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Polymorphism and linkage analysis of the prothoracicotropic hormone gene in the silkmoth, *Bombyx mori*

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Summary

We looked for polymorphism of the prothoracicotropic hormone gene locus (*Ptth*) among inbred strains of the silkmoth, *Bombyx mori*, by *in vitro* DNA amplification (polymerase chain reaction), and found three alleles, $Ptth^A$, $Ptth^B$ and $Ptth^C$. The $Ptth^A$ allele contained a third intron consisting of 680 bp and a fourth intron of 350 bp. $Ptth^B$ contained the same size third intron but a longer fourth intron of 490 bp, while $Ptth^C$ had a longer third intron of 1080 bp and a shorter fourth intron of 350 bp. In 29 strains which we examined, 9 strains had $Ptth^A$, 8 strains had $Ptth^B$, and 2 strains had $Ptth^C$. The other 10 strains had heterogeneous genotypes with the same 3 alleles.

Linkage analyses using these polymorphisms showed that *Ptth* was linked to the *or* gene on chromosome 22, and was independent of other chromosome markers. To determine the map position, we obtained an F_1 hybrid between the w41 strain ($Ptth^C + ^{or} + ^{mw}$) and the w22 strain ($Ptth^B$ *or mw*), and backcrossed the F_1 hybrid to females of the w22 strain. From the segregation of *or*, *mw* and *Ptth* in 861 progeny, recombination values were calculated as 18·5% between *or* and *mw*, 23·9% between *mw* and *Ptth*, and 7·3% between *Ptth* and *or*. Because *or* and *mw* have already been mapped at positions 22–8·9 cM and 22–25·2 cM, respectively, we mapped *Ptth* at 22–2·5 cM . The *Ptth* locus is independent of any loci which are known to control growth and molting.

1. Introduction

Molting and metamorphosis of insects are induced by ecdysteroids, and the release of ecdysteroids from the prothoracic glands is triggered by the prothoracicotropic hormone (PTTH) (Kataoka *et al.* 1987, 1991). Recently, the sequences of the mRNA and the gene coding for PTTH in the silkmoth, *Bombyx mori*, were determined (Kawakami *et al.* 1990; H. Ishizaki, personal communication). Many defective mutants for growth and molting have been found in *B. mori* (Doira, 1983; Japanese Society of Sericultural Science, 1986; Doira *et al.* 1992). Among them several non-molting mutants have been isolated, and examined physiologically. For example, the *nm-g* (non-molting glossy) mutant lacks ecdysteroids in the hemolymph and is considered to have an abnormality in the biosynthesis or release of ecdysteroids in the prothoracic glands (Nagata *et al.* 1987). There are also some variants for moltinism, numbers of larval molts (Morohoshi, 1957). Typical strains molt four times, and the final larval instar is the fifth instar, while some strains molt 2, 3, 5, or 6 times during their larval stages. Why non-molting mutants cannot molt, has not been clarified completely, nor have the mechanisms that determine the numbers of larval molts been investigated precisely.

It can be presumed that such variants involve differences in endocrine factors, such as biosynthesis, release and reception of ecdysteroids, juvenile hormones, and neuropeptides. PTTH is a primary candidate for a factor involved in these kinds of mutations because it regulates growth at the highest level in the endocrine cascade. Silkworm geneticists have mapped the non-molting mutants and moltinism genes onto the 28 conventional linkage maps. In order to clarify the relationship between such mutant loci and the PTTH gene, we searched for polymorphic

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Table 1. *Distribution of* Ptth *alleles*

