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Ancient Conservation of Trinucleotide Microsatellite Loci in Polistine Wasps

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Microsatellites have proven to be very useful genetic markers for studies of kinship, parentage, and gene mapping. If microsatellites are conserved among species, then those developed for one species can be used on related species, which would save the time and effort of developing new loci. We evaluated conservation of 27 trinucleotide loci that were derived from 2 species of *Polistes* wasps in cross-species applications on 27 species chosen from the major lineages of the Vespidae, which diverged as much as 144 million years ago. We further investigated cross-species polymorphism levels for 18 of the loci. There was a clear relationship between cladistic distance and both conservation of the priming sites and heterozygosity. However the loci derived from *P. bellicosus* were much more widely conserved and polymorphic than were those derived from *P. annularis*. The disparity in cross-species utility between these sets of loci means that caution should be used in generalizing from conservation rates derived from single species. We found no relationship between locus conservation or heterozygosity and GC content of flanks, repeat motif, repeat length, or heterozygosity in the original species, which suggests that generalizations from other studies reporting such patterns are premature. © 1998 Academic Press

Key Words: **microsatellites; trinucleotide repeats; population genetics; Vespidae; *Polistes;* phylogeny; evolution; DNA polymorphism.**

INTRODUCTION

DNA microsatellites have become the genetic marker of choice for studies of gene mapping, kinship, and parentage (e.g., Queller *et al.*, 1993; Schlötterer and Pemberton, 1994; Tautz, 1989). They are highly polymorphic, easily scored, single-locus markers that are readily applied to small or degraded DNA samples, including museum specimens and sperm from the spermathecae of female insects (Ellegren, 1991; Evans, 1993; Peters *et al.*, 1995). However, identifying microsatellites in a new species can be time consuming. If microsatellites are available for closely related species, they may save the effort of developing new loci. For microsatellites to be useful in cross-species applications, both the flanking sequence, necessary to achieve amplification, and variation in numbers of repeats must be conserved.

Thus far there is little consensus on what characteristics of microsatellite loci best predict utility in a given cross-species application. Candidate factors include divergence time, length of repeat, heterozygosity in original species, GC richness of the flanking regions, repeat motif, and product length (Glenn *et al.*, 1996; Strassmann *et al.*, 1996a, 1997a; Weber, 1990). Data on cross-species applications tend to come from a few dinucleotide loci originating from a single species and applied to a small set of other species. For example, microsatellites from swallows and pied flycatchers were most consistently polymorphic within the family, though one locus was present and often polymorphic in most birds evaluated (Primmer *et al.*, 1996). About half the microsatellite primers from these birds amplified in species with a divergence time of about 11 million years (Primmer *et al.*, 1996). In fish, priming sites of some loci were present in species that diverged 470 million years ago while polymorphisms were most likely to occur among more closely related species (Rico *et al.*, 1996). Microsatellites are also frequently polymorphic even in species with low rates of nucleotide substitution such as turtles (FitzSimmons *et al.*, 1995) or with low levels of overall genetic variability such as cheetahs (Menotti-Raymond and O'Brien, 1995). The turtle loci have been conserved over 300 million years (FitzSimmons *et al.*, 1995). In this study, we investigate trinucleotide microsatellite conservation and polymorphism in a wasp family, Vespidae, which probably arose in the mid Jurassic (Carpenter, 1993). The social subfamilies,

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TABLE 1

Social Subfamilies of the Vespidae

Subfamily	Tribe	Genus	Subgenus
1. Polistinae			
	2. Polistini	*Polistes*	
			3. *Fuscopolistes*
			4. *Aphanilopterus*
			5. *Polistes* sensu strictu
	6. Mischocyttarini	*Mischocyttarus*	
	7. Ropalidiini		
	8. Epiponini		
9. Vespinae			
10. Stenogastrinae			

Note. Numbers correspond to important nodes in Figs. 1 and 2.

Stenogastrinae, Vespinae, and Polistinae, contain about 800 species (Carpenter, 1991, 1993, 1996). We investigated 9 microsatellites discovered in *Polistes annularis* and 18 microsatellites from *Polistes bellicosus*. The present study extends previous studies of cross-species microsatellite conservation in several ways. (1) We investigated trinucleotide repeats, not the more commonly used dinucleotide repeats. Conservation may differ by repeat length, since amino acids are coded for by trinucleotides. Also, microsatellite-based neurological diseases in humans are all trinucleotide repeats (Richards and Sutherland, 1992). Trinucleotide repeats produce clearer, more easily scored, banding patterns. (2) We present our results in an explicit phylogenetic framework which makes it more clear at what point conservation of different primer sets falls off. (3) We look at conservation of sets of loci derived from two closely related species. By comparing conservation levels of these two sets of primers across the same phylogenetic distances, we can address the issue of the importance of the source species in microsatellite conservation.

METHODS

Selection of Microsatellite Loci

We assessed priming site conservation for 18 trinucleotide microsatellite loci from *P. bellicosus* (Tables 1 and 2; Strassmann *et al.*, 1997a) and 9 trinucleotide microsatellite loci from *P. annularis* (Hughes and Queller, 1993; Peters *et al.*, 1995; Strassmann *et al.*, 1997a). We assessed heterozygosity levels in cross-species applications for the 18 loci from *P. bellicosus*. We counted nonamplifying loci as missing for statistical analyses of polymorphism because lack of amplification does not imply that the repeat itself is not conserved or polymorphic. The three loci evaluated on fewer than 10 species were omitted from analyses of conservation of priming sites.

