Within- and Between-Individual Sequence Variation Among ITS1 Copies in the Meadow Grasshopper *Chorthippus parallelus* Indicates Frequent Intrachromosomal Gene Conversion

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Sequencing multiple copies of the ITS1 region revealed the coexistence of two or more haplotypes within the genome of *Chorthippus parallelus*. Using a PCR-RFLP approach, the ITS1 numbers and frequencies of haplotypes present in each of 40 individuals were investigated, revealing a consistent lack of homogeneity. For each individual, the level of intra-individual variation was estimated from a sample of 20 ITS1 copies. The level of differentiation in haplotype frequency among individuals was then estimated by maximum likelihood using models based on the Dirichlet distribution. This confirmed the existence of significant levels of variation among individuals within each population studied. The most likely turnover mechanism that could generate this pattern of variation is gene conversion, operating at the intrachromosomal level. Furthermore, the discovery of linkage disequilibrium among the ITS1 haplotypes of *C. parallelus* suggests that intrachromosomal gene conversion occurs more frequently than interchromosomal recombination. Subspecies of *C. parallelus* showed significantly different haplotype distributions following about 0.5 Myr of divergence. With respect to the process of concerted evolution, we show that homogenization of repeats is slow relative to speciation, and the standing variation among individuals is sufficient for selection to operate.

Introduction

Ribosomal DNA (rDNA) is a multicopy gene family ubiquitous to all living organisms. The tandemly repeated units of rDNA are organized into large blocks known as nucleolar organizer regions (NORs). In eukaryotes, each rDNA unit contains the genes of the 18S, 5.8S, and 28S ribosomal RNA (rRNA), interspersed by spacer regions. The two internal transcribed spacer regions, ITS1 and ITS2, are located either side of the 5.8S gene. Between the 28S and 18S genes are two external transcribed spacers (3′ETS and 5′ETS), separated by the intergenic spacer (IGS). Although these ITS and ETS regions are initially transcribed to form part of the rRNA precursor molecule, they are progressively cleaved and removed and do not, therefore, directly contribute to the construction of a ribosome (Reeder 1990). However, the transcribed spacers contain signals for processing the rRNA transcript (Hillis and Dixon 1991).

Ribosomal DNA units do not evolve independently but, instead, appear to evolve in a concerted fashion such that copies are similar within a species and different between species. Their evolution is dependent on the interactions among natural selection, genetic drift, and molecular turnover mechanisms (Dover 1982). The relative contributions of these phenomena are poorly understood and are likely to vary among species. Because of a lack of empirical data, the process of concerted evolution has primarily been investigated using mathematical models and computer simulations (Nagylaki and Petes 1982; Ohta and Dover 1983, 1984; Walsh 1987; Stephan 1989). The results of these studies suggest that molecular turnover mechanisms such as gene conversion and unequal crossing-over could play a significant role in the concerted evolution of repeated sequences but their impact depends heavily on the rates of these processes relative to recombination and mutation (reviewed by Elder and Turner 1995).

Although it has been shown that the ITS1 region is not freely evolving (Schlötterer et al. 1994), the level of selective constraint acting on this region is expected to be minimal. Consequently, the pattern of ITS1 sequence variation at the intra-individual level will primarily depend on the spread of base substitutions or sequence rearrangements caused by molecular turnover mechanisms. Among individuals, the level of variation observed will depend on the rate at which random chromosomal assortment (meiosis) disperses these rearrangements among the individuals of the next generation and on genetic drift. It has been proposed that the interaction between molecular turnover and sexual reproduction will maintain all individuals of a population in a similar state at any one point in time (Dover 1982). This theory has important implications because an absence of significant variation among individuals will remove the potential for selection to act, meaning that molecular turnover mechanisms could potentially become the driving force behind the evolution of regions such as ITS1. By quantifying sequence variation at the individual, population, and subspecies level, this study investigated whether the populations of ITS1 copies within individuals are in a similar evolutionary state among individuals of the meadow grasshopper, *Chorthippus parallelus* (Orthoptera: Acrididae). In addition, these data provide an insight into the way in which the ITS1 region is evolving and indicate which molecular turnover mechanisms are significantly contributing to this process.

