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ORIGINAL ARTICLE

Geographically localised bursts of ribosomal DNA mobility in the grasshopper *Podisma pedestris*

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We report extraordinary variation in the number and the chromosomal location of ribosomal DNA (rDNA) arrays within populations of the alpine grasshopper *Podisma pedestris*; even greater differences were found between populations. The sites were detected by *in situ* hybridisation of labelled rDNA to chromosomal preparations. The total number of rDNA sites in an individual varied from three to thirteen. In the most extreme case, individuals from populations only 10 km apart had no rDNA loci in common. A survey of the geographical distribution of this variation identified clusters of populations with relatively similar chromosomal distribution of rDNA loci. These clusters correspond to those identified earlier by analysis of rDNA sequences. To explain this geographical clustering, we reconstructed the post-glacial colonisation of the region by assuming that the species' distribution has ascended to its current altitudinal range as the climate warmed. The reconstruction suggests that each cluster is descended from a colonisation route up a different alpine valley. That history would imply rapid establishment of rDNA differences, conceivably during the last 10 000 years since the last glaciation. The proposal for rapid change is consistent with the extensive within-population variation, which indicates that the processes responsible for the change in rDNA's chromosomal location continue to occur at a higher rate. We discuss whether our reconstruction of colonisation routes implies movement of the hybrid zone, which would indicate that a neo-XY sex chromosome system has spread through extant populations.
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Introduction

Ribosomal DNA (rDNA) genes code for the RNA component of ribosomes, which is essential for their role in transcription (McLain *et al*., 1995). Eukaryotic genomes contain multiple copies of their rDNA genes, presumably because exceptionally high quantities of RNA transcripts are required; it is not atypical for 50% of all RNA exported from the nucleus to be rRNA (Russel and Zomerdijk, 2005). Ribosomal DNA genes are organised in tandem arrays, each transcribed from a single promoter by RNA polymerase I. Each repeating unit in an array consists of an intergenic spacer involved in regulation (Reeder, 1990) then, typically, three conserved genes (*18S*, *5.8S* and *28S*) separated by more variable intergenic spacers (ITS1 and ITS2) and an external transcribed spacer (Hemleben and Zentgraf, 1994). Transcription of rDNA takes place in the nucleolar organising region, which can be shown by the cytogenetic technique of silver staining.

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Ribosomal DNA sequence is extensively used for phylogenetic reconstruction for two reasons. One is that some parts of rDNA are among the most conserved sequences known (Eickbush and Eickbush, 2007), so homologues can be readily identified. Second, the different units within any one species tend to be homogenous (especially members of the same array) (Liao, 1999) a pattern termed concerted evolution (Zimmer *et al*., 1980). The homogenising processes that maintain the similarity between units within a species probably include unequal crossing-over and gene conversion (Eickbush and Eickbush, 2007), although the relative importance of each mechanism is not clear and their contribution may vary depending on the genome in question.

In contrast to this conventional picture, there have been recent reports of species that do not exhibit the high within-species rDNA homogeneity characteristic of concerted evolution. They include aphids (Fenton *et al*., 1998), flatworms (Carranza *et al*., 1999), mosquitoes (Li and Wilkerson, 2007), trypanosomes (Beltrame-Botelho *et al*., 2005), the phylum Apicomplexa (Rooney, 2004) and actinomycetous bacteria (Wang *et al*., 1997). We have recently described the case of extensive within-individual and between-individual variability in ITS1 rDNA sequences of the grasshopper *Podisma pedestris* (Keller *et al*., 2006, 2008). Individuals from the same, or neighbouring, populations had more similar sequences, suggesting that ancestral polymorphisms can persist for extended periods of time. However, bursts of rapid change in the frequency of certain rDNA variants also

seem to have occurred, as several individuals had an rDNA sequence composition that differed markedly from their population, or any other (Keller *et al*., 2008).