Strain	Maintaining institution	*Ptth* allele
UT17 Shou-msc	Univ. Tokyo[1]	$Ptth^A$
319 Koha	NISES[2]	$Ptth^A$
130 p^M *Ze L q*	Kyushu Univ.[3]	$Ptth^A$
n15 *st*	Kyushu Univ.	$Ptth^A$
928 *K U*	NISES	$Ptth^A$
UT10 *Obs*	Univ. Tokyo	$Ptth^A$
UT03 *oh w-2*	Univ. Tokyo	$Ptth^A$
UT04 *rb*	Univ. Tokyo	$Ptth^A$
UTW1 *B. mandarina* from Sakado	Univ. Tokyo	$Ptth^A$
UT11 Daizo	Univ. Tokyo	$Ptth^B$
p50 Daizo	Kyushu Univ.	$Ptth^B$
UT12 J106	Univ. Tokyo	$Ptth^B$
UT13 *w*-2	Univ. Tokyo	$Ptth^B$
751 $E^{Ns}+{}^p$ $Ptth^B$	NISES	$Ptth^B$
r01 $w\text{-}3^{oe}$	Kyushu Univ.	$Ptth^B$
o80 *oy*	Kyushu Univ.	$Ptth^B$
w22 *or mw*	Kyushu Univ.	$Ptth^B$
w41 *bl cts*	Kyushu Univ.	$Ptth^C$
UTW2 *B. mandarina* from Hangzhou	Univ. Tokyo	$Ptth^C$
322 Sekko	NISES	$Ptth^A$ and $Ptth^B$
881 *U*	NISES	$Ptth^A$ and $Ptth^B$
w30 p^M *Ze L q*	Kyushu Univ.	$Ptth^A$ and $Ptth^B$
912 *pe re ch*	NISES	$Ptth^A$ and $Ptth^B$
UT14 *gn ms*	Univ. Tokyo	$Ptth^A$ and $Ptth^B$
UT01 *bl bts lem*	Univ. Tokyo	$Ptth^A$ and $Ptth^B$
UT02 *nb tub*	Univ. Tokyo	$Ptth^A$ and $Ptth^B$
UT05 *or*	Univ. Tokyo	$Ptth^A$ and $Ptth^B$
UT15 *Ym*	Univ. Tokyo	$Ptth^A$ and $Ptth^B$
UT16 N_4	Univ. Tokyo	$Ptth^A$ and $Ptth^C$

For each strain, 2–15 individuals were examined.

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variants of the PTTH gene using the polymerase chain reaction (PCR) and mapped it onto a chromosome by genetic mating experiments.

2. Materials and methods

(i) *Insects*

The strains of *Bombyx mori* L. and *Bombyx mandarina* Moore that were used in this study are listed in Table 1. They were raised with mulberry leaves as food by the conventional method.

(ii) *DNA extraction*

Genomic DNA was extracted from larval posterior silkglands or from pupal whole bodies by the method of Bender *et al.* (1983). It was further purified by extraction with phenol/chloroform, precipitated by ethanol, and resuspended in TE buffer (10 mM Tris-HCl, pH 8·0, 1 mM EDTA).

(iii) *Primers*

We synthesized six primers for *in vitro* DNA amplification to detect polymorphism of the PTTH gene. Their sequences were as follows: BP5: GACTCCTGCGATTTAGTTTC [2665→2646, reverse]; BP6: TCATGATTACTCGACCGAT(ACT)AT [1101→1117, forward]; BP7: TTATTATATCGTAG(CT)TG(AG)TA [2767→2748, reverse], BP8: AAGTCTTCTATTTCTTTCTG [2241→2222, reverse]; BP9: CAAAGAAAGTTTATACAGTG [2326→2345, forward]; BP10: TTGCACTTGCAAATACAAGG [1562→1581, forward]. Bases listed in parentheses are mixed sites. Numbers indicate the corresponding bases in the PTTH gene sequence of the Kinshu strain (H. Ishizaki, personal communication). They were synthesized with a CYCLON DNA Synthesizer (Millipore Corp., Bedford, USA).

(iv) *Polymerase chain reaction*

In the polymerase chain reaction (PCR), each reaction was 30 μl in volume and contained 0·2 μg template DNA, 0·2 mM each dNTP (Pharmacia-LKB Biotech.), 0·5 μM reverse primer, 0·5 μM forward primer, 3·0 units *Taq* polymerase (Wako Pure Chemical Industries, Osaka, Japan), and the manufacturer's reaction buffer. We used a 'Zymoreactor II' machine (ATTO Co. Ltd, Tokyo, Japan). The PCR consisted of 40 cycles usually performed as follows: denaturation at 94 °C for 60 s, annealing at 50 °C for 60 s, and extension at 72 °C for 120 s. Only when we used BP6 and BP7 primers, annealing time was increased to 120 s, and extension time was 180 s. After PCR, 15 μl of amplified product was run on a 1 or 2% agarose gel.

(v) *Cloning and sequencing*

The PCR product was purified by extraction with phenol/chloroform (1:1), concentrated by ethanol precipitation, and dissolved in TE. DNA ends in the PCR product were blunted using T_4 DNA polymerase in the DNA Blunting Kit (Takara Shuzo Co. Ltd, Kyoto, Japan) according to the manufacturer's instructions. The blunted DNA was extracted with phenol/chloroform and precipitated by ethanol. The plasmid, pBluescript II SK+ (Stratagene Cloning System, La Jolla, USA) was digested with the endonuclease *Sma*I (Nippon Gene Co. Ltd, Tokyo, Japan), ligated with the blunted PCR product, and used to transform the *E. coli* strain JM109. Plasmids were isolated from the transformants and purified by CsCl-gradient ultracentrifugation (Sambrook *et al.* 1989). Double-stranded plasmids were denatured with alkali and sequenced by the dideoxy chain termination method of Sanger *et al.* (1977) using the Sequenase Version 2·0 DNA Sequencing Kit (United States Biochemical Corp., Cleveland, USA) and commercial primers.

