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Hybrid origin of a B chromosome (PSR) in the parasitic wasp *Nasonia vitripennis*

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Abstract. Little is known about the origin and evolution of supernumerary (B) chromosomes. This study utilizes molecular markers to examine the evolutionary history and microstructural organization of the supernumerary paternal-sex-ratio (PSR) chromosome of the parasitic wasp *Nasonia vitripennis*. Copies of the retrotransposon *NATE* were previously isolated from PSR and the genomes of *N. vitripennis* and related wasp species. A phylogenetic analysis of sequences representing 29 elements from PSR and seven wasp species, coupled with a hybridization analysis of elements in genomic DNA provides evidence that PSR was recently transferred into *N. vitripennis* from a species in the genus *Trichomalopsis*. A linear region of the PSR chromosome was compared by Southern blot analysis with genomic DNA from *N. vitripennis*, *Nasonia longicornis*, *Trichomalopsis americanus*, and *Trichomalopsis dubius*. A region organized similarly to the region on PSR was not evident in any of the species, thus a progenitor region was not identified. However, the hybridizations revealed that this region of PSR is primarily composed of repetitive sequences that appear dispersed in these wasp genomes, and might represent additional mobile elements. At least three different dispersed repeats are present in the 18 kb region of PSR. The abundance of tandem and dispersed repetitive sequences in this relatively small region provides additional evidence for the degenerate structure of the PSR chromosome.

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Introduction

Supernumerary (or B) chromosomes[1] have been described in all major groups of animals and plants (Jones and Rees 1982; Beukeboom 1994), and are fundamentally similar to the accessory chromosomes or plasmids that are found in lower eukaryotes and bacteria. The critical characteristic of supernumerary elements is that they are unnecessary for survival of the organism in which they occur. As the definition by Camacho and Parker explicitly states, the B chromosome "follows its own evolutionary pathway" (Beukeboom 1994). Because of this autonomy from the standard (or A) chromosomal complement, B chromosomes are excellent systems for studying evolutionary dynamics. Studies on the evolutionary dynamics of Bs have primarily concentrated on examining the maintenance of the chromosomes within populations (Jones 1991). Very little is known about other evolutionary aspects, such as their origins, processes of molecular evolution, and ultimate evolutionary fates.

Identifying the origins of B chromosomes has been a lingering problem. Two primary sources for B chromosomes have been considered (Jones and Rees 1982; Green 1990); either an intragenomic fragment acquires the characteristics of a B, or interspecific hybridization provides foreign DNA that evolves into a B chromosome. Both of these scenarios share the central feature that a DNA fragment released from selective constraints evolves toward independence as a B chromosome. The first scenario postulates that B chromosomes arise from duplicated or fragmented pieces within the genome. Apparent examples occur in the plant *Crepis capillaris* (Jamilena et al. 1994a, b, 1995) and the fly *Drosophila subsilvestris* (Gutknecht et al. 1995). In both cases, molecular analyses indicate that repetitive sequences on the Bs are shared with the A chromosomes, but progenitor

[1] B chromosome, "a dispensable supernumerary chromosome that does not recombine with the A chromosomes and follows its own evolutionary pathway". (J.P.M. Camacho and J.S. Parker, Beukeboom 1994)

regions have not been identified and direct sequence comparisons have not been performed. Another pathway for the evolution of a B from within the genome is the acquisition of B characteristics by sex-limited chromosomes (W or Y), as has been postulated for the New Zealand frog *Leiopelma hochstetteri* (Green et al. 1993; T.F. Sharbel, personal communication). The other proposed source for the origin of B chromosomes is foreign DNA introduced from a closely related species through hybridization. This scenario has been reproduced experimentally with two plant species, where chromosomes from *Coix gigantea* were incorporated as supernumeraries in plants with an intact *Coix aquatica* chromosomal complement (Sapre and Deshpande 1987). Also, molecular data are suggestive of an interspecific origin of the B in the Australian annual *Brachycome dichromosomatica* (John et al. 1991; Leach et al. 1995). A sequence isolated from the B chromosome cross-hybridized to genomic DNA from other *Brachycome* species, but not to the A chromosomes of *B. dichromosomatica*. Another possible example of B chromosome origin following interspecific hybridization is in the gynogenetic Amazon molly *Poeccilia formosa* (Schartl et al. 1995; McVean 1995).

Comparisons of DNA similarity are necessary for determining B chromosome origins, although several factors may dictate the pattern of similarity. Both the mechanism of origin and the age of the B chromosome will influence the composition of a B relative to the A chromosomal complement. Recent intragenomic or hybrid origins initially create different relationships between the B and resident chromosomal complement, but over time the initial pattern will be obscured. If a B chromosome arises from within the genome, initially it will be very similar to the progenitor region. Through time the B will diverge quantitatively through sequence evolution and qualitatively through rearrangements. Selection pressure to reduce pairing with the progenitor region during meiosis, which would cause genetic imbalance, may drive sequence divergence from the progenitor region in the A complement (Amos and Dover 1981). Even without the selective pressure to reduce B/A pairing, the absence of recombination and selection presumably dictates the loss of functional loci on B chromosomes (Green 1990). Under a hybrid origin, the B is originally dissimilar from the A complement. Over time, the B exchanges mobile elements with the new resident genome, thus becoming qualitatively more similar in repetitive DNA content. Although there are possible examples of both intragenomic and intergenomic origins for particular B chromosomes, none of the systems have been examined to the extent where the mechanism of origin and pattern of molecular evolution can be clearly documented.

Paternal sex ratio (PSR) is a B chromosome in the parasitic wasp *Nasonia vitripennis* (Nur et al. 1988; Werren 1991). The transmission pattern of this chromosome is an extreme example of the independent nature of B chromosomes. Only male wasps carry PSR and the chromosome is transmitted by sperm (Werren and van den Assem 1986; Beukeboom and Werren 1993a). Following fertilization with a PSR-bearing sperm, the maternal chromosomes and PSR undergo the first mitotic division in the zygote, whereas the standard paternal chromosomes form a chromatin mass that fails to divide (Werren et al. 1987; Reed and Werren 1995). Loss of the paternal chromosomes results in the transformation of a diploid zygote into a haploid zygote that develops into a male, owing to haplodiploid sex determination. Molecular analyses of PSR have characterized a number of repetitive sequences from this chromosome (Nur et al. 1988; Eickbush et al. 1992; McAllister 1995). Three different families of PSR-specific tandemly repeated sequences (PSR2, PSR18, PSR22), a tandem repeat (PSR79) that is also present in the A complement, and a retrotransposable element (*NATE*) have all been cloned and characterized from PSR. These isolated sequences indicate that the overall composition of PSR is distinct from the A chromosomes of *N. vitripennis*. Cytologically, PSR is also heterochromatic (Reed 1993).