Occasionally, studies have detected changes in the chromosomal location and size of specific rDNA arrays (loci). For example, the location of arrays differs between sibling species within the *Drosophila melanogaster* complex (Lohe and Roberts, 1990). More rapid change is suggested by differences between populations of the brown trout, *Salmo trutta* (Castro *et al*., 2001), and the grasshopper, *Eyprepocnemis plorans* (Cabrero *et al*., 2003). In *D. melanogaster,* the number of rDNA units on the X chromosome varied more than twofold between different lines, after 400 generations of divergence in the laboratory (Averbeck and Eickbush, 2005).

The variation in the dynamics of rDNA evolution between different species is puzzling, as the underlying molecular biology is presumed to be the same. One possibility is that general properties of the genome are relevant. The genomic environment of *P. pedestris* is quite different from a typical model organism. It contains 16.93 pg of DNA (Westerman *et al*., 1987), roughly 100 times more than the genome of *D. melanogaster*. It includes multiple copies of mitochondrial pseudogenes in the nuclear DNA (Numts). Analysis of Numts suggests that point mutations are more frequent than deletions in the *P. pedestris* nuclear genome, the inverse situation to that in *D. melanogaster* (Bensasson *et al*., 2001). By studying such atypical genomes we hope to understand how the internal genomic environment can affect its evolution.

Scientific interest in *P. pedestris* initially arose because it is subdivided into chromosomal races. It is a flightless grasshopper adapted to steppe-like climate. It is widely distributed in Northern Eurasia (Klochanova, 1953; Latchininsky, 1997). In the French Alps, the area of study, the species' range has retreated to higher altitudes in the last 10 000 years, because of post-glacial climate warming (Hewitt, 1999). Abundant populations are typically found between 1500 and 2500 m (Nichols and Hewitt, 1986). The sex chromosome system is XO for males and XX for females throughout most of the range of the species (the unfused race). In the Southern Alps, a Robertsonian fusion between the L3 autosome and the X chromosome has become established to produce a neo-XY system, with XY males and XX females (the fused race) (John and Hewitt, 1970; Hewitt, 1975).

In places where the two chromosomal races meet, a narrow cline in the frequency of the fusion is formed, which is 800 m wide (Barton and Hewitt, 1981b). The hybrid zone does not seem to coincide with an environmental gradient (Barton and Hewitt, 1981a; Nichols and Hewitt, 1988), indicating that it is a tension zone maintained by some form of selection against introgressing genotypes. This hypothesis is supported by crossing experiments, which detected inviability (Barton and Hewitt, 1981b). Before our rDNA sequence analysis, few fixed differences had been found between the races: one involved a restriction enzyme variant in rDNA (Dallas *et al*., 1988) and another was a 5% reduction in total genomic DNA (Westerman *et al*., 1987), which could conceivably involve rDNA. Both differences remained in strong linkage disequilibrium with the fusion in one well studied transect across the hybrid zone. No obvious size difference between the chromosomes of the two races has been discerned by light microscopy and no other clear differentiation between the races has been found in morphology or allozymes (Halliday *et al*., 1983; Nichols and Hewitt, 1986).

In this paper, we continue the investigation of the rDNA variation within *P. pedestris* by identifying the chromosomal location (loci) of the rDNA tandem arrays. We employ fluorescent *in situ* hybridisation (FISH) with an rDNA probe, to detect chromosomal 'sites', where there are rDNA arrays. We exploit a subset of the samples used by Keller *et al*. (2008) so that direct comparisons can be drawn with their results.

Materials and methods

Sample collection and preparation

Details of the sampling are provided in Keller *et al*. (2008). Coordinates are listed in the Supplementary information. Briefly, two areas of the Southern French Alps (one close to the village of Seyne and the other close to Casterino) were sampled, usually between 1500 and 2000 m, where *P. pedestris* is most prevalent and accessible. Male grasshoppers were collected within a 20 m radius for each population and dissected in the field. The testes were fixed in freshly prepared 3:1 ethanol:acetic acid solution, the fixative was replaced with ethanol and the samples were kept at 4 °C for long-term storage.

FISH technique and protocols

The FISH procedures were based on Schwarzacher and Heslop-Harrison (2000).

Probe information.