3. Results

(i) *Polymorphism in intron lengths of the PTTH gene*

Because PCR is a much simpler technique and less time-consuming than Southern hybridization, it is preferable for large-scale genetic experiments. Therefore, we adopted a PCR-based strategy to search for variants in the PTTH gene, which consists of 5 exons and 4 introns (H. Ishizaki, unpublished). We expected that intron sequences would be more variable than exons. First, we looked for variants of PCR products corresponding to the full-length gene, and secondly, we examined polymorphism of the introns.

We performed PCR using the most widely separated primers, BP6 and BP7, to amplify the nearly full length of the PTTH gene. This set of primers successfully amplified the PTTH gene as a single band. Twenty-nine strains were screened, and three types of allele were found (Table 1 and Fig. 1). We tested DNA from 2 to 15 individuals for each strain. Nine strains had the A-type allele ($Ptth^A$), which was approximately 1600 bp in length, eight strains had the B-type allele ($Ptth^B$), which was approximately 1750 bp in length, and two strains had the C-type allele ($Ptth^C$), which was approximately 2000 bp in length. In addition, 9 strains were heterogeneous for $Ptth^A$ and $Ptth^B$, and one strain carried $Ptth^A$ and $Ptth^C$. PCR products of $Ptth^A$ from *B. mandarina* in Sakado, $Ptth^B$ from strain UT11 (Daizo), and $Ptth^C$ from strain N_4 were cloned and both ends were sequenced. Over 300 bases were determined for each allele, and in all cases matched the sequence of the Kinshu strain (H. Ishizaki, personal communication) except for a few base mismatches which did not affect the amino acid sequence. This degree of matching indicated that the amplified DNA was specific to the PTTH gene.

Our preliminary studies on restriction maps of cloned PCR products suggested that the length differences of the full-length gene were caused by variations of the third and fourth introns. To determine the source of polymorphism in *Ptth* alleles, we used the primers BP10 and BP8 to amplify the third intron. This set of primers amplified approximately 680 bp DNA in the strains which had $Ptth^A$ and $Ptth^B$, whereas it amplified approximately 1080 bp DNA in the strains which had $Ptth^C$ (Fig. 2). We also

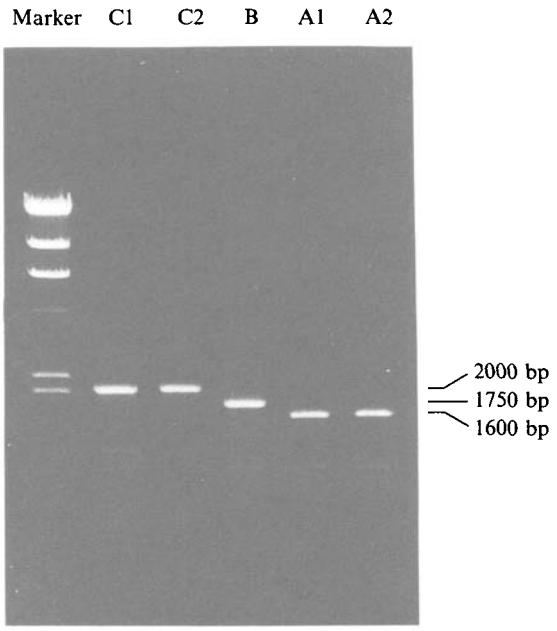

Fig. 1. Variation in the full length of the PTTH gene amplified with the primers, BP6 and BP7. C1, *Bombyx mandarina* from Hangzhou, China, and C2, N_4 strain of *B. mori* have a 2·0 kb PTTH gene designated as $Ptth^C$. B, UT11 Daizo strain of *B. mori* has $Ptth^B$. A1, n15 strain of *B. mori* and A2, *B. mandarina* from Sakado, Japan, have $Ptth^A$. Marker, λ phage DNA digested with *Hind* III was used as size markers.