In previous papers (Nur et al. 1988; Werren 1991; McAllister 1995), a hybrid origin of the PSR chromosome has been proposed. Based upon the geographic distribution of PSR (restricted to western North America in an area of sympatry with the sibling species *Nasonia longicornis*) and evidence that interspecific cytoplasmic incompatibility results in the generation of *de novo* chromosomal fragments (Ryan et al. 1985; Beukeboom and Werren 1993b), it was proposed that PSR arose as a chromosomal fragment from *N. longicornis* generated by interspecific hybridization with *N. vitripennis*. In this paper, a sequence analysis of the retrotransposon *NATE* is used to examine directly the evolutionary history of the PSR chromosome. Sequences of 29 elements isolated from PSR and the genomes of seven wasp species are analyzed. *NATE* appears useful for inferring the history of PSR, because the phylogenetic pattern exhibited by a larger sample of elements was consistent with vertical maintenance of the element in this group of wasps (McAllister and Werren 1997). Furthermore, in an attempt to identify the progenitor region from which PSR was derived, a linear region of PSR is used to examine the organization of cross-hybridizing sequences in the A complements of four wasp species. Results support a hybrid origin of PSR, but through hybridization between *N. vitripennis* and a wasp from the closely related genus *Trichomalopsis*.

Materials and methods

Genomic DNA. Nine species of parasitoid wasps were used in this study. These wasp species are *N. vitripennis* (with PSR and without PSR), *N. longicornis*, *Nasonia giraulti*, *Trichomalopsis* (=*Eupteromalus*) *americanus*, *Trichomalopsis* (=*Eupteromalus*) *dubius*, *Urolepis rufipes*, *Muscidifurax uniraptor*, *Muscidifurax raptorellus*, and *Muscidifurax raptor*. All of these species are classified in the tribe Pteromalini within the family Pteromalidae (Burks 1979). Wasps were cultured in the laboratory on *Sarcophaga bullata* or *Musca domestica* pupae. Total genomic DNA was extracted from pooled wasps of a single strain using standard techniques for phenol/chloroform extraction and ethanol precipitation as previously described (McAllister 1995; McAllister and Werren 1997).

Phylogeny of NATE. The sequences of *NATE* used in this study were previously used in a more extensive analysis of element phylogeny (McAllister and Werren 1997), and the sequences are available in GenBank (U29470–U29473, U69494, U69496–U69506, U69514–U69522, U69525–U69527, U69529). Procedures for isolating these sequences of *NATE* have been presented (McAllister 1995; McAllister and Werren 1997). Briefly, λ clones containing copies of *NATE* on the PSR chromosome were isolated from a genomic library containing DNA from PSR males in the MI strain of *N. vitripennis*. Two cross-hybridizing elements were isolated from a genomic library of the IV-14 strain of *N. longicornis*. An approximately 900 bp region encompassing the reverse transcriptase (RT) domain was sequenced from four PSR and two *N. longicornis* elements. Regions of sequence conservation were identified and two sets of primers were designed for amplifying elements from genomic DNA using the polymerase chain reaction (PCR). The g-primer set (N6, 5′ TAC ACC TCA AAG GCA CAG; N14, 5′ AGA CCA GCT TCG TTT ATC C) was designed to amplify a wide range of elements. The p primers (N12, 5′ GCC TCC CTC TCG TCT GCA; N13, 5′ CAA TGG GTT TGA CTC GTT CG) form a primer set designed preferentially to amplify elements more closely related to those on PSR. A 700 bp consensus sequence is shared by both reaction products, encompassing 240 bp preceding and 460 bp into the RT domain of the elements.

The previous study of 43 element sequences isolated from nine species of parasitoid wasps indicated that two major subfamilies (I and II) of *NATE* are present in these wasps (McAllister and Werren 1997). All of the elements isolated from PSR were contained in subfamily I, so this analysis concentrated on this subfamily. Sequences from 29 elements representing 7 species and PSR were analyzed. Elements were examined from PSR, *N. longicornis*, *N. giraulti*, *T. americanus*, *T. dubius*, *U. rufipes*, *M. raptorellus*, and *M. uniraptor*. Nomenclature of the element sequences is given by the species name, the method of obtaining the sequence (c=clone from genomic library, p=PCR product using the p-primer set, g=PCR product using the g-primer set), and a number identifying the sequence.

The sequences were visually aligned to minimize the number of gaps. A parsimony analysis to determine the relationships among the sequences was performed using PAUP ver. 3.1 (Swofford 1991). The heuristic search option was used with the sequences added randomly 50 times. Three sequences from subfamily II were designated as a user-defined outgroup for rooting the subfamily I sequences. To determine the confidence in each node in the phylogenetic tree, 500 bootstrap replications were performed using the heuristic search option with ten random additions. Pair-wise differences were also calculated using PAUP.

Southern blotting of NATE. The genomic DNAs were screened for the presence of *NATE* using hybridization. Genomic DNA from each wasp species was digested with *Alu*I following the manufacturer's recommendations (BRL). The quality and quantity of digested DNAs were visually compared by ethidium bromide staining following electrophoresis of a subset of the reactions. Equal amounts of digested DNAs were electrophoresed in a 1.5% agarose gel and transferred to a nylon membrane. An N6/N14 PCR product from a λ clone (λP16) containing a PSR-inserted *NATE* (PSR c16) was labeled with [α^{32}P]dATP using random primer labeling following the manufacturer's (BRL) suggestions and used as a probe. The hybridization solution contained 5×SSC, 5×Denhardt's solution, 125 mg/ml denatured calf thymus DNA, and 1% SDS (Ausubel et al. 1992). Hybridization was performed for 16 h at 65°C. The final wash of the membrane was at 65°C with a solution of 0.1×SSC and 0.1% SDS. (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate.)

Structure of PSR. The structure of an 18 kb cloned region from the PSR chromosome was examined. This region (λP17) of the PSR chromosome was isolated in a previous study (McAllister 1995), because it contains a partial copy of *NATE*. In addition to *NATE* (PSR c17), which was truncated by the cloning site at one end of the insert, the clone contains 11 kb flanking the intact end of this element. Subclones from λP17 were obtained in two rounds of double-digestion and ligating into Bluescript (Stratagene), one with *Eco*RI and *Hin*dIII and the other with *Eco*RI and *Hin*cII. A restriction map of this region was obtained by hybridizing a blot of fully and partially digested λP17 DNA with specific subclones from the insert.