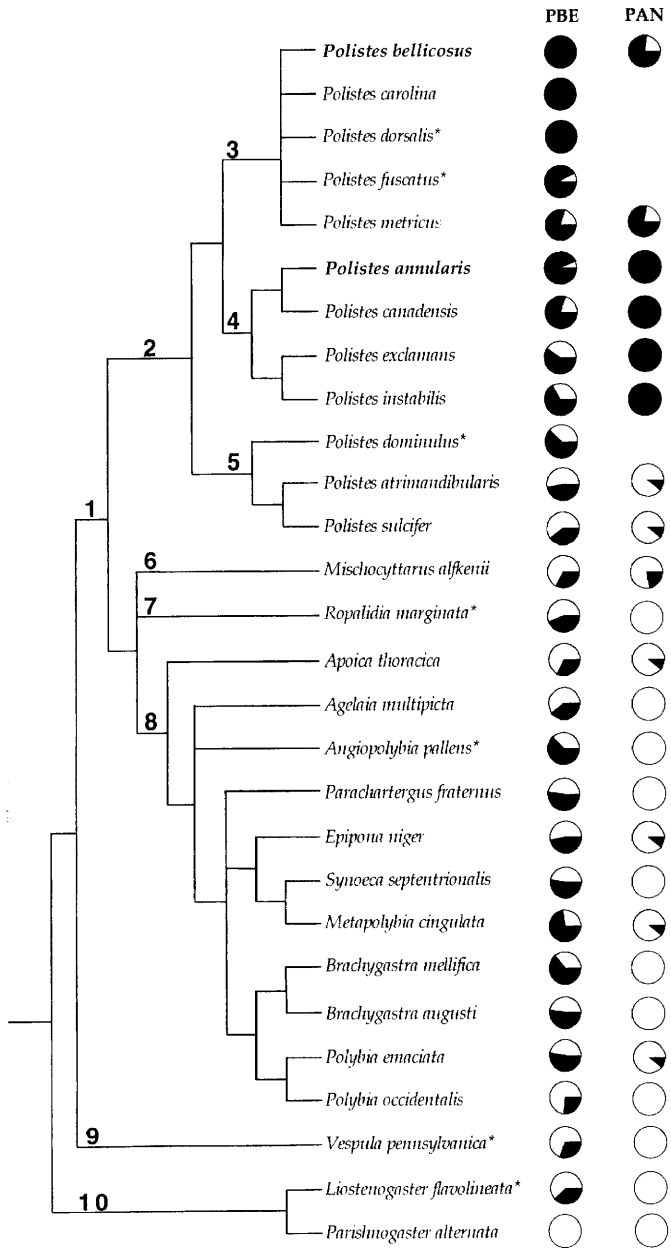

FIG. 1. Cladogram (after Carpenter, 1991) on which we indicate presence of microsatellite loci. The filled-in section of the pie diagrams indicates the proportion of loci that showed a product of appropriate size on agarose gels. Bold-faced species are those from which microsatellites were developed. (PBE:loci from *Polistes bellicosus*; Pan:loci from *P. annularis*.)

Selection of Species and Sample Sizes

To determine priming site conservation in cross-species applications, we selected 27 species of wasps from the three eusocial subfamilies of the Vespidae: Polistinae, Stenogastrinae, and Vespinae (Fig. 1, Tables 1 and 2). In the Polistinae, we chose 8 species of New World *Polistes* (species 1–8 in Table 1), 3 Old World

TABLE 2

Primers from *Polistes bellicosus*

Species	Pbe 80 AAG[a]	Pbe 102 TAG	Pbe 128 TAG[a]	Pbe 203 AAG	Pbe 205 AAG	Pbe 216 AAG[a]	Pbe 269 AAG[a]	Pbe 411 AAT[a]	Pbe 413 AAT[a]	Pbe 414 AAT	Pbe 424 AAT[a]	Pbe 430 AAG[a]	Pbe 433 TAG	Pbe 440 AAT[a]	Pbe 442 AAT	Pbe 475 AAT[a]	Pbe 484 AAT[a]	Pbe 492 AAT[a]
1. *Polistes carolina*	1	1	1	1	x	1	1	1	x	x	1	1	x	1	x	1	x	1
2. *Polistes dorsalis*[a]	1	1	1	1	1	1	1	1	1	x	1	1	x	1	1	1	1	1
3. *Polistes fuscatus*[a]	0	1	1	1	1	1	1	1	1	x	1	1	x	1	1	1	1	1
4. *Polistes metricus*	0	x	1	1	x	1	1	1	0	1	1	1	1	0	x	1	1	1
5. *Polistes annularis*	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6. *Polistes canadensis*	0	x	1	1	x	1	1	0	0	1	1	1	1	0	x	1	1	1
7. *Polistes exclamans*	0	x	0	1	x	1	1	1	0	1	0	1	0	0	x	1	1	1
8. *Polistes instabilis*	0	x	1	1	x	1	1	1	0	1	1	1	0	0	x	1	1	0
9. *Polistes dominulus*	1	1	1	1	0	1	1[c]	1	0	0	0	1[e]	1	1	0	0	0	1[b]
10. *Polistes atrimandibularis*	1	x	0	1	x	1	1	1	0	0	0	0	1	0	x	0	0	1
11. *Polistes sulcifer*	1	x	0	1	x	1	1	0	0	0	0	1	0	0	x	0	0	1
12. *Mischocyttarus alfkenii*	0	x	0	1	x	1	0	0	0	1	1	1	0	0	x	0	0	0
13. *Ropalidia marginata*[a]	0	0	1	1	0	1	0	0	0	0	0	1[d]	0	0	1[d]	0	1[c]	1
14. *Apoica thoracica*	1	x	0	1	x	1	0	0	0	0	1	0	0	0	x	1	0	0
15. *Agelaia multipicta*	0	x	1	1	x	1	0	1	0	0	1	0	0	0	x	0	1	0
16. *Angiopolybia pallens*[a]	0	0	1	1	1	1	1	1	0	0	1	1	0	1	1[d]	1	0	0
17. *Parachartergus fraternus*	1	x	1	1	x	1	1	1	0	0	1	0	0	0	x	1	0	0
18. *Epipona niger*	0	x	1	1	x	1	1	0	0	1	0	0	0	0	x	1	0	1
19. *Synoeca septentrionalis*	1	x	1	1	x	1	0	1	0	0	1	0	0	0	x	1	1	0
20. *Metapolybia cingulata*	1	x	1	1	x	1	1	1	0	0	1	0	0	1	x	1	1	1
21. *Brachygastra mellifica*	1	1	1	1	1	1	1	1	0	x	1	0	0	0	x	1	1	0
22. *Brachygastra augusti*	1	x	0	1	x	1	1	1	0	1	1	0	0	0	x	1	0	0
23. *Polybia emaciata*	0	x	0	1	x	1	1	1	0	0	1	0	0	0	x	1	1	1
24. *Polybia occidentalis*	1	x	0	1	x	1	0	1	0	0	0	0	0	0	x	0	0	0
25. *Vespula pennsylvanica*[a]	0	0	1	1	0	1[d]	0	0	0	0	0	1[d]	0	0	0	0	0	1
26. *Liostenogaster flavolineata*[a]	1[f]	0	0	0	0	1[b]	1	1[c]	0	0	0	1[d]	0	0	0	0	1[c]	0
27. *Parishnogaster alternata*	0	x	0	0	x	0	0	0	0	0	0	0	0	0	x	0	0	0