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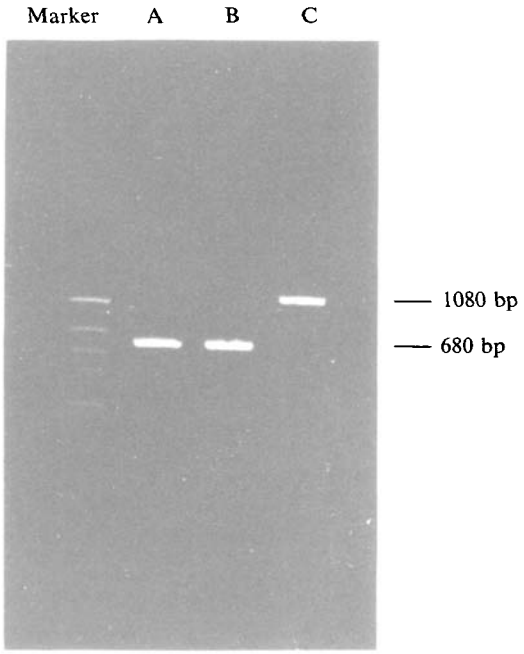

Fig. 2. Variation in the length of the third intron amplified with the primers, BP10 and BP8. A, *Ptth*^{A} of strain n15. B, *Ptth*^{B} of strain w22. C, *Ptth*^{C} of strain w41. Marker, ϕX174 DNA digested with *Hinc* II was used as size markers.

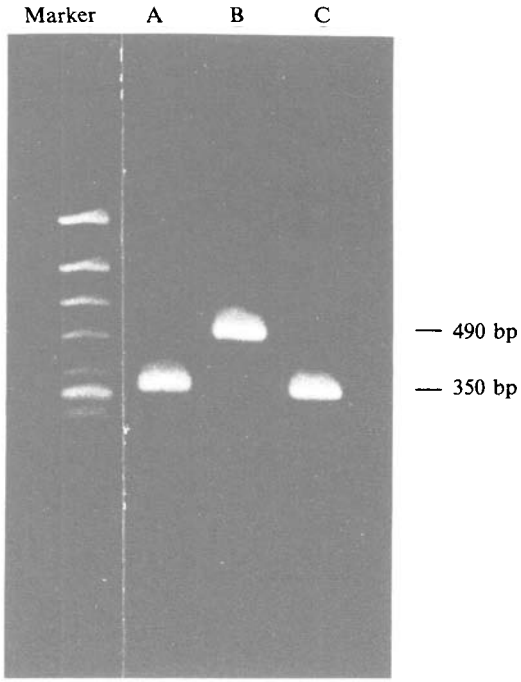

Fig. 3. Variation in the length of the fourth intron amplified with the primers, BP9 and BP5. A, *Ptth*^{A} of strain n15. B, *Ptth*^{B} of strain w22. C, *Ptth*^{C} of strain w41. Marker, ϕX174 DNA digested with *Hinc* II was used as size markers.

amplified the fourth intron using BP9 and BP5 as primers. As a result, a 350 bp fragment was amplified from the *Ptth*^{A} and *Ptth*^{C} strains, and a 490 bp fragment was amplified from the *Ptth*^{B} strains (Fig. 3). The difference of the full lengths among *Ptth*^{A}, *Ptth*^{B}

Table 2. *Linkage screening of* Ptth

Mating scheme	Phenotype of progeny	No. of progeny: *Ptth*^{C}	No. of progeny: *Ptth*$^{A/C}$ or *Ptth*$^{B/C}$	No. of progeny: *Ptth*^{A} or Ptth^{B} or *Ptth*$^{A/B}$	Chromosome (visible marker)	Linkage
(N4 *Ptth*^{C} ♀ × w30 p^{M} *Ze L Ptth*$^{A/B}$ ♂) ♀ × N4 ♂	p^{M} *Ze L*	0	1		2(p^{M}) 3(*Ze*) 4(*L*)	No
(N4 *Ptth*^{C} ♀ × 914 *pe ch Ptth*$^{A/B}$ ♂) ♀ × 914 ♂	*pe ch*		2	1	5(*pe*) 13(*ch*)	No
(N4 *Ptth*^{C} ♀ × 751 E^{Ns} + ^{p} *Ptth*^{B} ♂) ♀ × N4 ♂	E^{Ns} + ^{p}	3	1		2(+^{p}) 6(E^{Ns})	No
(N4 *Ptth*^{C} ♀ × 928 *K Ptth*^{A} ♂) ♀ × N4 ♂	*K*	2	1		11(*K*)	No
(N4 *Ptth*^{C} ♀ × 881 *U Ptth*$^{A/B}$ ♂) ♀ × N4 ♂	*U*	2	0		14(*U*)	No
(N4 *Ptth*^{C} ♀ × UT01 *bl bts lem Ptth*$^{A/B}$ ♂) ♀ × UT01 ♂	*bl bts lem*		1	0	15(*bl*) 17(*bts*) 3(*lem*)	No
(N4 *Ptth*^{C} ♀ × UT02 *nb tub Ptth*$^{A/B}$ ♂) ♀ × UT02 ♂	*nb tub*		1	0	19(*nb*) 23(*tub*)	No
(N4 *Ptth*^{C} ♀ × UT03 *oh w-2 Ptth*^{A} ♂) ♀ × UT03 ♂	*oh w-2*		1	0	20(*oh*) 10(*w-2*)	No
(N4 *Ptth*^{C} ♀ × UT04 *rb Ptth*^{A} ♂) ♀ × UT04 ♂	*rb*		1	1	21(*rb*)	No
(N4 *Ptth*^{C} ♀ × UT05 *or Ptth*^{A} ♂) ♀ × UT05 ♂	*or* +^{p}		0	14	22(*or*)	Yes
	+ ^{or} + ^{p}		13	0	2(+^{p})	No
(W4 *Ptth*^{C} ♀ × w22 *or mw Ptth*^{B} ♂) ♀ × w22 ♂	*or mw*		0	7	22(*or*)	Yes
	+ ^{or} + ^{mw}		7	0	22(*mw*)	Yes
(N4 *Ptth*^{C} ♀ × w30 *q Ptth*$^{A/B}$ ♂) ♀ × w30 ♂	*q*		1	0	7(*q*)	No