Four subclones from the λP17 region were used to examine the structure of cross-hybridizing sequences on PSR and in total genomic DNA of *N. vitripennis* (without PSR), *N. longicornis*, *T. americanus*, and *T. dubius*. Genomic DNA from each strain and λP17 was digested with *Hin*dIII; the samples were divided into four aliquots containing equal amounts of DNA and separated in an 0.8% agarose gel. After the DNAs had been transferred to a nylon membrane, the membrane was cut into four pieces, each containing *Hin*dIII-digested DNA from all the wasp strains and λP17. Inserts from subcloned regions of λP17 were amplified from plasmids using the T3 and T7 primer sites located at both ends of the polycloning region in Bluescript. These PCR products were labeled by random primer labeling and used as probes. Labeling of probes and hybridization were carried out under the same conditions as above. Each of the four probes was hybridized to one of the four membranes. Final washes of the membranes were in a solution of 0.2×SSC and 0.1% SDS at 65°C. This procedure provided an independent hybridization for each probe to identical DNA samples, which were electrophoresed under the same conditions.

Partial crude sequence was obtained from the region of λP17 flanking the copy of *NATE*. Approximately 300 bp was read into each end of the plasmid subclones, and most of the sequence was obtained by reading only a single strand. Sequencing reactions were performed on chemically denatured (NaOH) double-stranded plasmid DNA (Ausubel et al. 1992) using the T3 and T7 sequencing primers. Reactions were performed with the Sequenase kit (USB), labeling with [α^{35}S]dATP, and electrophoresing through buffer gradient gels. Manipulation of the sequence was performed using ESEE ver. 1.0 (Cabot and Beckenbach 1989) and GCG (Genetic Computer Group, Madison, Wis.). Homology searches of the sequence databases were performed with the BLASTN and BLASTX algorithms (Altschul et al. 1990; Gish and States 1993) using NCBI (National Center for Biotechnology Information) facilities accessed through the network server.

Results

Phylogenetic analysis of NATE *to determine PSR history*

This analysis of the relationships among these *NATE* sequences was performed to investigate the evolutionary history of PSR. The phylogenetic analysis revealed that elements on PSR form a very closely related group (Fig. 1). All but two of the PSR elements are contained in a monophyletic group that is supported by a relatively high (84%) bootstrap value, considering there are only two diagnostic nucleotides supporting this group. This indicates that these elements have recently replicated from a common ancestral element. Although the close relationship among these elements apparently represents a recent diversification, the seven PSR elements exhibit a 1.1% mean total pair-wise difference throughout the 700 bp region (Table 1). Therefore, a substantial amount of sequence divergence has occurred among these elements.

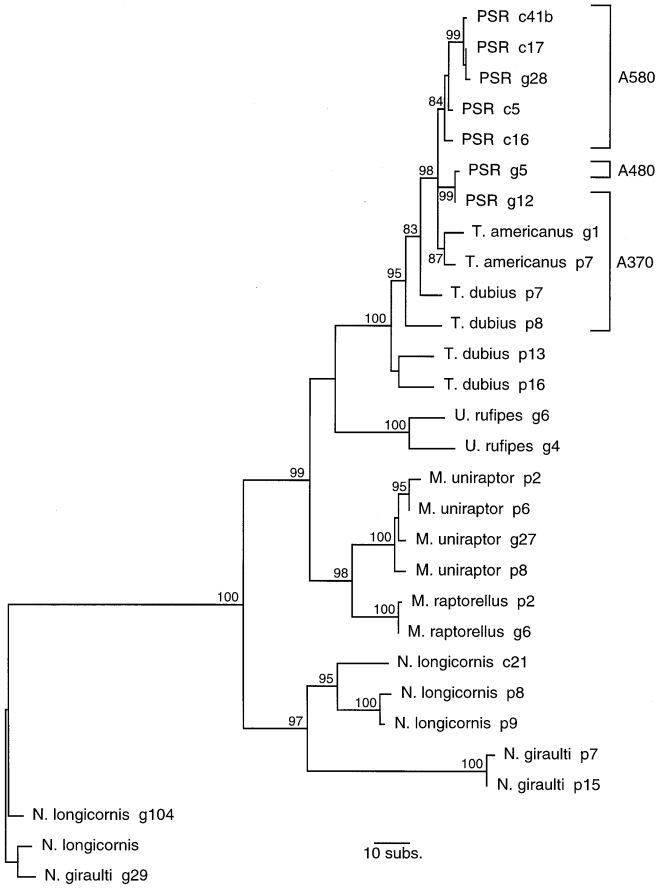

Fig. 1. Phylogenetic relationships among element sequences from the paternal-sex-ratio (PSR) chromosome of *Nasonia vitripennis* and seven wasp species. This is one of the three equally parsimonious trees obtained with a minimum length of 409 substitutions. Bootstrap values are presented for the nodes with greater than 80% support

Most of these elements designated as occurring on PSR have been independently verified as inserts on the chromosome. Four of the seven PSR elements were obtained in λ clones (identified with "c" in Fig. 1) by screening a genomic library, and unique PCR and/or Southern blot assays were developed to detect the presence of the regions containing these elements (McAllister et al. in preparation). By screening genomic DNA extracted from a strain of *N. vitripennis* with and without PSR, these elements have been specifically localized to the chromosome. The three elements obtained by PCR were not independently verified as inserts on this chromosome, but similar elements were not obtained upon amplification from genomic DNA isolated from the same *N. vitripennis* strain (MI) without PSR, using the p-element primers.

As seen in Fig. 1, element sequences from the genome of *T. americanus* are grouped as the closest relatives to the PSR elements; furthermore, the elements on PSR are contained in a clade represented by elements obtained from both species of *Trichomalopsis*. This clade of PSR/*Trichomalopsis* elements is strongly supported by a bootstrap value of 100%, indicating that this relationship is not due to chance. In contrast, elements from the *Nasonia* species were distantly related to the PSR elements (Fig. 1). Failure to amplify PSR-like elements in any of the three *Nasonia* species was not due to sampling error during cloning of the PCR products. Two different primer pairs were used to amplify elements from genomic DNA, and restriction digestion of these products and visualization in ethidium bromide-stained agarose gels revealed that PSR-like elements were not amplified from the genomes of any of the three *Nasonia* species. Therefore, *NATE* elements on PSR are more closely related to those found in *Trichomalopsis* than to those in *Nasonia*.