Ribosomal DNA probe: The rDNA probe was either a labelled pTa71 clone (Gerlach and Bedbrook, 1979) (a widely used standard rDNA probe), or a 35S PCR product amplified from *P. pedestris* 18S DNA. Preliminary two-colour FISH experiments using both probes simultaneously indicated that they resulted in the same labelling profile. This result indicates that the probes are detecting rDNA rather than non-specific hybridisation with repetitive sequences. The 35S PCR used the primers 18S_F (5′-ACGTTACTTGGATAACTGTGGT-3′) and ITS2_B (5′-TATGCTTAAATTCAGGGGGT-3′) (Beebe and Saul, 1995), which amplify a 2.5 kb region that includes most of the *18S* gene and extends until the very beginning of the *28S* gene. PCR amplifications were carried out using a PTC-220 DNA Engine DyadTM Peltier Thermal Cycler (MJ Research, Essex, UK). Each reaction (25 μl) contained approximately 5–10 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 pmol of each primer, 0.5 units of BIOTAQ DNA Polymerase (Bioline, London, UK) and 1 × NH_4^+ buffer. An initial denaturation step of 3 min at 94 °C was followed by 31 cycles of 30 s at 94 °C, 30 s at 52 °C, 3 min at 72 °C and a final elongation step of 10 min at 72 °C.

Telomeric probe: A telomeric probe was also used in the FISH survey, primarily as a marker to help interpret the configuration of the bivalents at meiosis, and hence to distinguish the chromosomes. The sequence of the telomeric repeat of all Orthoptera studied so far is $(TTAGG)_n$ (López-Fernández *et al*., 2006). The telomeric probe was generated by PCR reaction using the primers Telo_3 (5′-TAGGTTAGGTTAGGTTAGGT-3′) and Telo_4 (5′-CTAACCTAACCTAACCTAAC-3′) alone. They hybridize

with each other and hence produce a product in the absence of other template DNA (Sahara *et al*., 1999). The PCR reaction followed the protocol in Ijdo *et al*. (1991). PCR amplifications were carried out using a PTC-220 DNA Engine DyadTM Peltier Thermal Cycler (MJ Research). Each reaction (100 µl) contained 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 pmol of each primer, 2 units of BIOTAQ DNA Polymerase (Bioline) and 1 × NH_4^+ buffer. An initial denaturation step of 3 min at 94 °C was followed by 10 cycles of 60 s at 94 °C, 30 s at 52 °C, 60 s at 72 °C and 30 cycles of 60 s at 94 °C, 30 s at 52 °C, 90 s at 72 °C and a final elongation step of 5 min at 72 °C.

Nick translation: Probe labelling was performed by nick translation following the protocol provided in Schwarzacher and Heslop-Harrison (2000). Each reaction (50 µl) contained nick translation buffer (0.05 M Tris-HCl, pH 7.8, 0.005 M $MgCl_2$, 0.05 mg ml^{-1} bovine serum albumin, nuclease-free water (Sigma-Aldrich, Dorset, UK), 0.2 mM dithiothreitol, 0.005 mM of each dCTP, dGTP and dATP, and 2.5 units of DNA polymerase I and 0.002 units of DNase I (Invitrogen, Paisley, UK). For biotin labelling, 2.5 µl of 0.4 mM biotin-11-dUTP (Sigma, Dorset, UK), made up from powder in 100 mM Tris-HCl pH 7.5, were added to the reaction. For digoxigenin labelling, 1 µl of 0.35 mM digoxigenin-11-dUTP (Roche Biochemicals, Burgess Hill, UK) and 0.65 mM dTTP were added to the reaction. The product was stored at −20 °C until required, and could be used without further purification. The total DNA produced per reaction was judged by eye on an agarose gel and standardised to ∼1 µg.

Chromosome preparations: Meiotic chromosome squashes were prepared by squashing the fixed testes in 50–75% acetic acid on polylysine coated slides (VWR, Lutterworth, UK). The slides were frozen on a column of dry ice for 30 min and the coverslip was flicked off using a scalpel blade. The slides were air-dried and stored at −20 °C, typically for 2 weeks, before going through the FISH protocol.