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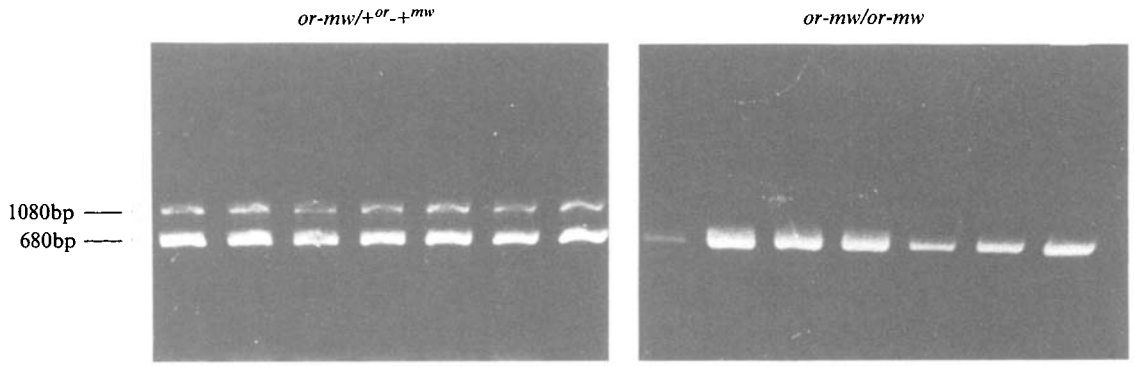

Fig. 4. Linkage between *or-mw* and *Ptth*. Each sample is a PCR product of the 3rd intron of the PTTH gene in progeny of the cross of $Ptth^B$-*or-mw*/$Ptth^C$-+^{or}-+^{mw}♀ × $Ptth^B$-*or-mw*/$Ptth^B$-*or-mw*♂. Left panel, progeny showing the wild-type phenotype. Right panel, *or-mw* homozygous progeny.

and $Ptth^C$ was explained by the length variations of the third and fourth introns.

We cloned the PCR products of the third intron from $Ptth^C$ in the strain N_4 and the fourth intron from $Ptth^B$ in strain UT11 (Daizo). Upon partial sequencing, we found very good coincidence with the Kinshu sequence though both introns contained insertions. We are now determining the complete sequences of the introns.

(ii) *Linkage screening of the PTTH gene*

To determine the linkage group to which the PTTH gene belongs, we performed large-scale mating experiments using visible chromosome markers. We used primer sets BP6/BP7 for full-length alleles and BP9/BP5 for the third intron. The mating schemes and the results are summarized in Table 2. First we crossed the N_4 strain, which had the $Ptth^C$ allele, with a strain that carried one or more chromosome markers and $Ptth^{A/B}$. We used a strain of N_4 which was genetically selected to contain only the $Ptth^C$ allele, although N_4 had originally carried $Ptth^C$ and $Ptth^A$ heterozygously. Next we crossed the F_1 hybrid with the marker strain to test for recessive visible markers, or with the N_4 strain to test for dominants.