Species	Pan 12 AAT	Pan 27 AAT	Pan 45 AAT	Pan 48 AAT	Pan 69 AAT	Pan 104 TAG	Pan 109 AAT	Pan 111B AAT	Pan 117 AAT
a. *Polistes bellicosus*	0	1	1	0	1	1	1	1	1
4. *Polistes metricus*	1	0	1	0	1	1	1	1	1
6. *Polistes canadensis*	1	1	1	1	1	1	1	1	1
7. *Polistes exclamans*	1	1	1	1	1	1	1	1	1
8. *Polistes instabilis*	1	1	1	1	1	1	1	1	1
9. *Polistes dominulus*	x	x	x	x	x	x	x	1	x
10. *Polistes atrimandibularis*	0	0	0	0	0	0	0	1	0
11. *Polistes sulcifer*	0	0	0	0	0	0	0	1	0
12. *Mischocyttarus alfkenii*	0	0	1	0	1	0	0	0	0
13. *Ropalidia marginata*	0	0	0	0	0	0	0	0	0
14. *Apoica thoracica*	0	0	0	0	1	0	0	0	0
15. *Agelaia multipicta*	0	0	0	0	0	0	0	0	0
16. *Angiopolybia pallens*	0	0	0	0	0	0	0	0	0
17a. *Parachartergus colobopterus*	x	0	x	x	0	0	x	0	x
17b. *Parachartergus fraternus*	0	0	0	0	0	0	0	0	0
18. *Epipona niger*	0	0	0	0	0	0	0	0	1
19. *Synoeca septentrionalis*	0	0	0	0	0	0	0	0	0
20. *Metapolybia cingulata*	0	1	0	0	0	0	0	0	0
21. *Brachygastra mellifica*	0	0	x	0	0	0	0	0	0
22. *Brachygastra augusti*	0	0	0	0	0	0	0	0	0
23. *Polybia emaciata*	0	0	0	0	0	0	0	0	1
24. *Polybia occidentalis*	0	0	0	0	0	0	0	0	0
25. *Vespula pennsylvanica*	0	0	0	0	0	0	0	0	0
26. *Liostenogaster flavolineata*	0	0	0	0	0	0	0	0	0
27. *Parishnogaster alternata*	0	0	0	0	0	0	0	0	0

Note. Success of amplification by microsatellite primers on new species where 1 indicates appropriately sized product, 0 indicates failure to yield appropriately sized product, and x indicates not tried.

[a] Combinations of starred species and loci were tried at annealing temperatures ranging from 37.5 to 62.5 (see text).

[b] Product obtained at an annealing temperature 2–2.5°C lower than optimized for *P. bellicosus*.

[c] Product obtained at an annealing temperature 5–8°C lower than optimized for *P. bellicosus*.

[d] Product obtained at an annealing temperature 10–15°C lower than optimized for *P. bellicosus*.

[e] Product obtained at an annealing temperature 2–2.5°C higher than optimized for *P. bellicosus*.

[f] Product obtained at an annealing temperature 10°C higher than optimized for *P. bellicosus*.

Polistes (species 9–11), 1 species each from the tribes Mischocyttarini (species 12) and Ropalidiini (species 13), and 11 species from Epiponini (species 14–24). We sampled 1 species from the Vespinae (species 25) and 2 species from the most distantly related Stenogastrinae (species 26 and 27). We used at least three individuals per species.

We used the annealing temperature found to be optimum in the species from which we cloned the locus. For a subset of species-by-locus combinations, those starred in Table 2 and on Fig. 1, we also investigated whether or not varying annealing temperatures in the PCR increased the number of amplifying loci (Table 2). We changed annealing temperatures in 2.5 to 5°C increments from 37.5 to 62.5°C.