C. parallelus represents an ideal model system, as the recent evolutionary history of the species has been intensively studied and, in contrast to *Drosophila*, the genome of this species contains multiple NORs. During the last ice age(s) this species was repeatedly confined to southerly refugia (Hewitt 1993). When *C. parallelus* expanded its range northwards again at the end of the

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last ice age (~10,000 years before present (YBP)), hybrid zones formed where the different refugial populations met. The level of divergence that had accrued was sufficient for the French population (originating from the Balkan refuge) and the Spanish population (derived from a refuge in southern Spain) to be regarded as distinct subspecies, namely *Chorthippus parallelus parallelus* (*Cpp*) and *Chorthippus parallelus erythropus* (*Cpe*), respectively (Reynolds 1980). Based on mitochondrial sequence data (COI), the estimated time of divergence for these two species is 500,000 years (Lunt, Ibrahim, and Hewitt 1998), which is equivalent to 500,000 generations.

A diagnostic difference between *Cpp* and *Cpe* involves their haploid complement of NORs. Both subspecies have two autosomal NORs (on chromosomes L_2 and L_3), but *Cpp* has an additional NOR located on its X chromosome (Gosalvez et al. 1990). It is currently unclear whether the common ancestor had two or three NORs, but the discovery that Italian *C. parallelus* have two NORs (Flanagan et al. 1999) suggests that a NOR gain may have occurred recently in the ancestor of *Cpp*.

Sequencing multiple ITS1 copies from several *C. parallelus* individuals (Parkin 2002) revealed the presence of polymorphic nucleotide sites that will be referred to in this paper as "within-individual polymorphisms" (WIPs). Four of these WIPs were targeted in this study (identified as *WIP1, 2, 3*, and *4*), chosen because they are present in more than one individual with the coexisting base types occurring at intermediate frequencies. These polymorphisms represent the transition period when a new mutation has arisen in one rDNA copy and begun to spread to other copies in the genome. Depending on the rate of spread, these substitutions may now be species, subspecies, or population-specific or may be shared across species.

Materials and Methods

Samples

Adult grasshoppers were collected from four sites: Tourniac (Massif centrale, France; 2°13′E, 45°12′N), Aunat (Pyrenees-Orientales, France; 2°6′E, 42°47′N), Bellver (Lleida, Spain; 1°46′E, 42°22′N), and Cervera de Pisuerga (León, Spain; 4°30′W, 42°52′N). The Spanish and French populations represent the subspecies *C. p. erythropus* and *C. p. parallelus*, respectively, with the Aunat and Bellver sites close to but not in the hybrid zone where they meet. For each study population, DNA was extracted from five females and five males using a "salting-out" method (Sunnucks and Hales 1996). Only a single foreleg was used.

ITS1 PCR

The ITS1 region was amplified using the primers ITS1A (5′-CCTTTGTACACACCGCCCGT) and ITS1D (5′-GTTCATGTGTCCTGCAGTTCAC). This primer pair was originally designed for use on mosquitoes (Sharpe, Harbach, and Butlin 2000) and targets the flanking rRNA gene regions (18S and 5.8S) to produce a PCR product of 602 bp in *C. parallelus* (GenBank accession number AY 585651). All PCR amplifications were performed on a Hybaid OmniGene thermal cycler and carried out in 25 μl volumes overlaid with one drop of mineral oil. Each 25 μl volume contained: approximately 5 ng genomic DNA template, 3.0 mM $MgCl_2$, 12.5 pmol each primer, 0.12 mM dNTPs, 0.25 units Thermoprime Plus DNA polymerase (ABgene) with the associated ReddyMix PCR buffer at 1× concentration. After an initial denaturation of 5 min at 95°C there were 35 cycles of amplification (1 min at 94°C, 1 min at 67°C, and 2 min at 72°C) before a final incubation of 10 min at 72°C. The products were purified on columns (Promega) prior to cloning or sequencing.