The results of the crosses indicate independent assortment for p^M (chromosome 2), *Ze* (3), *lem* (3), *L* (4), *pe* (5), E^{Ns} (6), *q* (7), *w*-2 (10), *K* (11), *ch* (13), *U* (14), *bl* (15), *bts* (17), *nb* (19), *oh* (20), *rb* (21), *tub* (23) and sex (1), as shown in Table 2. On the other hand, we found clear linkage between the *Ptth* locus and the *or* (r-translucent) gene at position 8·9 on chromosome 22, which expressed translucent larval skin. In the offspring of the cross (N_4 $Ptth^C$♀ × $Ptth^A$ *or*♂)♀ × $Ptth^A$ *or*♂, all of the 14 *or*/*or* individuals had the $Ptth^A/Ptth^A$ genotype, while all of the 13 *or*/+ ones had $Ptth^A/Ptth^C$. This segregation shows that the PTTH gene is located on chromosome 22. We also examined the relationship to *mw*, another gene on chromosome 22, by the cross (w41 $Ptth^C$♀ × w22 $Ptth^B$ *or mw*♂)♀ × w22♂, and reconfirmed complete linkage (Fig. 4).

Table 3. *Three-point cross among* Ptth, or, *and* mw

	No. of individuals					
Phenotypes\Batch #	1	2	3	4	5	Total
$Ptth^{B/C}$ +^{or} +^{mw}	67	87	84	101	91	430
$Ptth^B$ *or mw*	33	22	58	35	69	217
$Ptth^B$ +^{or} +^{mw}	6	5	5	8	7	31
$Ptth^{B/C}$ *or mw*	2	3	7	6	6	24
$Ptth^{B/C}$ +^{or} *mw*	10	14	15	18	18	75
$Ptth^B$ *or* +^{mw}	13	21	18	13	11	76
$Ptth^B$ +^{or} *mw*	0	0	0	0	0	0
$Ptth^{B/C}$ *or* +^{mw}	3	0	0	5	0	8
Total	134	152	187	186	202	861

The mating scheme was: w22 $Ptth^B$ *or mw*♀ × (w22♀ × w41 $Ptth^C$ +^{or} +^{mw}♂). $Ptth^{B/C}$ means double bands of the B-type and C-type.

(iii) *Three-point cross*

To localize the PTTH gene on chromosome 22, we performed a three-point cross using *or* (22–8·9) and *mw* (minute wings, 22–25·2) as markers (Doira *et al.* 1978, 1992; Doira, 1983). Primers BP5 and BP9 were used to amplify the third intron. The mating scheme was as follows: $Ptth^B$ *or mw*♀ × ($Ptth^B$ *or mw*♀ × $Ptth^C$ +^{or} +^{mw}♂)♂. Numbers of individuals scored in the next generation are shown in Table 3. Recombination values were calculated as 18·5% between *or* and *mw*, 23·9% between *mw* and *Ptth*, and 7·3% between *Ptth* and *or*. Because the distance between *or* and *mw* has been determined to be 16·3 cM (Doira *et al.* 1978), we calculated the locus of the PTTH gene as 8·9 − [7·3 × (16·3/18·5)] = 2·5 (cM). Although the expected number of double recombinants was 861 × 0·185 × 0·073 = 11·6, only 8 double recombinants ($Ptth^B/Ptth^C$ *or*/*or mw*/+ and $Ptth^B/Ptth^B$ *or*/+ *mw*/*mw*) were obtained. We calculated the coincidence coefficient as 8/11·6 = 0·69. This value

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indicates that there is approximately 30% interference to the occurrence of double crossovers.

4. Discussion

We found three size variants of the PTTH gene and ascertained that they involve variations of the third and fourth introns. We speculate that the third intron in $Ptth^C$ and the fourth intron in $Ptth^B$ contain insertion sequences consisting of 400 and 140 bp, respectively, and that these insertions do not affect the function of the PTTH gene. *B. mandarina* from China carries $Ptth^C$, whereas *B. mandarina* from Japan has $Ptth^A$ (see Fig. 1 and Table 1), suggesting that these variants might have already existed in the original population of *B. mandarina* and were introduced to *B. mori* during domestication (Yoshitake, 1968).

The PTTH gene is located on chromosome 22 (see Fig. 5). The genes *Ict-E* (Inhibitor of chymotrypsin E), *or* (r-translucent), *sku* (skunk), and *mw* (minute wing) have been already mapped onto this chromosome (Doira *et al.* 1978, 1992). The *sku* mutant is known to involve an endocrine aberration because the injection of 20-hydroxyecdysone can rescue lethality in individuals homozygous for *sku* (Yoshitake *et al.* 1978*a*, *b*). We think, however, that the *sku* mutation is not directly caused by a structural defect of the PTTH gene since the distance between *sku* and *Ptth* is as great as 13·5 cM. The other loci, *Ict-E*, *or*, and *mw*, also do not seem to be related to the function of the PTTH gene.