Table 1. Mean (±SD) pair-wise sequence differences within and among element groups

	PSR-7[a]	T. am.-2	T. dub.-4	U. ruf.-2	M. uni.-4	M. rel.-2	N. lon.-3	N. gir.-2	N. lon.-2[b]	N. gir.-1[b]
PSR-7	0.011 (±0.007)	0.018 (±0.003)	0.034 (±0.008)	0.091 (±0.002)	0.092 (±0.003)	0.091 (±0.002)	0.115 (±0.003)	0.161 (±0.003)	0.141 (±0.002)	0.147 (±0.002)
T. am.-2		0.012 –	0.036 (±0.008)	0.092 (±0.002)	0.093 (±0.002)	0.094 (±0.002)	0.117 (±0.003)	0.165 (±0.004)	0.146 (±0.002)	0.152 (±0.002)
T. dub.-4			0.033 (±0.004)	0.085 (±0.002)	0.084 (±0.005)	0.083 (±0.005)	0.111 (±0.002)	0.156 (±0.002)	0.145 (±0.006)	0.152 (±0.006)
U. ruf.-2				0.003 –	0.094 (±0.002)	0.091 (±0.001)	0.116 (±0.006)	0.158 (±0.005)	0.146 (±0.003)	0.154 (±0.006)
M. uni.-4					0.009 (±0.003)	0.042 (±0.003)	0.115 (±0.002)	0.156 (±0.003)	0.154 (±0.003)	0.161 (±0.003)
M. rel.-2						0.001 –	0.109 (±0.002)	0.148 (±0.002)	0.142 (±0.002)	0.150 (±0.001)
N. lon.-3							0.030 (±0.021)	0.105 (±0.003)	0.146 (±0.003)	0.153 (±0.003)
N. gir.-2								0.003 –	0.174 (±0.002)	0.180 (±0.002)
N. lon.-2									0.016 –	0.015 (±0.003)

T. am., *Trichomalopsis americanus*; T. dub., *T. dubius*; U. ruf., *Urolepsis rufipes*; M. uni., *Muscidifurax uniraptor*; M. rel., *M. raptorellus*; M. lon., *Nasonia longicornis*; N. gir., *N. giraulti*

[a] Number of elements in group
[b] Outgroup elements

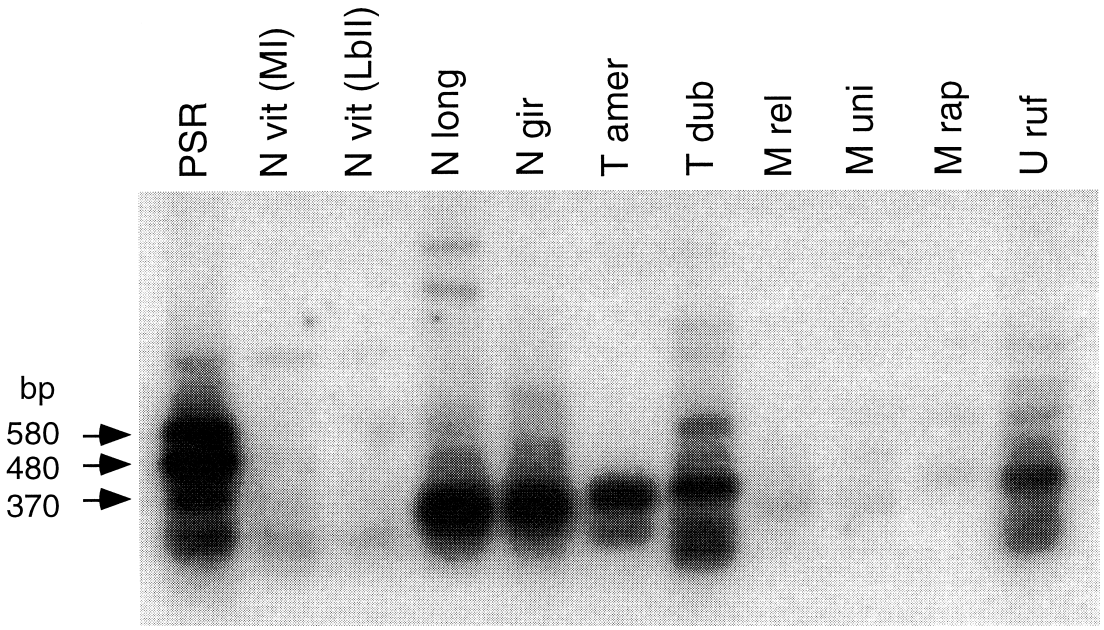

Fig. 2. Hybridization of the reverse transcriptase (RT) region of *NATE* to restriction enzyme-digested genomic DNA from 11 wasp strains. Each genomic DNA was digested with *Alu*I, and the membrane was probed with the RT region of PSR c16. Fragment sizes are indicated for the A580, A480 and A370 subgroups from PSR. N vit, *N. vitripennis*; M. rap, *M. raptor*; other abbreviations as in Table 1

The grouping of elements on PSR with elements from the two species of *Trichomalopsis* was the only example where element relationships were not representative of taxonomic status (Fig. 1). In this and the previous phylogenetic analysis (McAllister and Werren 1997), the relationships among elements are very consistent with taxonomic boundaries, indicating that *NATE* has been transmitted vertically during the divergence of the tribe Pteromalini.

Detection of elements in genomic DNA through Southern hybridization

To confirm that elements were present in genomic DNA and to detect the presence of restriction-site defined subgroups, a Southern blot analysis was performed. Probe selection and stringency requirements were designed to concentrate the analysis on elements closely related to those on PSR. The probe consisted of the region analyzed in the sequence analysis and was derived from PSR c16. Genomic DNA was digested with *Alu*I, because this restriction enzyme exhibited phylogenetically informative restriction patterns based on the sequence data.

All genomic DNAs exhibited at least faint hybridization to the probe (Fig. 2). Strong hybridization was observed to genomic DNA from the MI strain of *N. vitripennis* when the PSR chromosome was present, but only faint hybridization was observed in the absence of PSR (Fig. 2). A European strain (LabII) of *N. vitripennis* that was not carrying PSR exhibited similarly faint hybridization to the probe. Strong hybridization dependent on the presence of PSR confirms that elements are restricted to this chromosome and are not present on the A chromosomes of *N. vitripennis*.

By examining *Alu*I restriction fragments that hybridized to the probe, the presence of PSR-like elements could be visualized. Three different *Alu*I variants were identified among the element sequences on the PSR chromosome. These element subgroups are referred to as A580, A480, and A370 (Fig. 1), and each is expected to produce a major hybridizing band of 580, 480, and 370 bp, respectively, upon digestion with *Alu*I. The A580 and A480 subgroups were only identified among element sequences isolated from PSR, whereas the A370 subgroup was present among PSR, *T. americanus* and *T. dubius* elements (Fig. 1). Based on the hybridization results, it appears that only elements producing a 370 bp fragment are present in the genome of *T. americanus* (Fig. 2). Strong hybridization was also observed to the 370 bp *Alu*I fragment in *T. dubius*; however, additional bands were also present in genomic DNA from this species (Fig. 2). Hybridization was detected at bands that apparently correspond to the 580 and 480 bp restriction fragments, although hybridization intensity was lower than that at the 370 bp restriction fragment. Hybridization to the 580, 480, or 370 bp fragments was not apparent in *N. vitripennis*, *N. longicornis*, or *N. girault*i.