To investigate heterozygosity in cross-species applications we used 11 species concentrated in *Polistes* but also including representatives of the other tribes (Fig. 1, Table 1). Samples from very small or inbred populations are likely to give microsatellite results that are not representative for the species as a whole. Therefore we tried to use common species or species whose population structure we had previously investigated and found to be outbred (Carpenter *et al.,* 1993; Choudhary *et al.,* 1994; Hughes *et al.,* 1993a, 1993b; Queller *et al.,* 1988; Strassmann *et al.,* 1989, 1991, 1994). We sampled populations of three species that were close to the edge of their range. *Polistes instabilis* and *Brachygastra mellifica* came from the northern edge of their range and *Polistes annularis* came from an isolated population at the western edge of its range (Richards, 1978; Strassmann, 1979). We used approximately 10 individuals from at least 5 different colonies; sample sizes for *P. bellicosus, P. instabilis* and *P. dominulus* were larger and those for *Brachygastra mellifica* smaller.

DNA Extraction, PCR, and Visualization

Methods followed protocols from Strassmann *et al.* (1996b). Each PCR reaction mix consisted of 2 μL diluted genomic DNA (about a nanogram), 2 μL of primer mix (2.5 μM), 0.1 μL 10 mM dNTP mix, 1 μL 10× buffer (500 mM KCl, 100 mM Tris–HCl, pH 9.0, 1% Triton X-100), 4.08 μL dH_2O, 0.62 μL 25 mM $MgCl_2$, and 0.05 μL *Taq* polymerase (5 units/μL) under an oil overlay. Radioactive PCR was similar to nonradioactive PCR except for the addition of 1–2 microcuries of ^{35}S-dATP (Amersham) to internally label the PCR product. We ran 35 to 40 cycles of 92°C denaturing for 60 s, 60 s annealing (at a temperature optimized for the primers used, see Hughes and Queller, 1993; Strassmann *et al.,* 1997) and 45 s extension at 72°C, followed by 5 to 10 extra minutes at 72°C to allow for the complete extension of all PCR fragments.

We evaluated priming site conservation primarily on agarose gels. Nonradioactive PCR products were electrophoresed through a 2% agarose gel and the DNA was visualized under UV light with ethidium bromide. Allele lengths were measured by comparing them with PCR Marker (Promega) and positive controls (DNA from the original species). We evaluated polymorphism levels by incorporating ^{35}S into the PCR and then running the PCR product on a denaturing polyacrylamide gel and exposing the dried gel to X-ray film. We determined allele sizes by comparison to an M13 sequencing reaction (Strassmann *et al.,* 1996b).

To verify the restricted applicability of primers from *P. annularis,* we repeated all runs using fresh oligonucleotides, enzymes, buffers, chemicals, and DNA extracted from a fresh set of exemplars. Results of the new run were similar to those of the initial run, and so are not reported separately.

Interpretation of PCR Products

Products that on agarose gels were fuzzy, very faint, had multiple products, or were of a size more than 200 bp away from that in the original species were considered failed amplifications, indicating changes in either the priming site or large insertions or deletions in new species. Products that give a single, clear band within 200 bp of the original length are considered to be positive amplifications. A positive amplification is an indication that the priming sites have been conserved. When we state that a locus is present, it means that the priming sites have been conserved and no large insertions or deletions have occurred between them. Loci were considered present with radioactive PCR if a single band, or set of polymorphic bands at 3-bp intervals, appeared within 200 bp of the product size from the original species. Positive controls from the original species, either *P. bellicosus* or *P. annularis,* confirmed the absence of technical problems for nonamplifying loci.

Cross-Species Homology of Loci

If a PCR product appears to be a variable microsatellite locus in a new species, it is most likely to be the same locus. Priming sites may be conserved even if the locus does not have a variable number of repeats. The best way to verify the match of a microsatellite locus in a new species is to sequence the PCR product obtained from the cross-species application. Previous studies have used this technique to investigate identity of microsatellite loci (Primmer *et al.,* 1996; Rico *et al.,* 1996). In all 20 cases the microsatellite locus in the new species matched that in the original species. We confirmed the general finding that conserved priming sites indicate that the same locus is being amplified for seven of our loci in a total of 45 cross-species applications (Table 3). For all but 2 of the 45 sequences, we sequenced PCR products directly using cycle sequencing with the thermo sequenase radiolabeled (^{33}P) cycle sequencing kit (Amersham). For the other 2 we used a TA cloning kit (Invitrogen) and Sequenase kit (United

TABLE 3

Base Pair Differences between Cross-Species Sequences and *P. bellicosus* Sequences of the Same Loci

Locus	Average % of base pair (bp) changes ± SE	Average # bp ± SE sequenced	# of species sequenced
Pbe203AAG	15.2 ± 4.3	91 ± 4	7
Pbe216AAG	13.9 ± 2.1	129 ± 4	16
Pbe269AAG	10.6 ± 9.2	97 ± 12	5
Pbe411AAT	7.3 ± 0.8	120 ± 10	9
Pbe430AAT	21.6 ± 3.6	152 ± 7	3
Pbe440AAT	5.2 ± 0.02	134 ± 20	2
Pbe484AAT	14.0 ± 4.2	174 ± 23	3

States Biochemical). We calculated the percentage of bp differences between the original sequence from *P. bellicosus* and cross-species applications of the primers. We did not count the few insertions or deletions between the aligned sequences that we found. All 45 sequences indicated that the microsatellite primer pairs used in cross-species applications were amplifying the same locus (Table 3). We generally assessed primer pairs on agarose gels because it is an efficient way of screening many loci and optimizing annealing temperatures. We investigated the correspondence between loci scored as present on agarose gels and loci that gave products that looked like microsatellites on polyacrylamide gels and found that 110 of 116 (95%) loci scored as positives on agarose were also positive on acrylamide. Cases where positives on agarose were negative on polyacrylamide gels were usually the result of a clear band on agarose looking like a smear or multiple bands on polyacrylamide. PCR products run on denaturing polyacrylamide gels are likely to be the same locus particularly if they give a single product with the stutter band appearance of microsatellites and have multiple alleles separated in length by the number of bases in the repeat motif, which in our case is three.