Cloning

Purified PCR products were cloned using the pGEM-T Easy Vector system (Promega) and transformed into JM109 competent cells. Negative controls (water) were used to check for contamination. For each transformation, which represents an individual grasshopper, ITS1 PCR products were generated from 20 randomly chosen clones. A second ITS1 PCR (mismatch PCR) was performed using the same colonies, but the ITS1A primer was replaced by the primer ITS1-4 (5′-GTCTCAAAACTAG-CAAACTCGGTATCAT) and the annealing temperature lowered to 55°C. ITS1-4 is a *C. parallelus*-specific primer designed specifically for studying *WIP4*. This primer anneals to a region immediately upstream of the *WIP4* site but deliberately differs from the DNA template by two mutational changes in order to create a restriction site (*Nde* I) that encompasses the *WIP4* site. Without this modification, the *WIP4* site could not be investigated, as no commercial restriction endonuclease is available that could be used to determine the base type at that site.

Sequencing

For each population, a single female grasshopper was chosen from among the sample of 10 individuals. ITS1 PCR products from between five and 11 clones per individual were sequenced in both directions, using the ITS1A and ITS1D primers. Sequence reactions were performed with *Taq*FS dye-terminator fluorescent chemistry (Applied Biosystems) and run on an Applied Biosystems Model 373 automated sequencer.

Restriction Digests

Digests were performed to determine the base type at the four target ITS1 nucleotide sites (*WIP1, 2, 3*, and *4*). For each clone, three digests were performed using the restriction endonucleases: *Bsr* I (NEB), *Tai* I, and *Nde* I (MBI Fermentas). Following the manufacturer's instructions, 5 μl of PCR product was digested in a total reaction volume of 15 μl. The *Tai* I and *Bsr* I digests were performed using PCR products consisting of the entire ITS1 region, whereas the *Nde* I digest could only be performed on the modified products generated by the mismatch PCR. For all digests there were additional restriction sites within the ITS1 region that were expected to consistently cut, providing a positive control for the reaction. The digested PCR products were visualized on ethidium bromide-stained 2.5% agarose gels. All 15 μl of the digest reaction was loaded, and to ensure accuracy in

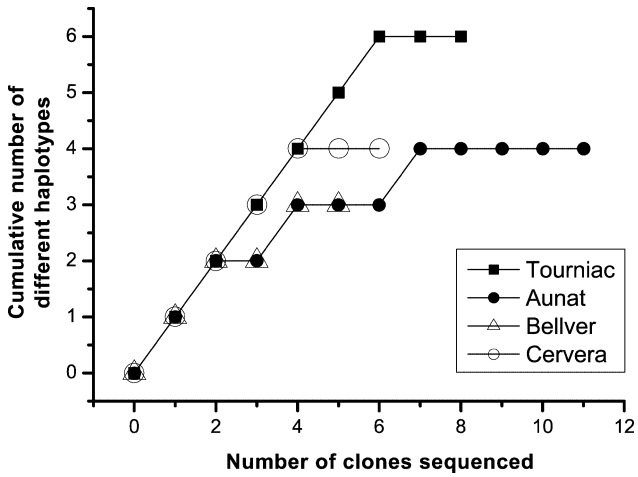

FIG. 1.—The number of different haplotypes observed among a sample of ITS1 copies amplified from four female *C. parallelus* individuals. The females from Tourniac and Aunat represent *C. p. parallelus* and those from Bellver and Cervera are *C. p. erythropus* individuals.

interpreting the RFLPs, a selection of standards was run in every gel. These standards were composed of digested PCR products whose full ITS1 sequence, and therefore the restriction endonuclease cutting sites, were known. Any fragments whose digest patterns could not be interpreted were sequenced. WIP patterns determined from clones were compared with restriction digests of the original PCR products from selected individuals as an additional check for contamination.