In situ hybridisation: Typically, 12 slides were treated at a time. Washes were made in Coplin jars (six slides each). The slides were treated with 100 µg ml^{-1} RNase A (Sigma, Dorset, UK) in Tris-HCl for 1 h at 37 °C in a humid chamber. They were then washed with 2 × saline-sodium citrate (SSC) (0.3 M NaCl, 0.03 M sodium citrate) and treated with 0.25 µg ml^{-1} pepsin (Sigma-Aldrich) in 0.01 M HCl for 5–15 min, depending on the amount of cytoplasm remaining around the meiotic chromosomes. After washing with 2 × SSC, the slides were fixed in freshly depolymerised paraformaldehyde 0.025% (w/v) at room temperature. They were then washed in 2 × SSC and dried in an ethanol series.

Next, 0.5–1.2 µl of biotin- or digoxigenin-labelled DNA (0.1 µg ml^{-1}) was added to the probe solution (50% formamide, 0.1% dextran sulphate, 0.01 × SSC, 0.0375% (w/v) sodium dodecyl sulphate). The probe mix was denatured at 76 °C for 10 min before hybridisation and kept on ice. It was applied to the slides directly and hybridisation was performed on the hot plates of a PTC-220 DNA Engine DyadTM Peltier Thermal Cycler (MJ Research) using the following program: 72 °C for 7 min, 55 °C for 2 min, 50 °C for 30 s, 45 °C for 1 min, 42 °C for 2 min, 40 °C for 5 min, 38 °C for 5 min, 37 °C thereafter (until the product was transferred to the humid chamber, typically after 1 h). The slides were incubated overnight at 37 °C in a humid chamber.

Post-hybridisation washing: The slides were washed in the following: 2 × SSC for 2 min at room temperature, a stringent wash (20% formamide in 0.1 × SSC) at 42 °C for 10 min, 2 × SSC at 42 °C for at least 10 min and then washed at room temperature in 4 × SSC Tween (0.6 M NaCl, 0.06 M sodium citrate, 0.002% v/v Tween). Sites of hybridisation of the digoxigenin- and biotin-labelled probes were detected simultaneously (two-colour FISH) using 10 µg ml^{-1} antidioxigenin-FITC (Roche Biochemicals) and 5 µg ml^{-1} avidin-Cy3 (Amersham Pharmacia, Buckinghamshire, UK), which were added to the slide and incubated at 37 °C for 1 h. A drop of Vectashield including DAPI (Vector laboratories, Peterborough, UK) was added on each slide to counterstain the chromatin and to stabilise the fluorochromes.

Imaging: Fluorescence signal was viewed with a Leica DMRA2 microscope, captured with a Hamanabu ORCA-ER digital camera and processed with the software Openlab V3.15 (Improvision Ltd, Coventry, UK) on a Power Macintosh computer. Images were exported in Tiff format (publication quality) to Adobe Photoshop (Adobe Systems, Uxbridge, UK) for further manipulation. The images were treated uniformly by changing contrast, brightness and colour balance only. Only individuals that provided at least three images from different cells, each with clear probe localisation, were included in the results.

Inferring colonisation routes

The past distribution of *P. pedestris* populations was assessed by starting from a map of the 1000 m altitudinal range (between 1500 and 2500 m, Figure 2d), in which most *P. pedestris* populations are currently found in the region. This band was displaced downwards to represent colder climates (Figures 2b and c). The assumption is that, within this range, there would have been suitable habitat, which would have been colonised rapidly compared with the rate of climate change. The actual range would have been a subset of the area indicated, because of other ecological limitations. Nevertheless, the reconstruction is sufficient to suggest (a) the possible colonisation routes and (b) the subdivision of the range.

Altitude and GPS data were manipulated in ArcView-GIS v9.2. The elevation data for the map were kindly provided by Patrick Coquillard. The GPS data for the years 2003–2005 were estimated from maps whereas the 2006 sampling locations were obtained directly in the field by a GPS device.