Nonamplifying Alleles

Nonamplifying alleles (also called null alleles) at microsatellite loci are caused by mutations in priming sites (Pemberton *et al.* 1995). Since the other alleles at that locus amplify, and most individuals are heterozygotes at good microsatellite loci, nonamplifying alleles do not usually result in blank lanes on gels. Nonamplifying alleles are detected in ours and most other studies only by detailed pedigree analyses. In most cases they can be visualized (i.e., turned into amplifying alleles) by designing new primers or occasionally by lowering the annealing temperature since nonamplifying alleles are usually the result of mutations in one of the priming sites. Here we report the frequency and identity of nonamplifying alleles in *P. bellicosus* and in cross-species applications of *P. bellicosus* loci on *P. dorsalis, P. fuscatus* and *P. carolina.*

RESULTS

Presence of Microsatellite Loci

Microsatellite locus priming sites derived from *P. bellicosus* frequently amplified in the Polistinae, while those loci derived from *P. annularis* were restricted to New World *Polistes* (Fig. 1, Table 2). The loci from *P. bellicosus* were no more frequent in the two European *Polistes* species than they were for the other polistine tribes, Epiponini, Ropalidiini, and Mischocyttarini. Some loci amplified in the other subfamilies in the exemplars we tested; there was substantially less amplification only in one of the most phylogenetically distant genera, *Parischnogaster* (Fig. 1, Table 2).

Annealing Temperature Changes

Only a minority of primers that did not anneal at the annealing temperature used in the original species annealed at a different (and usually lower) temperature (Table 2). Seventeen of 88 (19%) species by locus combinations amplified at temperatures 2 to 15°C different from the original annealing temperature. Half of these were within 5°C of the original amplification temperature.

Is Cross-Species PCR Amplifying the Same Locus?

We sequenced cross-species PCR products for 45 species using primers from 7 of the microsatellite loci from *P. bellicosus* (Table 3). In all we sequenced 5514 bp. Sequence of the regions flanking the microsatellite showed a clear correspondence for all 45 cross-species applications. Depending on the locus, between 79 and 93% of basepairs in the cross-species sequence corresponded to the original sequence from *P. bellicosus.* This indicates that the PCR primers amplified the same locus as opposed to an unrelated secondary product or PCR artifact (Table 3; aligned sequences available from the senior author). The species we chose to sequence generally came from more distant nodes relative to *P. bellicosus* (Fig. 1). We sequenced only 8 species from *Polistes,* while we sequenced 22 from the Epiponini, 8 from the Mischocyttarini, and 7 from the Ropalidiini (Table 1). The clear match of flanking regions supports our premise that a product of approximately the same size in a new species is likely to be the same locus. Three of the sequences that proved to be the same locus as in *P. bellicosus* actually fell outside our accepted size range (two very short, one apparently too long) and so were scored as not matching (0 score in Table 2). Therefore the results in Table 2 may be viewed as conservative. There was also a very close correspondence between primer pairs giving a product on agarose gels and those doing so on denaturing polyacrylamide gels. The many loci which proved to be polymorphic in new taxa is a further indication that the microsatellite

TABLE 4

Heterozygosity and Number of Alleles of Microsatellite Loci from *P. bellicosus* in New Species

Locus	*Polistes bellicosus*	*Polistes dorsalis*	*Polistes fuscatus*	*Polistes carolina*	*Polistes annularis*	*Polistes instabilis*	*Polistes dominulus*	*Brachy-gastra mellifica*	*Angi-polybia pallens*	*Ropalidia marginata*	*Vespula penn-sylvanica*	*Liosteno-gaster flavolineata*
Pbe80AAG	0.65 (6)[a]	—	—	0.76 (11)	0.00 (1)	X	—	—	—	—	—	—
Pbe102TAG	0.71 (6)	0.69 (6)	0.76 (6)	0.76 (8)	0.33 (2)	X	.73 (6)	0.25 (4)	—	—	—	—
Pbe128TAG	0.54 (6)	0.60 (4)	0.65 (5)	0.68 (5)	0.00 (1)	0.18 (2)	.72 (5)	0.00 (1)	0.42 (3)	0.17 (2)	0.54 (4)	—
Pbe203AAG	0.65 (13)	0.76 (8)	0.80 (7)	0.81 (11)	0.00 (1)	0.00 (1)	.56 (2)	0.00 (1)	0.00 (1)	0.00 (1)	0.00 (1)	—
Pbe205AAG	1.00 (6)	0.77 (6)	0.58 (4)	X	0.19 (2)	X	—	0.00 (1)	0.53 (4)	—	—	—
Pbe216AAG	0.04 (2)	0.67 (5)	0.52 (3)	0.22 (3)	0.00 (1)	0.14 (2)	0.00 (1)	0.00 (1)	0.17 (2)	0.00 (1)	—	X
Pbe269AAG	0.45 (8)	0.86 (10)	0.78 (6)	0.74 (7)[a]	0.76 (3)	0.65 (8)	.65 (5)	0.00 (1)	0.00 (1)	—	—	—
Pbe411AAT	0.21 (7)	0.81 (8)	0.82 (8)	0.76 (6)	0.92 (6)	0.76 (7)	.29 (3)	0.00 (1)	0.00 (1)	—	—	0.00 (1)
Pbe413AAT	0.40 (5)	0.86 (9)	0.78 (7)	X	— (0)	X	—	—	—	—	—	—
Pbe414AAT	0.21 (6)	X	X	X	—	0.71 (6)	—	X	X	X	X	X
Pbe424AAT	0.74 (7)	0.85 (9)	0.88 (10)	0.84 (16)	0.78 (7)	0.88 (10)	—	0.80 (6)	0.71 (5)	—	—	—
Pbe430AAG	0.29 (4)	0.76 (6)	0.38 (3)	0.42 (3)	0.00 (1)	0.34 (3)	.86 (9)	—	0.00 (1)	—	0.00 (1)	—
Pbe433TAG	0.04 (2)	X	X	X	0.00 (1)	X	0.00 (1)	—	X	X	X	X
Pbe440AAT	0.55 (10)	0.72 (4)[a]	0.88 (10)	0.79 (9)	—	X	.74 (6)	—	0.58 (3)	—	—	—
Pbe442AAT	0.70 (9)	0.83 (10)	0.77 (8)	X	0.00 (1)	X	—	X	0.00 (1)	0.53 (3)	—	—
Pbe475AAT	0.50 (5)	0.83 (8)	0.84 (10)[a]	0.42 (2)	0.00 (1)	0.51 (3)	—	(2)[b]	0.00 (1)	—	—	—
Pbe484AAT	0.05 (2)	0.89 (11)	0.67 (4)	X	0.53 (3)	—	—	0.00 (1)	—	0.35 (2)	—	0.00 (1)
Pbe492AAT	0.43 (9)[a]	0.81 (7)	0.41 (4)[a]	0.80 (9)	0.91 (9)	0.49 (2)	.82	—	—	0.79 (6)	0.72 (6)	—
Average	0.453	0.781	0.701	0.667	0.295	0.466	.537	0.117	0.219	0.307	0.315	0
SD	0.272	0.082	0.162	0.199	0.376	0.293	.324	0.269	0.283	0.314	0.371	0