Results

The ITS1 region of *C. parallelus* spans 334 bp, based on comparisons to *Drosophila* (Tautz et al. 1988; Parkin 2002). A total of 30 ITS1 copies obtained from four female *C. parallelus* individuals were sequenced and aligned using ClustalX (Thompson et al. 1997) (see Supplementary Material online). All unique mutations (a total of 14) were removed, as no reliable distinction can be made between singleton WIPs and PCR artifacts. The true level of intra-individual variation will, therefore, be underestimated.

In total, nine WIPs were identified among the 30 sequences. Four of these WIPs—identified as *WIP1, 2, 3*, and *4*—were subsequently investigated using the PCR-RFLP approach and occur at nucleotide sites 58, 312, 15, and 124 of the *C. parallelus*' ITS1 sequence, respectively. Intra-individual variation was consistently found, with several haplotypes coexisting in the genome of each individual (fig. 1). Only one WIP (*WIP1*) was consistently found in all four individuals. From these sequence data the remaining eight WIPs appear to be subspecies-specific. However, the RFLP results show that the nucleotides at the *WIP2, 3*, and *4* sites are actually polymorphic in both subspecies, although in each case the second base type is relatively rare in one of the subspecies (table 1). This suggests that each mutational change arose prior to the divergence of the two subspecies and that homogeneity has yet to be restored at any of these nucleotide sites after at least 500,000 generations.

Twenty ITS1 copies were sampled from each individual, and for each ITS1 copy the base type present at each of four target nucleotide sites (*WIP1, 2, 3*, and *4*) was determined from the RFLP results. The base type frequencies among individuals within each population typically appear very variable (fig. 2). To establish if this variation was a consequence of the sampling strategy employed, a further 20 ITS1 copies were sampled from three of the individuals using new DNA extractions from a single leg. The base type frequencies of these additional 20 copies did not differ significantly from those of the first 20 copies (data not shown). Together with the controls described above, the repeatability of these results means the variation found among individuals cannot simply be attributed to the methodology employed.

When listed in order of name, the base types found at the *WIP1, 2, 3*, and *4* sites were used to form a haplotype for each ITS1 copy sampled. Multiple haplotypes were found in the genome of each individual, but the TCCA haplotype exhibited a universal occurrence, suggesting that it is ancestral (fig. 3). The CTCA and CCCG haplotypes are subspecies-specific for *Cpp* and *Cpe*, respectively, although there is an individual in both the Aunat and Bellver populations that does not conform to this pattern. Because these two populations are close to the hybrid zone, it is possible that the appearance of haplotypes that are *Cpe*-specific in *Cpp* individuals, and vice versa, may be the result of introgression.

In *Cpp* there is a NOR on the X chromosome. This means that the diploid complement of NORs differs between males and females, due to the XO/XX sex determining system of this species, and the proportion of ITS1 copies derived from the X NOR differs between the sexes (2/6 in females, 1/5 in males, if the copy number per NOR is constant). The variation observed among the *Cpp* individuals could, therefore, be a consequence of this difference. An AMOVA was calculated in Arlequin (Schneider, Roessli, and Excoffier 2000) to test this hypothesis and revealed that the sex of the individual

Table 1
The Mean Base Type Frequencies at Four ITS1 Nucleotide Sites (*WIP1, 2, 3, and 4*) in the Study Populations of *C. p. parallelus* (*Cpp*) and *C. p. erythropus* (*Cpe*)

		WIP1		*WIP2*		*WIP3*		*WIP4*	
Subspecies	Population	C	T	C	T	C	T	A	G
Cpp	Tourniac	0.305	0.695	0.715	0.285	0.910	0.090	0.975	0.025
	Aunat	0.470	0.530	0.620	0.380	0.850	0.150	0.995	0.005
Cpe	Bellver	0.595	0.405	0.975	0.025	1.000	0.000	0.475	0.525
	Cervera	0.675	0.325	1.000	0.000	0.995	0.005	0.305	0.695