Results

Extreme rDNA locus variability

There was great variation in both the number and location of chromosomal sites detected by the rDNA probe. We found differences between populations, and often between individuals within the same population. We shall use the term 'sites' for a chromosomal region

where signal was detected; hence at any one locus there could be none, one or two sites. If there was only one site, the locus was classified as monosomic. When calculating the total number of rDNA sites in an individual, a homologous pair of sites was counted as two observations irrespective of any difference in size, whereas a monosomic locus was counted as one. The FISH method may not detect small numbers of rDNA units, hence sites with small numbers of units cannot be distinguished from those with none.

The rDNA sites were categorised as being on large, medium or small bivalents, following John and Hewitt (1970) and as being proximal (near to the centromere), or distal (close to the far chromosome end; see Figure 1). All *P. pedestris* chromosomes seem to be telocentric, with the exception of the fused X chromosome. Details of the FISH data are provided in Supplementary file S1. Individuals with as few as three and as many as 13 rDNA sites were found. Surprisingly, monosomic rDNA loci were in the majority in some populations. Consequently, the maximum possible number of sites may be greater than the observed maximum of 13 rDNA sites.

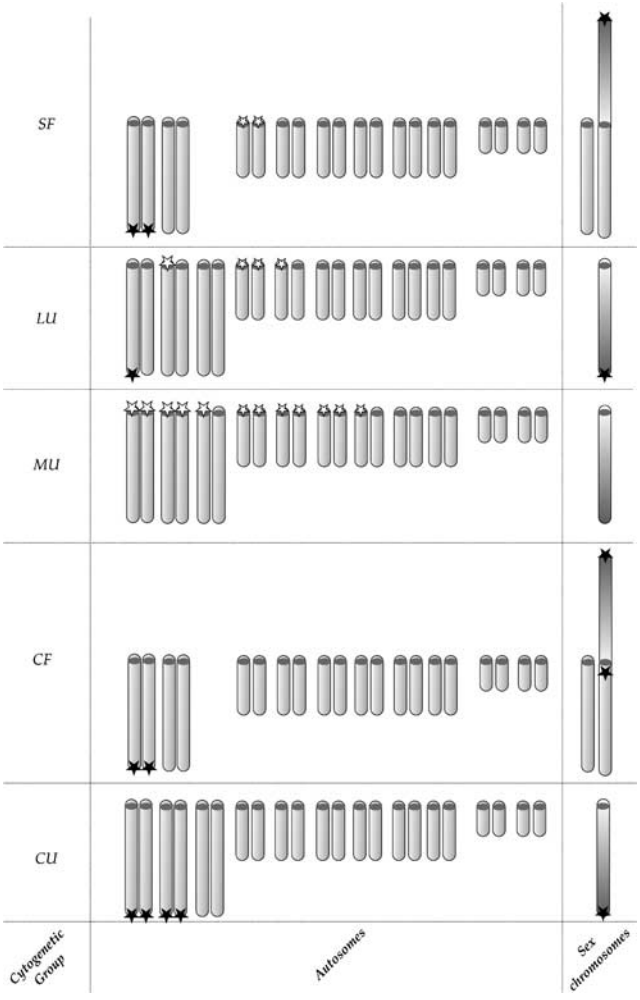

Figure 1 Ideograms of a karyotype from each of the cytogenetic groups (original images are in Supplementary Figure S2). Black stars show rDNA sites that are present in most members of a group. White stars indicate rDNA sites that are absent in some members of the group. The darker chromosome arm is the ancestral X chromosome. Note that because the chromosomes within each size category (small, medium and large) cannot be distinguished, loci can only be provisionally classified as consistently present or not. The second letter of each cytogenetic group code indicates the nature of the X chromosome: F = fused; U = unfused.