Although several non-molting mutants have been found in *B. mori*, *nm* (11–11·6) (Umeya & Karasawa, 1930; Yokoyama, 1936; Shimizu *et al.* 1980), *nm-b* (2–25·1) (Banno *et al.* 1985), *nm-d* (9–16·3) (Doira *et al.* 1984), *nm-k* (4–26·8), *nm-m* (13–27·9) (Shimizu *et al.* 1983), and *nm-g* (17–39·1) (Nagata *et al.* 1987) are located on other chromosomes. Some variants for moltinism have also been isolated. The major locus controlling moltinism is the *M* locus (6–3·0) (Ogura, 1931, 1932, 1933; Shimodaira, 1947), which contains M^3 (Trimolting), *M* (wild type, Tetramolting) and M^5 (pentamolting) alleles. Additional factors affecting moltinism are the *rt* locus (recessive trimolting, 7–9·0) (Hirobe, 1952) and the *mod* locus (dimolting, 11–25·2) (Oota *et al.* 1957; Ninaki *et al.* 1980). All these genes are located on other chromosomes.

It is known that some genes control the rate of larval growth. *Lm* (Late maturity, 1–2·0) (Nagatomo, 1941; Morohoshi, 1957) controls the timing of pupation as well as moltinism and voltinism. *rm* (retarded molting, 3–6·5, Doira *et al.* 1992) also regulates larval growth. They have been mapped on chromosomes other than the 22nd. Embryonic and larval lethal mutations have also been mapped on different chromosomes (Doira, 1983; Japanese Society of Sericultural Science, 1986; Doira *et al.* 1992).

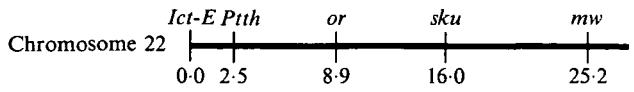

Fig. 5. Revised linkage map of chromosome 22 including the *Ptth* locus. See text for symbols.

We conclude that none of the known mutations and variants which affect larval growth and molting involve functional defects of the PTTH gene. Therefore, it is likely that all the genes described above control processes other than synthesis of PTTH, for example, the release of PTTH, function of the prothoracic gland, or reception of ecdysteroids at target tissues.

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References

Banno, Y., Kawaguchi, Y. & Doira, H. (1985). Genetical studies of the N-methyl-N-nitrosourea induced 'non-molting b' mutation in *Bombyx mori*. *Journal of Sericultural Science of Japan* **54**, 227–231 (in Japanese).

Bender, W., Spierer, P. & Hogness, D. S. (1983). Chromosome walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the bithorax complex in *Drosophila melanogaster*. *Journal of Molecular Biology* **168**, 17–33.

Doira, H. (1983). Linkage maps of *Bombyx mori* – status quo in 1983. *Sericologia* **23**, 245–269.

Doira, H., Chikushi, H. & Kihara, H. (1978). Linkage studies of *Bombyx mori*: Discovery of a new linkage group *or-mw*. *Journal of Sericultural Science of Japan* **47**, 27–31 (in Japanese).

Doira, H., Kihara, H. & Banno, Y. (1984). Genetical studies on the 'non-molting dwarf' mutations in *Bombyx mori*. *Journal of Sericultural Science of Japan* **53**, 427–431 (in Japanese).

Doira, H., Fujii, H., Kawaguchi, Y., Kihara, H. & Banno, Y. (1992). *Genetical Stocks and Mutations of* Bombyx mori: *Important Genetic Resources*. Institute of Genetic Resources, Faculty of Agriculture, Kyushu University, 73 pp.

Hirobe, T. (1952). A preliminary report on inheritance of the recessive trimolter in the silkworm, *Bombyx mori*. *Technical Data, Department of Sericulture, Ministry of Agriculture and Forestry, Japan* **33**, 23 (in Japanese).

Japanese Society of Sericultural Science (1986). Nomenclature and symbols of the genes in the silkworm, *Bombyx mori*. *Journal of Sericultural Science of Japan* **55**, 95–111 (in Japanese).

Kataoka, H., Nagasawa, H., Isogai, A., Tamura, S., Mizoguchi, A., Fujiwara, Y., Suzuki, C., Ishizaki, H. & Suzuki, A. (1987). Isolation and partial characterization of prothoracicotropic hormone of the silkworm, *Bombyx mori*. *Agricultural and Biological Chemistry* **51**, 1067–1076.