Note. Data are presented as expected heterozygosity (number of alleles) except for *P. bellicosus, P. annularis,* and *B. mellifica,* for which observed heterozygosities are reported. X, not attempted. —, no PCR product in correct size range. *P. bellicosus* data are from Strassmann *et al.* 1997.

[a] Detailed study indicated null allele(s) present.

[b] Two loci of same size, one variable.

primers are amplifying the same microsatellite-containing locus (Table 4).

Factors Influencing Microsatellite Conservation

Substantial variation exists among the loci in level of conservation of amplification. The *P. annularis* TAG locus and the eight *P. annularis* AAT loci amplified in only 21 and 24% of species, respectively. The two *P. bellicosus* TAG loci amplified in 39% of species. The nine *P. bellicosus* AAT loci amplified in 47% of species and the five AAG loci were most conserved, appearing in 72% of species assayed. The *P. annularis* AAT loci are significantly less conserved than the *P. bellicosus* AAT or AAG loci ($P = 0.011$ and 0.0001, two sample t test). Therefore, species of origin is an important predictor of conservation of amplification in our study. In regressions run separately for *P. bellicosus* and *P. annularis* loci, we failed to find an effect on conservation of priming sites for primer length, annealing temperature, GC content of the primer region, or GC content of flanking regions. The total length of amplified flanking sequence, calculated by subtracting the length of the repeat in the original clone from the expected product size including the primers, was negatively correlated with amplification for the *P. bellicosus* loci ($P < 0.005$). Combining all *P. bellicosus* loci for this last analysis appears free of bias as the average length of the AAT and non-AAT loci is similar (146 vs 145 bp).

Polymorphism in Cross-Species Microsatellite Amplifications

The proportion of clearly scoreable loci that were polymorphic decreased with increasing phylogenetic distance, ranging from 100% for the most closely related species to 0% for the most distant. Within the same species group, all microsatellite loci were variable in all species tested (Tables 4 and 5). The heterozygosity of microsatellite loci derived from *P. bellicosus* was very high in other species (Fig. 2, Table 4). In the three species most closely related to *P. bellicosus,* heterozygosity of all loci that were present was between 0.67 and 0.78, a range well above that of 0.45 average heterozygosity in *P. bellicosus* itself. Average heterozygosity was equal to or above that found in *P. bellicosus* for all species of *Polistes* that we investigated except *P. annularis.* When only polymorphic loci were included, heterozygosities of all species were at or above that found in *P. bellicosus* itself (Fig. 2). This was even true for the polymorphic loci in *Vespula pennsylvanica,* which is in a different tribe.

To determine whether levels of polymorphism in *P. bellicosus* could be used as predictors of variability in other species we ran linear regressions comparing the observed heterozygosity for the original and target species. Taken singly, significant positive relationships were found for *P. carolina, P. dominulus,* and *Ropalidia*

TABLE 5

Allele Size Ranges of Microsatellite Loci from *P. bellicosus* in New Species

Locus	No repeat	*Polistes bellicosus*	*Polistes dorsalis*	*Polistes fuscatus*	*Polistes carolina*	*Polistes annularis*	*Polistes instabilis*	*Polistes dominulus*	*Brachygastra mellifica*	*Angiopolybia pallens*	*Ropalidia marginata*	*Vespula pennsylvanica*	*Liostenogaster flavolineata*
Pbe80AAG	195	169–211	—	—	179–206	187	X	—	—	—	—	—	—
Pbe102TAG	75	109–112	115–130	109–145	109–139	112–115	X	111–132	109–124	—	—	—	—
Pbe128TAG	109	165–180	165–174	162–174	162–174	150	150–153	150–171	148	171–177	135–141	147–159	—
Pbe203AAG	111	132–231	108–165	144–165	141–180	123	139	150–156	118	114	119	108	—
Pbe205AAG	180	231–247	228–243	228–240	X	226–229	X	—	231	201–231	—	—	—
Pbe216AAG	150	174–180	165–177	171–177	172–199	164	164–170	176	147	144–147	161	—	X
Pbe269AAG	120	142–181	159–186	144–168	147–165	160–166	145–169	152–164	126	124	—	—	—
Pbe411AAT	134	161–182	159–180	162–189	159–177	173–194	164–194	167–173	157	157	—	—	154
Pbe413AAT	208	227–245	233–263	224–248	X	—	X	—	—	—	—	—	—
Pbe414AAT	124	172–196	X	X	X	—	124–154	—	X	X	X	X	X
Pbe424AAT	152	194–224	213–258	189–243	172–232	188–227	195–234	—	194–209	166–193	—	—	—
Pbe430AAG	155	191–203	190–205	199–205	194–200	182	177–186	208–253	—	178	—	178	—
Pbe433TAG	200	221–224	X	X	X	249	X	248	—	X	X	X	X
Pbe440AAT	191	213–258	222–294	216–258	220–244	—	X	178–196	—	251–257	—	—	—
Pbe442AAT	138	175–205	166–202	175–202	X	164	X	—	X	123	152–176	—	—
Pbe475AAT	116	138–153	129–153	144–198	150–162	152	155–161	—	144–162	138	—	—	—
Pbe484AAT	161	191–194	178–235	187–196	X	183–189	—	—	240	—	241–244	—	287
Pbe492AAT	107	141–194	150–168	156–174	146–176	134–173	126–129	124–142	—	—	123–159	111–138	—