Division of the populations into cytogenetic groups

Some rDNA loci were consistently present in almost all individuals from a sampled population. These loci are indicated in bold in Table 1 and were used to divide the populations into geographically contiguous groups. First, the populations were divided into fused and unfused as indicated by the second letter of the cytogenetic group name (F or U in Table 1). The fused populations were further subdivided into the Casterino fused (CF) group, which had a proximal rDNA site on the X chromosome, and the Seyne fused group, which did not. The Mélèzes unfused populations had no distal rDNA sites on the X chromosome (and very few on autosomes). The remaining unfused populations could be subdivided on the basis of the median number of distal autosomal rDNA sites, two in the case of Lac Noir unfused group and four for Casterino unfused group. Figure 1 illustrates an individual from each cytogenetic group. FISH images from the same individuals are provided in Supplementary File S2.

The cytogenetic groupings are compared with those of Keller *et al*. (2008) in Figure 2. Shown in Figure 2a is a principal component analysis plot based on SNP variants in rDNA, in which each symbol represents an individual. The genetic clusters are distinguished by colour and the same colours were used to show the geographical distribution of each group on the adjoining maps (Figures 2b–d). With one exception, the groupings obtained from the new cytogenetic data correspond to those identified earlier from the molecular genetic data. The one exception is a population (Colle della Maddalena) geographically isolated from the rest (see Discussion).

Hybridisation of the telomere specific $(TTAGG)_n$ probe was observed on the centromeres of all fused X chromosomes. It is conceivable that telomeric DNA was left at this location after the fusion between two telocentric chromosomes (the ancestral X chromosome and an L3 autosome) and has subsequently been retained.

Discussion

Extensive variation in number and location of rDNA sites

We have found extensive variation in the number and location of rDNA sites both between and within cytogenetic groups of *P. pedestris* (Table 1). A few comparable reports exist from a wide range of taxa, including beetles of the genus *Zabrus* (Sánchez-Gea *et al*., 2000), the red abalone *Haliotis rufescens* (Gallardo-Escárate *et al*., 2005), the fish *Astyanax scabripinnis* (Mantovani *et al*., 2005), the grasshopper *E. plorans* (Cabrero *et al*., 2003) and onions *Allium* spp. (Schubert and Wobus, 1985). *P. pedestris* stands out for two reasons. One is the extent of variation in the number of rDNA sites and of monosomicity (the occurrence of loci at which rDNA occurs on only one of the two homologous chromosomes), especially in the Mélèzes unfused populations, where there were from five to thirteen sites, of which 0–75% were monosomic. Second, variation in rDNA loci (in number, location and monosomicity) was

Table 1 Summary statistics of the five different cytogenetic groups

Cytogenetic group	*X sites*		*Distal sites*		*Proximal sites*		*Sample size*
	Distal	*Proximal*	*Number*	M_o	*Number*	M_o	
SF	**1**	0	**2**	0	0.71	0.74	34
CF	**1**	**1**	**2.08**	0.08	0.15	1	13
LU	**1**	0	**1.94**	0.06	2.41	0.52	17
MU	0	0	0.2	1	9.7	0.33	20
CU	**1**	0	**3.94**	0.02	0	0	16

Abbreviations: CF, Casterino fused; CU, Casterino unfused; LU, Lac Noir unfused; MU, Mélèzes unfused; SF, Seyne fused.
The putatively expressed (i.e. consistently present) loci are indicated in bold and were used to define the cytogenetic groups. Number is the mean number of rDNA sites per individual. M_o is the monosomicity, calculated as the proportion of autosomal rDNA loci that are monosomic.

extremely localised geographically. The Casterino area displayed little variation from population to population, and limited polymorphism within cytogenetic groups. By contrast, the most extreme example of localised variation is provided by the Mélèzes unfused and Seyne fused cytogenetic groups. They occur only 10 km apart, yet include individuals with no rDNA loci in common.

Just as high levels of heterozygosity would be observed at a conventional locus with a high-mutation rate, the high levels of rDNA locus monosomicity in *P. pedestris* could be explained by high rates of origin and loss of rDNA arrays. As these changes have persisted, they do not seem to be strongly deleterious, perhaps, because they involve non-expressed rDNA. Indeed, Keller *et al*. (2006) estimated that 75% (range 71–78%) of the total rDNA is methylated or has atypical DNA sequence, using Southern blot experiments. Hence, it is conceivable that whole rDNA loci are composed of constitutively inactive repeats or pseudogenes.