The sequence analysis revealed that elements on PSR are primarily grouped into a single clade and most closely related to elements in *T. americanus*. However, hybridization (Fig. 2) indicates that elements in the A580 and A480 subgroups (Fig. 1) are present in the genome of *T. dubius*. This finding is important for the relationship among the PSR elements, because it indicates these

elements do not truly form a monophyletic group descended from a single element on PSR. In other words, multiple elements could have been present on the PSR progenitor fragment prior to its transfer from *Trichomalopsis* into *N. vitripennis*. An alternative possibility is that A370 elements in the *T. dubius* genome have lost either of two *Alu*I restriction sites, thus yielding elements with 580 and 480 bp restriction fragments. These may not be more similar to the PSR elements in overall sequence, but may only share the lost *Alu*I restriction sites. Based on the intensity of the hybridization, however, many elements in the *T. dubius* genome must have lost these sites.

Strong hybridization was observed to genomic DNA from *N. longicornis* and *N. giraulti*; however, the hybridizing restriction fragments at approximately 310 bp (Fig. 2) apparently correspond to sequences of subfamily II elements that were distantly related to those on PSR (Fig. 1). The subfamily I elements sequenced from *N. longicornis* and *N. giraulti* are approximately 88% and 84% identical, respectively, to the probe (PSR c16) sequence (Table 1). The outgroup elements (subfamily II) in *N. longicornis*/*N. giraulti* are about 85% identical (Table 1). Because such strong hybridization was observed to genomic DNA from *N. longicornis* and *N. giraulti*, given about a 13% sequence difference between the probe and hybridizing elements, this suggests that *NATE* is very abundant in these two species. Faint hybridization was observed to genomic DNA from the three species of *Muscidifurax* (Fig. 2), which is consistent with a relatively low copy number in these species, given the observed similarity (91%) to the probe sequence (Table 1). The probe hybridized relatively strongly to *U. rufipes* (Fig. 2), and these elements are also about 91% similar to the probe (Table 1).

Structure of PSR in relation to the A chromosomes

Additional regions were studied to investigate the origin and structure of PSR. For this purpose, the structure of a linear region on the PSR chromosome, containing a copy of *NATE* (PSR c17) and undefined sequences flanking the element, was examined. An approximately 18 kb region of PSR was present as an insert in λP17. About 7 kb of one end of the insert is comprised of *NATE*, and the cloning site at this end falls within the element, causing its truncation. The structure of the DNA flanking the intact end of *NATE* was investigated. Plasmid subclones were obtained throughout the insert of λP17. A restriction map of the intact *NATE* end (3′ or right) and the region flanking the element is presented (Fig. 3A).

Except for the copy of *NATE*, the content of the λP17 region was previously unknown. No cross-hybridization was detected when the region was probed with tandemly repeated sequences that have been previously described on the PSR chromosome (Eickbush et al. 1992; McAllister 1995). Four nonoverlapping subclones were obtained that represented most of the flanking region. Using these four subclones as probes, Southern blots were performed to determine the organization of cross-hybridizing sequences on PSR and the A chromosomes of *N. vitripennis*, *N. longicornis*, *T. americanus*, and *T. dubius*.

Southern blots with the probes from the region flanking *NATE* revealed that most of this region comprises repetitive sequences. Three of the four probes cross-hybridized to repetitive sequences in genomic DNA from *N. vitripennis*, *N. longicornis*, *T. americanus*, and *T. dubius*. Subclone p17-1 hybridizes to a 2.1 kb *Hin*dIII fragment from PSR corresponding to the fragment from which it was derived (Fig. 3B). Hybridization at a similar-sized band was not observed in any of the other genomic DNAs. Comparisons between *Hin*dIII-digested DNA from *N. vitripennis* with and without PSR reveal the presence of other cross-hybridizing sequences on PSR (Fig. 3B). Cross-hybridizing sequences are also present in the A complement of *N. vitripennis*; however, most of the hybridization is concentrated at an approximately 800 bp *Hin*dIII fragment. This 800 bp *Hin*dIII fragment is also present in *N. longicornis*, *T. americanus*, and *T. dubius*. In addition to hybridization at this 800 bp fragment, many larger restriction fragments hybridized to p17-1. The two *Trichomalopsis* species exhibited much stronger hybridization than the two *Nasonia* species and PSR, providing additional evidence for similarity between PSR and the genomes of the two *Trichomalopsis* species.

The p17-2 subclone is separated from p17-1 by about 300 bp and it apparently hybridizes to the same 800 bp *Hin*dIII fragment as p17-1, but also hybridizes to additional regions (Fig. 3C). Again, this 800 bp fragment is shared by the two *Nasonia* and two *Trichomalopsis* species. A *Hin*dIII restriction site is present in p17-2, so two adjacent *Hin*dIII fragments of 2.1 and 3.1 kb hybridize to this probe on PSR (Fig. 3C). Other than these two fragments, very similar hybridization patterns were exhibited in the presence or absence of PSR with the *N. vitripennis* A complement. This indicates that few or no other cross-hybridizing sequences are present on PSR. The p17-2 probe hybridized very strongly to genomic DNA from both *Nasonia* and *Trichomalopsis* species.

The p17-3 subclone, further across this region, appears to be single copy on PSR. A single 3.4 kb *Hin*dIII fragment hybridizes to the probe in the presence of the PSR chromosome (Fig. 3D). Cross-hybridization was not detected in genomic DNA from *N. vitripennis*, *N. longicornis*, *T. americanus*, and *T. dubius*. The image in Fig. 3D resulted from exposure for 2 days, and following exposure for 9 days no signal was detected in the other genomic DNAs. Two different methods were used in attempts to isolate a region in the A complement that is homologous to p17-3. Genomic libraries from *N. longicornis* and *N. vitripennis* (with PSR) were screened with p17-3, and no clones representing homologs were isolated. From partial sequence obtained from this region, four different primers were synthesized. These primers were used in four different combinations at medium and low stringency reaction conditions in attempts to amplify the region from *N. vitripennis*, *N. longicornis*, *N. giraulti*, and *T. dubius*. Although the target sequence was always amplified in the presence of PSR, neither a product of similar size nor one that cross-hybridized