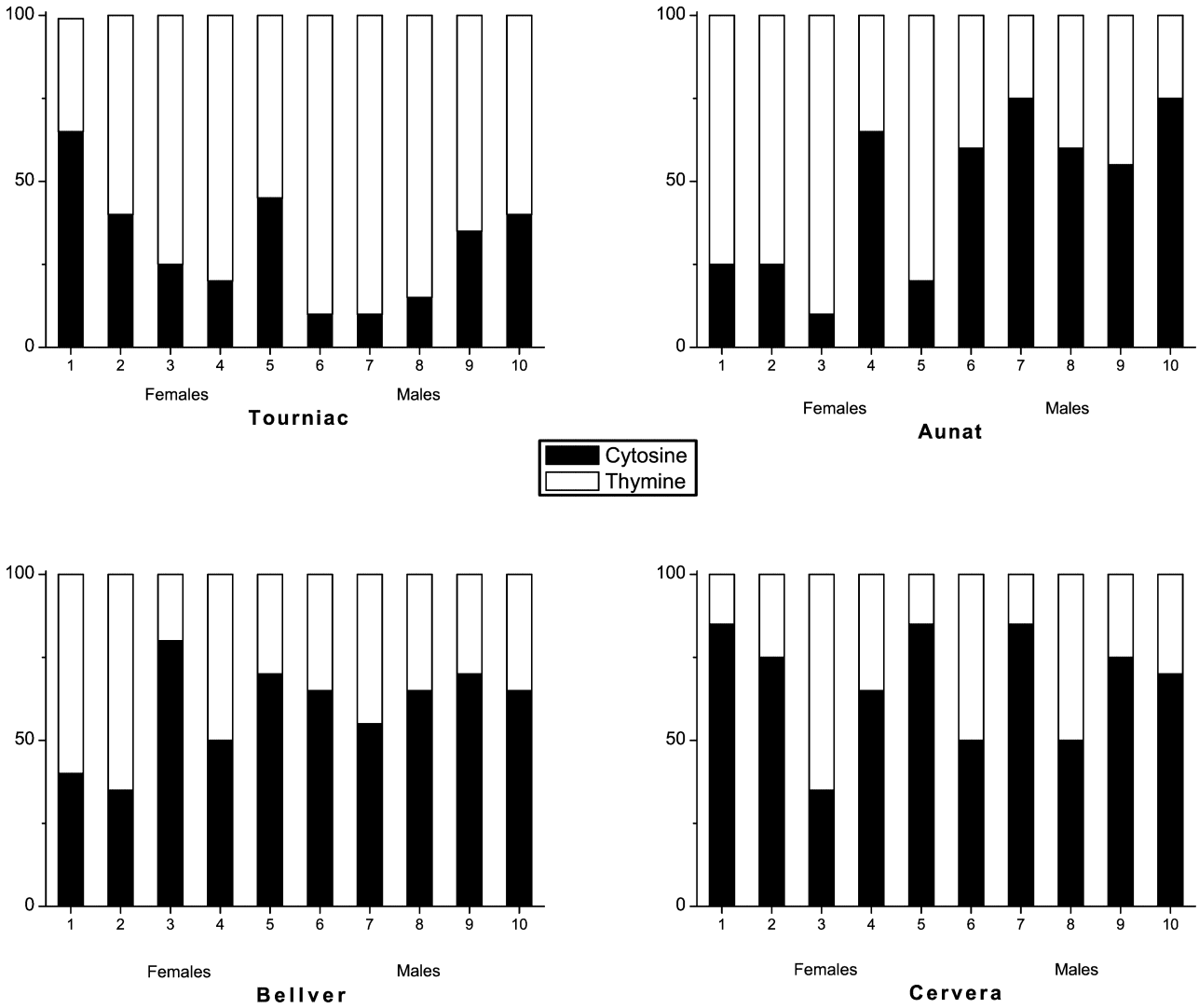

FIG. 2.—Base type frequencies at the WIP1 ITS1 nucleotide site within each of the 40 individuals taken from the four study populations of *C. parallelus* (Tourniac, Aunat, Bellver, and Cervera). Individuals 1 to 5 are female, 6 to 10 are male. See online Supplementary Material for data on the other three WIPs.

does not explain a significant proportion of the variation observed (table 2). This suggests that the X NOR in *Cpp* does not contain distinctive haplotypes compared to the autosomal NORs. This may mean that there is enough interchromosomal gene conversion to prevent divergence, but an alternative explanation is that the X NOR was recently derived from an autosomal NOR and has not yet diverged in ITS1 sequence.