Note. No Repeat, expected product size of an allele with zero repeats in *P. bellicosus*. X, not attempted. —, no product.

marginata (Linear regressions, $P = 0.013$, 0.01, and 0.035, respectively). However the lack of a significant relationship for the other eight species, coupled with the fact that the Bonferroni corrected probability for 95% confidence with 11 tests is $P < 0.005$, indicates that polymorphism in the source species is probably not a very useful predictor of polymorphism in cross-species applications.

We evaluated how much can be inferred regarding the presence of useful polymorphic loci from one cross-species application to one involving another related species. Of five *P. bellicosus* loci that were polymorphic in *P. annularis,* three were present and polymorphic in the closely related *P. instabilis* but another five loci that were not polymorphic in *P. annularis* were also polymorphic in *P. instabilis* (Table 4). Of the three loci that were polymorphic in *B. mellifica,* one was polymorphic in *A. pallens* but another four loci that were not polymorphic in *B. mellifera* were also polymorphic in *A. pallens*. Thus, polymorphism cannot be inferred from one cross-species application to a related cross-species application with much confidence.

Within the genus *Polistes,* alleles for the *P. bellicosus* loci were typically approximately the same size in all species, which may indicate that they all had similar numbers of repeats. Fifteen of 19 monomorphic products outside of *Polistes* were shorter than the shortest allele seen in *P. bellicosus*. Most variable loci in these genera overlapped the allele size range seen in *P. bellicosus*. These results indicate a reduction of the repeat region in monomorphic loci.

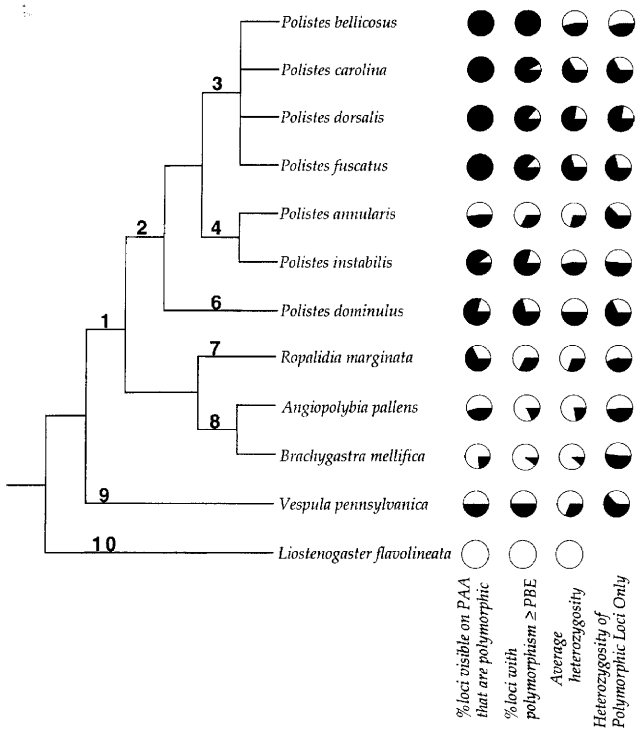

FIG. 2. Cladogram of species evaluated for polymorphism in microsatellite loci. We present four measures of polymorphism as proportions of filled-in sections of pie diagrams: percentage of loci resolved on polyacrylamide gels that are polymorphic; percentage of polymorphic loci with heterozygosities greater than or equal to that found in the species from which the loci were cloned, *P. bellicosus;* average heterozygosity of all loci; and average heterozygosity of polymorphic loci. Numbers on nodes refer to taxa specified in Table 1.

Several loci show nonamplifying alleles for specific species (Table 5), which we detected as missing alleles in pedigree analyses. We have used some of the polymorphic loci derived from *P. bellicosus* for detailed studies in *P. bellicosus* (7 loci, Arévalo *et al.,* 1998; Field *et al.,* 1998), *P. dorsalis* (7 loci, Arévalo *et al.,* 1998), *P. fuscatus* (9 loci, Peters *et al.,* in prep.), and *P. carolina* (7 loci, Seppä *et al.,* in prep.). We found 4 of the 30 loci (13.3%) applied to a new species had nonamplifying alleles and 2 of 7 loci (29%) in *P. bellicosus* itself had nonamplifying alleles (Table 4). Pbe492AAT had nonamplifying alleles in both *P. bellicosus* and in *P. fuscatus;* there was no other overlap among species for loci with nonamplifying alleles. No species had more than 2 loci with nonamplifying alleles, and the nonamplifying alleles themselves tended to be uncommon. One nonamplifying allele became visible when the annealing temperature of the PCR was dropped (Arévalo *et al.,* 1998). Nonamplifying alleles are no more common in cross-species applications than they are in the species from which primers were designed.