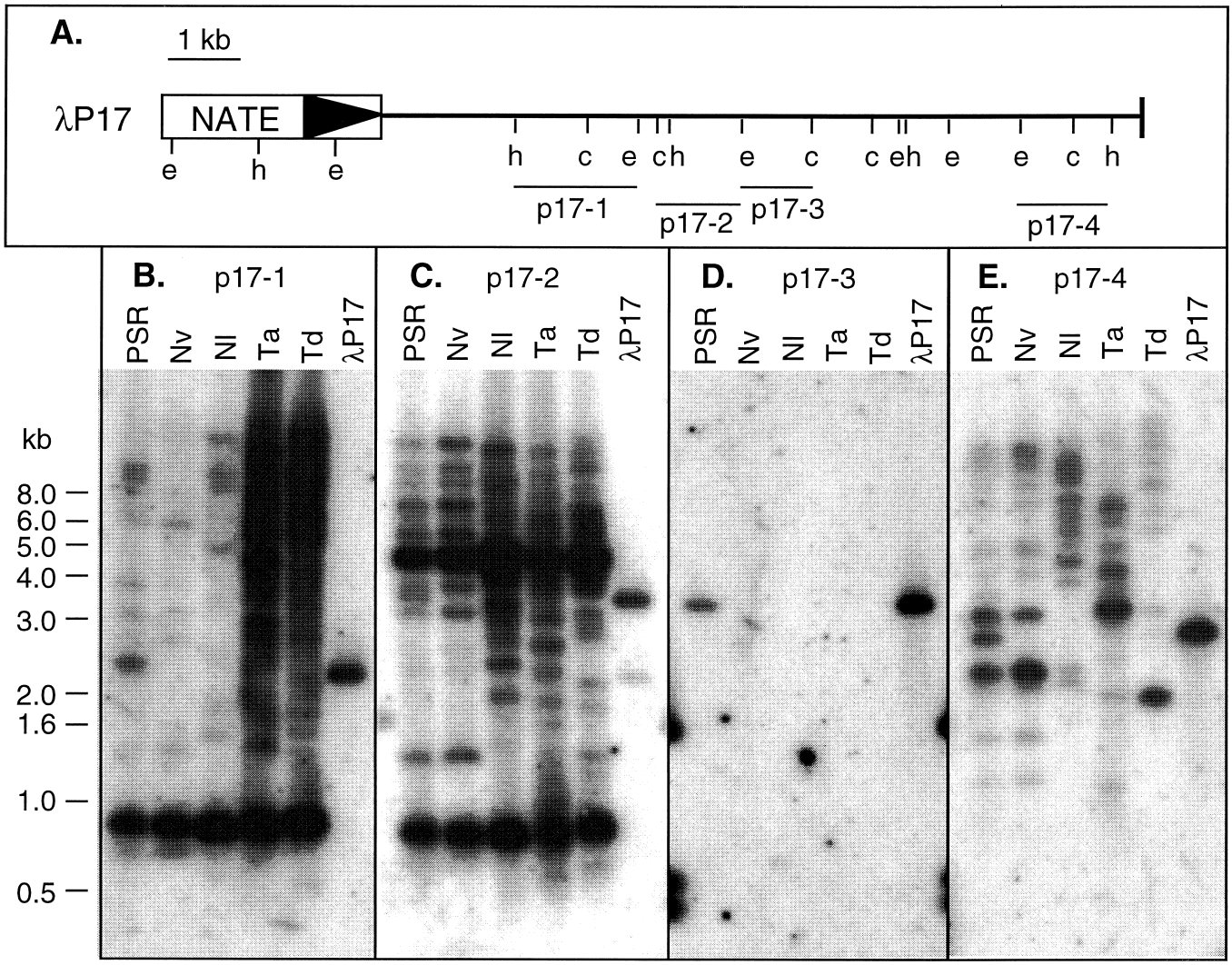

Fig. 3A–E. Organization of a cloned region of PSR and hybridization of subclones to genomic DNAs. **A** Restriction map and location of subclones used in the hybridization analysis. Restriction enzymes are: *c Hin*cII, *e Eco*RI, *h Hin*dIII. **B–E** Autoradiograms following hybridization of each subclone to genomic DNAs and the λP17 clone. Each of the panels contains *Hin*dIII-digested DNA from *N. vitripennis* with PSR (*PSR*), *N. vitripennis* without PSR (*Nv*), *N. longicornis* (*Nl*), *T. americanus* (*Ta*), *T. dubius* (*Td*), and a λ clone containing this region of PSR (*λP17*)

with the target sequence was amplified from the four genomic DNAs. So far, a region from an A chromosome that is homologous to p17-3 has not been isolated; thus the origin of this single-copy fragment remains elusive.

Another repetitive sequence is located in the most terminal section of this region. The subclone p17-4 was derived from a 2.7 kb *Hin*dIII fragment on PSR and this is visualized in the hybridization (Fig. 3E). There apparently are few or no additional cross-hybridizing sequences on PSR, because, other than the 2.7 kb fragment, very similar hybridization patterns were observed in the presence or absence of PSR with the *N. vitripennis* A complement. Cross-hybridizing sequences were detected in genomic DNA from all four of the wasp species, although each species exhibited a unique hybridization pattern (Fig. 3E).

Throughout this entire region, there is no detectable restriction site conservation between PSR and the cross-hybridizing regions in the *Nasonia* or *Trichomalopsis* genomes. The content of this region appears to be a mixture of different repetitive sequences, except for the small single-copy region (Fig. 3D). In this 18 kb region on the PSR chromosome, there is a copy of *NATE* and at least two additional repetitive elements that appear to be dispersed in the genomes of these wasp species. At least one repetitive element is present in the sequence encompassed by p17-1 and p17-2 and a different element in the region of p17-4 (Fig. 3A–C, E).

A genetic database search using sequence from these apparently repetitive sequences identified similarity to known repetitive elements. Similarity to a group of LTR (long terminal repeat) retrotransposons was revealed in three different stretches of amino acids inferred from the partial nucleotide sequences from the p17-4 subclone and adjacent subclones. The *412* retrotransposon received the top score (P=7.5×10^{-18}) in the BLAST search, and two other elements (*osvaldo* and *TED*) in the same retrotransposon group were included in the top five

scores. This group of retrotransposons is very distantly related to *NATE* (McAllister 1995). Putative nucleotide identity between the p17-1/p17-2 region and different tRNAs was identified. Although the significance of the similarity was relatively low, the repetitive sequence in this region could potentially represent a SINE-like element, since these elements have tRNA homology (Deininger 1989).