A second AMOVA revealed that the variation observed within each subspecies was primarily within individuals, with a significant component of variation among individuals but no significant variation among populations of either subspecies (table 3).

The Dirichlet distribution was used to obtain maximum likelihood estimates of the mean haplotype frequencies (P_i) within each population and subspecies together with an additional parameter (F) that measures the level of differentiation among individuals. Since this study considers the correlation that exists between the rDNA copies within an individual, relative to the rDNA loci of the total population (or subspecies), the F parameter provides an analogue of the F_{st} statistic (Weir 1996, pp. 83–86). For all populations and subspecies, F was consistently greater than zero, thereby confirming that significant variation exists among individuals (fig. 4).

Within each subspecies, nearly all individuals had the same two predominant haplotypes, namely CTCA and TCCA for *Cpp* and CCCG and TCCA for *Cpe* (fig. 3). In both instances, the two predominant haplotypes are two mutational steps apart, indicating a lack of independence between WIPs, that is, that they are in linkage disequilibrium within individuals. Tests of association confirm this conclusion for the two WIPs that occur at intermediate frequency in each subspecies: *WIP1* and *WIP2* in *Cpp* and *WIP1* and *WIP4* in *Cpe* (chi-square test with Yates' correction, $P < 0.01$ in 15 out of 20 individuals in *Cpp* and in 18 out of 20 individuals in *Cpe*).

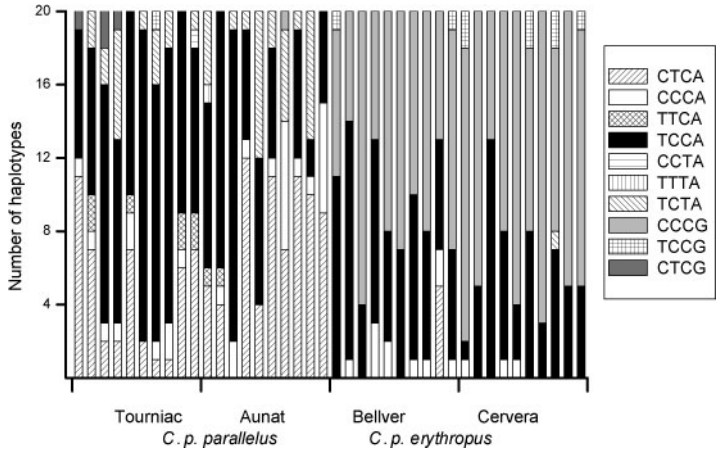

FIG. 3.—ITS1 haplotype frequencies found within 40 *C. parallelus* individuals representing four populations: Tourniac (France), Aunat (France), Bellver (Spain) and Cervera (Spain).

Discussion

ITS1 variation was consistently found within *Chorthippus parallelus* individuals (see fig. 3), with different nucleotide base types predominating at specific WIP sites between populations and among individuals within a population (fig. 2). The level of intra-individual variation proved higher than expected, as the consensus ITS1 sequence for these individuals had typically revealed only one clear WIP (Parkin 2002). These findings have significant implications for the use of ITS sequences as markers in phylogeographic or species-level phylogenetic studies. They demonstrate the importance of sampling multiple ITS1 copies from each individual so that shared polymorphisms can be differentiated from putative substitutions. If the level of intra-individual variation is not directly studied, then caution should be applied when making phylogenetic inferences from consensus ITS1 sequences, especially among closely related species.

In *C. parallelus* the populations are evolving cohesively, in that individuals typically share the same ITS1 haplotypes (fig. 3). However, contrary to some predictions (Ohta and Dover 1983), the presence of significant levels of variation among individuals reveals that individuals are not in a similar state at any one point in time (fig. 4). Thus, molecular turnover mechanisms in highly repeated gene families need not preclude the operation of selection. The phenotypic effects of substitutions in ITS1 are unlikely to be sufficient to generate substantial fitness difference between individuals with varying mixes of haplotypes. However, for other repeated loci, including the rDNA coding regions, such differences could well be important.