DISCUSSION

The 45 loci that we sequenced were clearly the same loci as those sequenced from the original species (Table 2). The 20 loci that were sequenced across species in birds and fish showed the same pattern (Primmer *et al.,* 1996; Rico *et al.,* 1996). In all cases the sequence similarity indicated that the same locus was involved. This supports the view that when primer pairs are used on new species, a single PCR product of approximately the expected size represents the same locus.

We did not find that nonamplifying alleles were more frequent in cross-species applications. There were no more nonamplifying alleles in *P. bellicosus* loci used in *P. dorsalis, P. fuscatus,* and *P. carolina* than there were in *P. bellicosus* itself.

Phylogenetic distance had a clear effect on priming site conservation. Priming sites of loci cloned from both *P. annularis* and *P. bellicosus* were broadly conserved within both lineages of New World *Polistes* that we investigated. Divergence times between these two groups are unknown but are likely to be between 10 and 80 million years (James Carpenter, pers. com.). None of the loci from *P. annularis* were conserved in Old World *Polistes* or any more distantly related taxa, which indicates that their priming sites have diverged from those in *P. annularis* in the last 84 to 106 million years when Africa separated from South America (James Carpenter, pers. com.). Priming sites from *P. bellicosus* loci were frequently conserved throughout the subfamily Polistinae, particularly when annealing temperatures were adjusted as we did in *P. dominulus* and *A. pallens*. Divergence times for the tribes of the Polistinae are hard to fix, but must be greater than that within *Polistes* and less than that for the subfamilies of the Vespidae, and so are probably between 80 and 175 million years. Some of the *P. bellicosus* loci were also conserved in the other subfamilies of the Vespidae. The Vespinae/Polistinae split probably occurred in the mid Jurassic (about 175 million years ago), though the only fossil evidence is from the Cretaceous (James Carpenter, pers. com.; Carpenter and Rasnitsyn, 1990; Brothers, 1992). These fossils indicate a cosmopolitan distribution of the Vespinae by 90 million years ago at the latest and an origin of the split much earlier (James Carpenter, pers. com.). These results put *P. bellicosus* microsatellites among the most anciently conserved, particularly when their short annual generation time is taken into account. Longer absolute times are reported for fishes (Rico *et al.,* 1996), and turtles (FitzSimmons *et al.,* 1995), but they probably represent fewer generations.

The causes of the differences in priming site conservation between *P. annularis* and *P. bellicosus* are not clear. There are no consistent differences in characteristics of the loci themselves. The *P. annularis* primers are slightly lower in GC content (39% vs 45%), but did not differ appreciably in length (22 bp vs 23 bp), or optimal annealing temperature (52.2°C vs 52.7°C). There was no significant relationship between any of these parameters and amplification within species. Reexamination of the design of *P. annularis* primer pairs failed to reveal any other differences in features like degree of annealing between the primers that might decrease their conservation. The controls and replicate runs of the *P. annularis* loci rule out any purely technical reasons for nonamplification.

The lack of amplification using *P. annularis* primers on other members of the Vespidae is an indication that the flanking regions around these microsatellite repeats have changed more rapidly in this species. Our sample of *P. annularis* came from an isolated cliff population at the western edge of its range while our sample of *P. bellicosus* came from coastal prairie not near the edge of its range. Perhaps the isolated population of *P. annularis* is undergoing more rapid molecular evolution because of its small size, similar to reports for Hawaiian *Drosophila* (DeSalle and Templeton, 1988) and consistent with Ohta's nearly neutral model for molecular evolution (Ohta, 1976). In this case, the priming sites of *P. annularis* microsatellites may have changed more rapidly than those from *P. bellicosus*.

We found no predictive role in priming site conservation in either species for repeat motif, primer length, annealing temperature, or GC content of either the primer region or the flanking regions. Shorter amplified sequences were more conserved in *P. bellicosus*.

To be useful, both priming sites and repeat number polymorphism must be conserved. The loci from *P. bellicosus* were highly polymorphic in new species (Fig. 2, Table 3). In fact, average heterozygosities were much higher in the three most closely related species

(*P. dorsalis, P. fuscatus,* and *P. carolina*) than they were in *P. bellicosus* itself. When only polymorphic loci were included, *P. bellicosus* itself had the lowest level of heterozygosity of any species tested (Fig. 2, Table 3). It is interesting that this population of *P. bellicosus* also had the fewest polymorphic allozyme loci when compared to other *Polistes* species from the same habitat (Hughes *et al.,* 1993a). This species is generally restricted to coastal prairies, whereas *P. metricus, P. carolina,* and *P. exclamans* nest in a wider variety of habitats. This might explain why we did not see the typical pattern of decreased heterozygosity in cross-species amplifications (e.g., Primmer *et al.,* 1996).

We did not find that the level of polymorphism in cross-species applications predicted similar polymorphism levels in related cross-species applications. *P. annularis* and *P. instabilis* are closely related and yet exhibit different patterns of heterozygosity. *B. mellifica* and *A. pallens* are in the same tribe and also exhibited different patterns of heterozygosity (Table 3). In general, monomorphic loci in distant relatives of *Polistes* had product sizes consistent with total or near total loss of the repeat (Table 4). In sum, we found no support for any of the previously published hypotheses regarding predictors of priming site or heterozygosity conservation in either species or in the comparison between species.

The 57 microsatellite locus sequences and priming sites we have published or listed in GenBank will be useful for many studies on wasps from the subfamily Polistinae and may be useful in some members of the Vespinae (Hughes and Queller, 1993; Choudhary *et al.,* 1993; Strassmann *et al.,* 1996a, 1997a, b).

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