Discussion

It was previously hypothesized that the PSR chromosome recently originated in *N. vitripennis* following an interspecific hybridization with *N. longicornis* (Nur et al. 1988; Werren 1991; McAllister 1995). The PSR chromosome has a very limited distribution, and other evidence indicated that a centromeric fragment of the *N. longicornis* genome could have been transferred into *N. vitripennis* following a natural hybridization. This analysis was performed to determine whether sequences on the PSR chromosome supported the theory by sharing a high degree of similarity with homologous sequences in the *N. longicornis* genome. Comparison of the DNA content of B chromosomes with standard genomes is generally the method used for assessing the evolutionary history of Bs. Often these comparisons are difficult to interpret, because the sequences used in the analyses are repetitive and exhibit complex evolutionary patterns. A thorough analysis is required to determine the pattern exhibited by the marker before it can be used to infer the history of the B chromosome. By examining the phylogenetic relationships among the many copies of *NATE* isolated from the PSR chromosome and from the genomes of these parasitoid wasps, the evolutionary history of PSR has been further elucidated. The evolutionary pattern exhibited by *NATE* provides clear evidence that the underlying DNA that constitutes PSR was recently transferred into *N. vitripennis*. Contrary to previous predictions, however, the donor is apparently a member of the wasp genus *Trichomalopsis*.

For phylogenetic reconstruction, it would be preferable to compare a single-copy sequence on PSR and the homologous region from potential donor species. Such a marker has yet to be identified in the regions cloned from PSR. Although using *NATE* as a phylogenetic marker was not ideal, potential problems have been addressed in the analysis. One problem with the use of *NATE* is that multicopy elements often exhibit complex evolutionary patterns. Problems arise because the complement of elements in the genomes of a group of species may be comprised of paralogous elements that diverged prior to speciation events and orthologous elements that diverged because of speciation events (Cummings 1994). In other words, only the element lineages that diverged because of speciation events truly reflect phylogenetic history. Ancient duplications prior to speciation events and loss of element lineages can obscure evolutionary history. McAllister and Werren (1997) sequenced and analyzed 43 copies of *NATE* to determine its evolutionary pattern in this group of parasitic wasps. Both orthologous and paralogous lineages were evident among these elements. However, assays for isolating elements from genomic DNAs were designed to concentrate on elements that were similar to those isolated from PSR, so that the lineage of elements encompassing those from PSR was thoroughly represented.

The phylogenetic relationships among *NATE* elements indicate that all those isolated from the PSR chromosome form a closely related group. Because these elements belong to the same monophyletic group as elements isolated from the two species of *Trichomalopsis*, the elements on PSR are apparently derived from an ancestral element or elements in the genome of a member of this genus. One possibility is that, because *NATE* is a retrotransposon, the elements established themselves on PSR following horizontal transfer of a single element. However, this explanation has a few unlikely requirements. First, sequence evolution of the elements on PSR must be greatly accelerated. A large number of nucleotide substitutions have occurred in the RT region among the elements on PSR, with preferential accumulation at synonymous sites (McAllister 1995). Although error-prone replication during retrotransposition may increase the substitution rate (Preston et al. 1988; Roberts et al. 1988; Li and Loeb 1992), a large number of replications would be required to generate the amount of sequence diversity observed among the elements on PSR, but there are only about nine copies of *NATE* on PSR (McAllister et al., in preparation). In addition to sequence divergence among elements in this internal region, sequence differences between the two LTR sequences from a single element (PSR c16) indicate that this element inserted into its present location approximately 120,000 years ago (McAllister 1995). The time required to generate the observed sequence diversity among and within elements on PSR following horizontal transfer of a single element is inconsistent with the limited geographic distribution of this chromosome. Second, the founder element must have transferred to a male *N. vitripennis* that carried PSR, and inserted into the PSR chromosome without inserting elsewhere in the genome. This is very unlikely, because in the limited region where PSR occurs, only about 5% of males carry PSR (Skinner 1983; Werren 1991; Beukeboom 1992) and this chromosome only constitutes about 5.7% of the total genome (Nur et al. 1988; Reed 1993). Horizontal transfer of a single element and establishment of this element on PSR is a convoluted explanation of the pattern exhibited by *NATE*.

The most plausible explanation for the relationship between copies of *NATE* on PSR and in the genomes of the two *Trichomalopsis* species is that the chromosomal fragment that constitutes PSR was transferred from a species of *Trichomalopsis* into *N. vitripennis*. Presence of multiple elements on a chromosomal fragment that was transferred into *N. vitripennis* accounts for the observed divergence among the elements on PSR. Support for this conclusion is also provided by the hybridization data (Fig. 2). Although the PSR element sequences are most closely related to sequences isolated from *T. americanus*, the hybridization data suggest the presence of undetected elements in the *T. dubius* genome that are more

similar to the PSR elements. Among the sequenced elements, five of the seven elements on the PSR chromosome form a closely related group and one character that is shared by this group is a base substitution causing the loss of an *Alu*I restriction site. This subgroup was referred to as the A580 subgroup, because of the 580 bp *Alu*I restriction fragment in the RT region. Faint hybridization was observed to a similar-sized restriction fragment in genomic DNA from *T. dubius*. Presence of this restriction fragment in *T. dubius* may represent elements that were undetected when obtaining sequences. Hybridization to the 370 bp fragment, indicative of the elements that were amplified and sequenced, was more intense than hybridization to the 580 bp fragment; thus these elements would preferentially amplify by PCR. This interpretation indicates that elements contained in the A580 subgroup are present in the *T. dubius* genome, and these elements are not specific to the PSR chromosome. The same pattern was also exhibited for the A480 subgroup. However, these elements from *T. dubius* or other species of *Trichomalopsis* have not yet been isolated and sequenced.

Although multiple copies of NATE may have been present on the chromosomal fragment that was transferred from *Trichomalopsis* into *N. vitripennis*, some of the current elements on PSR may be derived through replication of elements on this chromosome. It is not known whether there are active elements on PSR. At the very least, the elements on PSR appear to be derived from a recently active lineage, because some pairs have extremely high similarity and an excess of synonymous over nonsynonymous substitutions (McAllister 1995). If active elements are present, however, the inheritance pattern of PSR prevents transfer of elements to the A chromosomes in the general population of *N. vitripennis*. The A complement of each male that carries PSR is destroyed after fertilization, owing to PSR action. Therefore, the standard genome of *N. vitripennis* that has been a potential recipient of elements from PSR was extinguished following exposure to this chromosome. This means that transposon flow between PSR and the standard *N. vitripennis* genome is unidirectional–PSR can potentially acquire elements from A chromosomes, but the reverse is prevented. Three iso-female lines of *N. vitripennis* obtained from the geographic region where PSR occurs were screened by Southern hybridization for the presence of PSR-like elements (B. McAllister, unpublished). No PSR-like elements were detected in the *N. vitripennis* lines, supporting the notion that the transmission mechanism of PSR prevents the movement of elements from PSR to the A chromosomes.