The high level of variation among *C. parallelus* individuals indicates that there is substantial heterogeneity in haplotype composition within at least one population of NOR-carrying chromosomes. This means that significant differences typically exist between the maternal and paternal NORs of an individual, with respect to their haplotype distributions. Reciprocal recombination would tend to break down such differences, reducing the heterogeneity in the population. This must be outweighed by the spread of new variants within chromosomes, most likely by intrachromosomal gene conversion. Although gene conversion may only involve a small number of rDNA copies, it has been shown that it can be very effective at altering variant frequencies, especially if it is biased in nature (Hillis et al. 1991).

Ohta and Dover (1984) investigated the cohesive population genetics of molecular drive within multigene families such as rDNA. Their models express the outcome in terms of the variance in copy number relative to the mean copy number in the population of chromosomes. They find that this relative variance (*RV*) is dependent on the relative rates of recombination and intrachromosomal gene conversion (unequal crossovers were not considered). To compare our data with the predictions of these models,

Table 2
AMOVA Results Calculated in Arlequin (Schneider, Roessli, and Excoffier 2000) Using the Four-Base Haplotype Data Partitioned by Sex

	Percentage of Variation			
Subdivision	Tourniac	Aunat	Bellver	Cervera
Between the sexes	2.19 ($P = 0.186$)	10.46 ($P = 0.038$)	−0.87 ($P = 0.487$)	−2.65 ($P = 0.866$)
Among individuals of the same sex	9.26* ($P < 0.001$)	7.95* ($P < 0.001$)	5.92 ($P = 0.019$)	9.69* ($P = 0.003$)
Within individuals of the same population	88.56	81.59	4.95	92.96

NOTE.—* indicates that result is significant after Bonferroni correction.

Table 3
AMOVA Results Calculated in Arlequin (Schneider, Roessli, and Excoffier 2000) Using the Four-Base Haplotype Data Partitioned by Population

	Percentage of Variation	
Subdivision	*C. p. parallelus*	*C. p. erythropus*
Among populations	1.74 ($P = 0.141$)	2.54 ($P = 0.071$)
Among individuals within populations	12.45* ($P < 0.001$)	6.49* ($P < 0.001$)
Within individuals	85.81	90.97

NOTE.—* indicates that result is significant after Bonferroni correction.

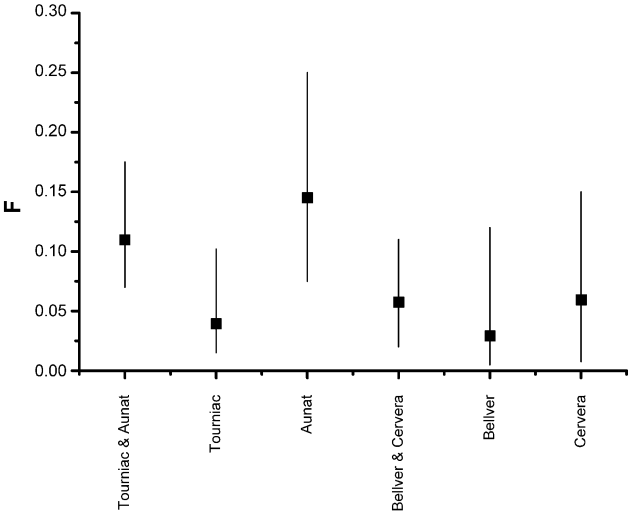

FIG. 4.—Maximum likelihood estimates of F representing the level of differentiation among *C. parallelus* individuals within each population (Tourniac, Aunat, Bellver, and Cervera) and subspecies (Tourniac and Aunat = *C. p. parallelus*; Bellver and Cervera = *C. p. erythropus*). Bars correspond to 95% confidence intervals.

sample estimates of the base type frequencies associated with each WIP (table 1) were used to derive measures of sample relative variance (among individuals). By simulating the sampling strategy employed to generate these data from *Cpe* populations, the relationship between sample relative variance at the individual level and the underlying relative variance in the population of chromosomes (*RV*) was established (see Parkin [2002] for details). Our data suggest a population relative variance of approximately 0.1 in terms of variant frequency, with a lower 95% confidence limit of approximately 0.01.