Kataoka, H., Nagasawa, H., Isogai, A., Ishizaki, H. & Suzuki, A. (1991). Prothoracicotropic hormone of the silkworm, *Bombyx mori*: amino acid sequence and dimeric structure. *Agricultural and Biological Chemistry* **55**, 73–86.

Kawakami, A., Kataoka, H., Oka, T., Mizoguchi, A., Kimura-Kawakami, M., Adachi, T., Iwami, M., Nagasawa, H., Suzuki, A. & Ishizaki, H. (1990). Molecular cloning of the *Bombyx mori* prothoracicotropic hormone. *Science* **247**, 1333–1335.

Morohoshi, S. (1957). *Physiological Studies on Moltinism and Voltinism in* Bombyx mori: *A New Hormonal Balance Theory on the Growth*. Japan Society for the Promotion of Science, Tokyo, Japan, 202 pp.

Nagata, M., Tsuchida, K., Shimizu, K. & Yoshitake, N. (1987). Physiological aspects of *nm-g* mutant: an ecdysteroid-deficient mutant of the silkworm, *Bombyx mori*. *Journal of Insect Physiology* **33**, 723–727.

Nagatomo, Y. (1941). On the sex-dependent expression of the trimolting property. *Journal of Sericultural Science of Japan* **12**, 171–183 (in Japanese).

Ninaki, O., Doira, H. & Chikushi, H. (1980). Genetical studies of the 'dimolting' mutant in *Bombyx mori*. *Journal of Sericultural Science of Japan* **49**, 347–351 (in Japanese).

Ogura, S. (1931). Erblichkeitsstudien am Seidenspinner *Bombyx mori* L. I. Genetische Untersuchung der Kokonfarbe. *Zeitschrift für induktiv Abstammungs-und Vererbungslehre* **58**, 122–156.

Ogura, S. (1932). Erblichkeitsstudien am Seidenspinner *Bombyx mori* L. II. Genetische Untersuchung der Hautung. *Zeitschrift für induktiv Abstammungs-und Vererbungslehre* **61**, 315–408.

Ogura, S. (1933). Erblichkeitsstudien am Seidenspinner *Bombyx mori* L. III. Genetische Untersuchung der Hautung. *Zeitschrift für induktiv Abstammungs-und Vererbungslehre* **64**, 205–268.

Oota, S., Watanabe, A. & Tokunaga, H. (1957). Genetical study on a spontaneous mutant, two molter, in the silkworm, *Bombyx mori*. *Journal of Sericultural Science of Japan* **26**, 77–81 (in Japanese).

Sambrook, J., Frisch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd Ed. New York: Cold Spring Harbor Laboratory Press.

Sanger, F., Miklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**, 5463–5467.

Shimizu, K., Enokijima, M., Fujimaki, T., Fujimori, H. & Matsuno, M. (1983). Inheritance of a new mutant, 'Matsuno non-molting' in *Bombyx mori*. *Journal of Sericultural Science of Japan* **52**, 348–353 (in Japanese).

Shimizu, K. Tanaka, N. & Matsuno, M. (1980). Linkage analysis of a non-molting mutant of *Bombyx mori* and its application to the stock maintenance. *Journal of Sericultural Science of Japan* **49**, 7–12 (in Japanese).

Shimodaira, M. (1947). Studies of linkage in the silkworm. I. Relation between VI and VIII linkage group. *Japanese Journal of Genetics* **22**, 82–84 (in Japanese).

Umeya, Y. & Karasawa, Y. (1930). A silkworm strain with a factor inhibiting development. *Japanese Journal of Genetics* **6**, 188–194 (in Japanese).

Yokoyama, J. (1936). Histological observations on a non-molting strain of silkworm. *Proceedings of the Royal Entomological Society, London* (*A*) **11**, 35–44.

Yoshitake, N. (1968). Phylogenetic aspects of the origin of Japanese race of the silkworm, *Bombyx mori* L. *Journal of Sericultural Science of Japan* **37**, 83–87 (in Japanese).

Yoshitake, N., Kobayashi, M. & Miyashita, T. (1987*a*). On the 'skunk' mutant in the silkworm. *Journal of Sericultural Science of Japan* **47**, 32–34 (in Japanese).

Yoshitake, N., Kobayashi, M. & Ogawa, Y. (1987*b*). On a smell factor existing in faeces from the skunk silkworm, *Bombyx mori*. *Journal of Sericultural Science of Japan* **47**, 161–165 (in Japanese).