The best explanation for the transfer of a chromosomal fragment from a species of *Trichomalopsis* to *N. vitripennis* is through copulation and fertilization. This requires two factors: ecological association of the two species, and breakdown in pre-zygotic isolating mechanisms. The genus *Trichomalopsis* (previously *Eupteromalus* in North America) contains many species and is distributed in both Nearctic and Palearctic regions (Burks 1979; Boucek and Rasplus 1991). Strains of *T. dubius* and *T. americanus* used here were not collected from the geographic region where PSR is present in *N. vitripennis*. The *T. americanus* strain was isolated as a parasite on muscoid flies (*M. domestica* and *Stomoxys calcitrans*) occurring in a feedlot near Lincoln, Nebraska, USA (J. Petersen, pers. commun.) and *T. dubius* was isolated from a dairy near Ithaca, New York, USA (Hoebeke and Rutz 1988). There are no records of either species being collected from the Great Basin region of North America, although the distribution of *T. americanus* includes the northwestern United States (Burks 1979). During censuses of natural populations of *Nasonia*, however, wasps tentatively identified as *T. dubius* were collected in the region where PSR is found (J. Werren, unpublished data). In the Great Basin region, *T. dubius* was occasionally collected from baits placed near carcasses and in feedlots, the primary habitats for *N. vitripennis*. Other than these limited observations on distributions and habitats, indicating a slight but not overwhelming ecological association, there is little known about potential contact between *N. vitripennis* and species of *Trichomalopsis*. In the event that a species of *Trichomalopsis* was in contact with *N. vitripennis*, copulation and fertilization were required to transfer the chromosomal fragment. In the laboratory, attempts at crossing either *T. dubius* or *T. americanus* with *N. vitripennis* have been unsuccessful. Males of either genus would court females of the other; however, females were not receptive (B. McAllister, unpublished). This suggests that strong pre-zygotic isolating mechanisms are present, so failure of these isolating mechanisms must be rare.

Although hybridization between a *Trichomalopsis* species and *N. vitripennis* may have been an extremely rare event, the phylogenetic pattern of *NATE* is strong evidence for hybridization and transfer of a chromosomal fragment that constitutes PSR into *N. vitripennis*. One major issue remains unresolved from the analysis of *NATE*. Was the chromosomal fragment a component of the normal chromosomal complement of the donor species prior to transfer, or did the male wasp that hybridized with *N. vitripennis* carry a fully functional PSR chromosome? The first scenario will be referred to as the Hybrid Generation model. Under this model, hybridization resulted in the fragmentation of the paternal (*Trichomalopsis*) genome; a haploid (male) zygote containing the maternal (*N. vitripennis*) genome plus the fragment developed, and the fragment eventually evolved into the extant PSR chromosome. Generation of centromeric fragments during failed development is not unprecedented in *Nasonia* (Ryan et al. 1985). The evolution of PSR from a chromosomal fragment requires rapid evolution of the ability to destroy the paternal genome, because if the haploid supernumerary was transmitted to females it would suffer substantial loss during meiosis (Ryan et al. 1985; Nur et al. 1988; Beukeboom and Werren 1993b). Hybrid Transfer of PSR is the alternative model for originating PSR in *N. vitripennis*. According to this model, PSR is not unique to *N. vitripennis*, but the B chromosome is also present in at least one species of *Trichomalopsis*. A male wasp of this *Trichomalopsis* species carried PSR and hybridized with a *N. vitripennis* female, thus transferring the chromo-

some. The action of PSR (destruction of the paternal genome) would have eliminated the effects of hybrid inviability.

Verification of either of the above models will be difficult, but the microstructural comparison between PSR and the genomes of the *Nasonia* and *Trichomalopsis* species provides an initial assessment of the Hybrid Generation hypothesis. Examination of a linear region of PSR in comparison with genomic DNA from these species indicated that a similar region was not present in the A chromosomes. Under the Hybrid Generation model, PSR was recently derived from a genomic region in a donor species. Similarity should be relatively high between PSR and the progenitor region. To account for the lack of similarity between PSR and the A chromosomes of these wasps, either the donor species has yet to be identified or rapid structural evolution has occurred on PSR and/or the region where PSR was derived. Both of these requirements are entirely plausible.

The microstructural analysis indicated that the linear region of PSR contained an abundance of repetitive sequences. All regions of the chromosome thus far examined contain dispersed or tandemly repeated sequences, except for the small single-copy region identified in this study. Furthermore, PSR has several sequences that are entirely unique. No cross-hybridization has been detected between the single-copy region of PSR and the chromosomes from the *Trichomalopsis* and *Nasonia* species. Also, three families of tandemly repeated sequences on PSR have not been identified in standard genomes of the *Nasonia* or *Trichomalopsis* species (Eickbush et al. 1992; McAllister, unpublished). Based on the molecular organization of PSR, the chromosome may be in the later stages of molecular degeneration, characterized by the accumulation of repetitive elements, deletions, and duplications (Amos and Dover 1981; Green 1990; Steinemann and Steinemann 1992). Evidence for a degenerate PSR chromosome could favor the Hybrid Transfer model, suggesting PSR is not recently derived from a standard chromosomal fragment, but an old B chromosome that recently transferred species. This model is also logistically more simple, because PSR would have presumably possessed the ability to maintain itself as a B chromosome. The Hybrid Generation model requires drive to evolve.

Under either model, PSR had to originate at some point. The difference is whether the de novo origin of PSR occurred at the same time as transfer into *N. vitripennis*, or whether de novo origin predates this event. Whichever hypothesis is correct, further analysis of this problem will provide insight into the process of molecular degeneration. Although the phenomenon of molecular degeneration is commonly exhibited by B chromosomes and sex-limited chromosomes, the population genetic forces underlying the process remain unknown (Rice 1987; Charlesworth 1991, 1996). Theory and an artificial simulation of Y-chromosome evolution indicate that the absence of recombination is a major factor (Rice 1994). Additionally, studies of the neo-Y chromosome of *Drosophila miranda* show that transposable elements have accumulated on that degenerating chromosome (Steinemann and Steinemann 1991, 1992). The study of molecular degeneration on B chromosomes will provide valuable insights into the process of molecular degeneration, because B and Y (or W) chromosomes are affected differently by selection. Whereas Y chromosomes are subject to purifying selection on genetic loci that are shared with the X (Fisher 1935), B chromosomes have no selection pressure to maintain unnecessary genetic loci. Therefore, further studies of PSR and other B chromosomes will provide useful examples for examining the effects of different population genetic forces on the process of molecular degeneration.

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