To make comparisons to the Ohta and Dover (1984) models, the *RV* estimate must be converted to a measure based on numbers of variants, rather than variant frequency, by multiplying the estimate by the number of rDNA copies within the genome. For *C. parallelus* there is evidence that the rDNA copy number may exceed 1,000 per genome (Parkin 2002), which suggests an extremely high *RV* value of 100 (lower limit 10). Even with the rDNA copy number reduced to 200, to match *Drosophila* (Long and Dawid 1980), the resultant *RV* estimate of 20 (lower limit 2) still exceeds the range of predicted values derived from the models of Ohta and Dover (1984). *RV* is expected to increase as the rate of intrachromosomal gene conversion increases relative to the rate of recombination. Crossing-over between homologous chromosomes tends to equalize the frequencies of haplotypes among the chromosomes, reducing the variance among individuals. The high estimates of *RV* in *C. parallelus* indicate that gene conversion is regularly altering variant frequencies at the intrachromosomal level, and this process more than counteracts the homogenizing effects of interchromosomal recombination. The lowest value of relative variance consistent with our data ($RV = 2$, $n = 200$, lower confidence limit of *RV*) requires the per copy rate of intrachromosomal gene conversion (λ in Ohta and Dover [1984]) to be of the same order as the per family rate of interchromosomal recombination per generation ($[n - 1]\beta$ in Ohta and Dover [1984], where n is the copy number and β is the per copy recombination rate).

Our approach did not enable us to compare the haplotypes present in the different NORs, except to the extent that this would be revealed from differences between the sexes of *Cpp*. Variation among NORs is expected because gene conversion is likely to be more common between homologous than nonhomologous chromosomes and has been observed in other species (Copenhaver and Pikaard 1996). However, variation among NORs cannot generate the differences in haplotype distributions that we observe among individuals. This is because each individual contains a sample of each of the NORs (two copies of the L_2 NOR and two copies of the L_3 NOR in *Cpe*). Our sample of 20 ITS1 copies from each individual therefore contains a mix of copies from the two NORs, but this mix should not vary among individuals, except due to sampling effects.

Linkage disequilibrium among the ITS1 haplotypes within individuals of *C. parallelus* also suggests that intrachromosomal gene conversion occurs more frequently than recombination between chromosomes. This is because gene conversion can spread intact haplotypes within repeat arrays, whereas recombination tends to break the association between WIPs. This conclusion was also reached by Schlötterer and Tautz (1994) and Polanco et al. (1998), based on their discovery that the rDNA arrays on the X and Y chromosomes of *Drosophila melanogaster* are homogenized for different polymorphic alleles. A similar pattern emerged between the two rDNA arrays of *Arabidopsis thaliana*, with specific polymorphic alleles largely homogeneous in each array. The coexisting polymorphic alleles within these arrays are organized into homogeneous adjacent units (Copenhaver and Pikaard 1996), suggesting that intrachromosomal events spread variants rapidly along an array. Based on the results of this study, the expectation is that the chromosomes of *C. parallelus* are predominantly populated by different ITS1 haplotypes. This would mean that the ITS1 region is evolving along independent chromosome lineages because the chromosome is, in effect, acting as a barrier to the spread of new mutations.

In *C. parallelus* significant differences were observed between the ITS1 haplotype distributions of the two subspecies. These differences have evolved in a period of ~500,000 generations. Molecular turnover mechanisms

have not resulted in rapid divergence because a common haplotype is still shared between subspecies, but new haplotypes have spread gradually over this long time period. Although populations of *C. parallelus* are now large, the species has a history of range expansion and contraction (Hewitt 1993), so genetic drift may have played a significant role in generating the observed divergence.

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