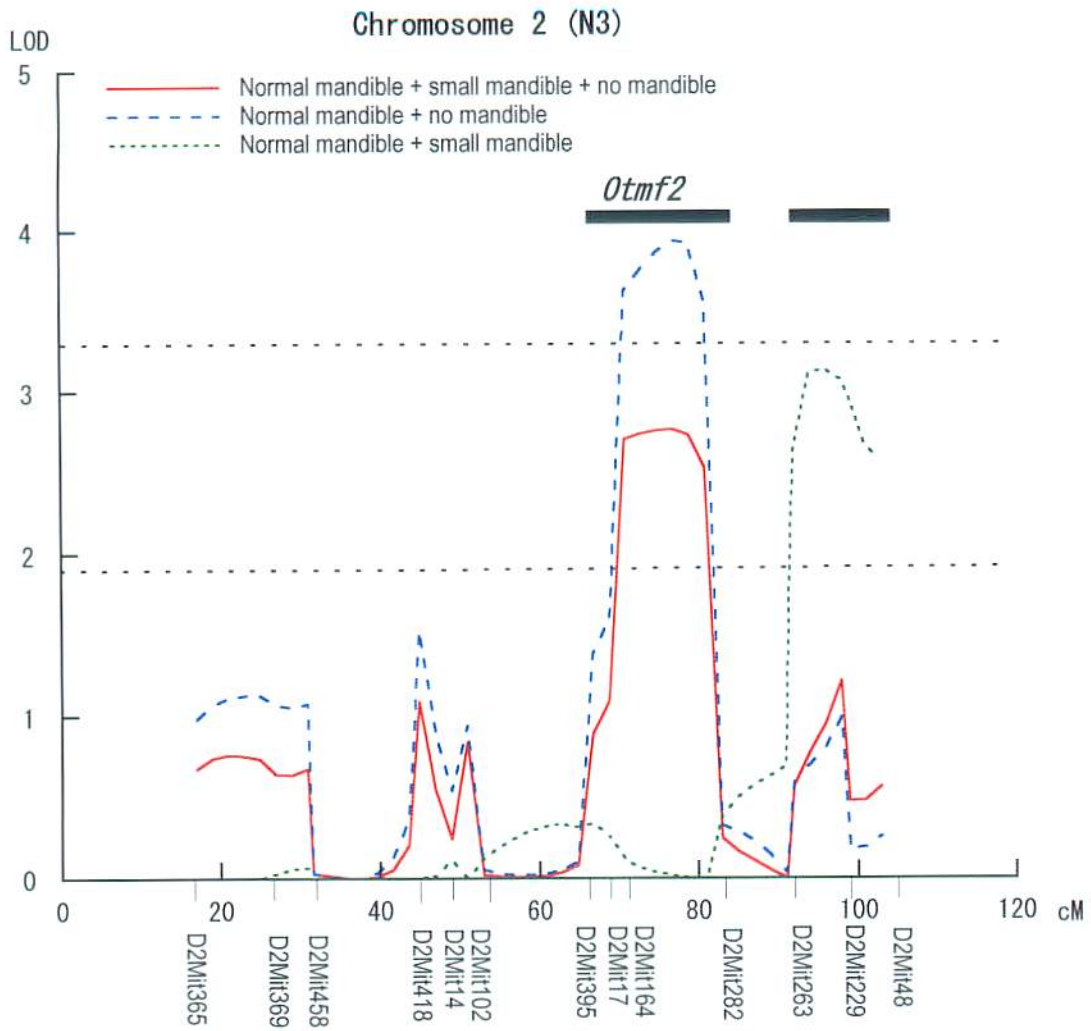

Fig. 7. Interval mapping of the modifier locus in the N3 analysis on chromosome 2.

The vertical and horizontal axes show LOD scores and the relative positions of the markers along the chromosomes from centromere (Left) to telomere (Right) in cM, respectively (determined by QTL cartographer). Two dashed horizontal lines display the LOD scores (1.9 and 3.3), which represent suggestive and significant linkages (Lander and Kruglyak, 1995), respectively. A red line represents values obtained from mutant embryos displaying phenotypes of no mandible, small mandible and normal mandible. A blue dashed line represents values obtained from mutants exhibiting no mandible and normal mandible phenotypes, and a green dashed line represents values obtained from those mutants characterized by small mandible and normal mandible phenotypes, respectively. The most likely position for each locus, determined by its two LOD support interval, is indicated by the closed bar above the plot.

Table 4. Summary of the genetic linkages.

Locus	Chr	Position	LOD	Phenotypes	Origin	Candidates
Otmf2	2	77.0	3.93 (N3)*	No mandible	B6 (recessive)	*Alx4*
Otmf18	18	11.1	3.33 (N2)	Small mandible and no mandible	CBA (dominant)	

Listed are the provisional nomenclature of loci that significantly modify the otocephalic phenotypes of *Otx2* heterozygous mutnat mice (Locus), the chromosomes are on which they are located (Chr), the distance from the centromere in cM (Position), the maximum LOD scores (LOD), phenotypes that controlled by the modifier locus (Phenotypes), origin of regulatory alleles (Origin), and candidates genes (Candidates).
*A LOD score was generated by mutant mice exhibiting normal mandible and no mandible.