We conjecture that the consistently present loci are likely to be the most active (that is, transcribed to produce the RNA component of ribosomes) because of selection against their removal. This interpretation corresponds with earlier published results, based on silver staining, showing that the (consistently present) distal locus of the X chromosome is active. The results were more equivocal for the autosomes and not readily interpretable (Gosálvez *et al*., 1988, J Gosálvez, C Lopéz-Fernández and JL Bella, personal communication). However, rDNA mobility is not confined to inactive loci: an evolutionary perspective allows us to conclude that active rDNA must have moved in some cases. In particular, active sites must have moved as the Mélèzes unfused and Seyne fused groups diverged, because they have no rDNA loci in common.

Recolonisation after the last glacial maximum

The subdivision of the study populations into different groups using the cytogenetic data reported here (number and location of rDNA sites) coincides with the subdivision based on rDNA sequence variation (Figure 2). This genetic structure is probably the result of repeated extinction and recolonisation events. The area was recently glaciated (Hewitt, 1999) and before that, there were multiple climatic cycles, which would have caused population expansion and contraction (every 100 000 years (Hays *et al*., 1976)). The geographical distribution of the cytogenetic groups can be explained by different colonisation routes along which the grasshoppers expanded into their current range after the last glacial maximum (arrows in Figures 2b and c). As *P. pedestris* rDNA harbours extensive within-individual and within-population diversity, the allele frequencies could diverge by genetic drift. Range expansion can cause rapid genetic drift as populations are founded and grow at the expanding margin of a species range (Nichols and Hewitt, 1994; Ibrahim *et al*., 1996; Excoffier and Ray, 2008). The cytogenetic and molecular differences could both have become established during the same expansions, which would explain why they correspond. The only minor discrepancy is a population to the northeast, Colle della Maddalena (marked by asterisk in Figure 2). Its rDNA sequence composition differs from Casterino unfused group (its cytogenetic group) in being fixed for six polymorphic sites that are common in other unfused populations. Perhaps, it accumulated differences from other Casterino unfused populations during an expansion from the Italian side of the border.

The chromosomal fusion may also have become established through genetic drift potentially during population expansion from a glacial refugium. The cytogenetic and molecular differences between the different fused populations could then have accumulated over a number of cycles. Attributing the current distribution of the fused race exclusively to extinction and recolonisation processes is not entirely straightforward, however, the current position of the hybrid zone does not seem to be at the junction of two expansions. If the expansion marked (i) in Figure 2b was comprised of unfused individuals it would seem to be inevitable that the route marked (ii) would also have been unfused; yet, fused populations are currently observed further up that valley. It is possible to propose alternative explanations; for example, a Northern unfused refuge in valley (i) from which the unfused regions were populated whereas the fused race arrived from the South (iii) from where it could also have reached the Casterino area and the other populations known to be fused (routes (iv)).

An alternative explanation is that the fused chromosomes spread northwards once unfused populations had become established. Conventional hybrid zone theory suggests the zone would not move, once formed (Hewitt, 1988), however, evidence that some zones do actually move is accumulating (reviewed in Buggs, 2007) and recent theoretical developments suggest that the spread of an X-autosome fusion could be explained by the sexually antagonistic selection expected in early sex chromosome evolution (Veltsos *et al*., 2008). The reduced recombination between the centromeric region of the neo-Y and the fused X chromosomes (John and Hewitt, 1970) would facilitate the accumulation of alleles with sexually antagonistic effects (Charlesworth, 1996). There is some preliminary evidence consistent with the spread of the fusion into an unfused genetic background at Casterino: individuals from the hybrid zone grouped with unfused individuals based on rDNA sequence variation (Keller *et al*. (2008) Supplementary material).

Most interesting is the distinction between the groups Mélèzes unfused and Lac Noir unfused for which the reconstruction suggests expansion along different valleys ((v) for Mélèzes unfused and the large valley to the north of (vi) for Lac Noir unfused group; Figure 2c) from the

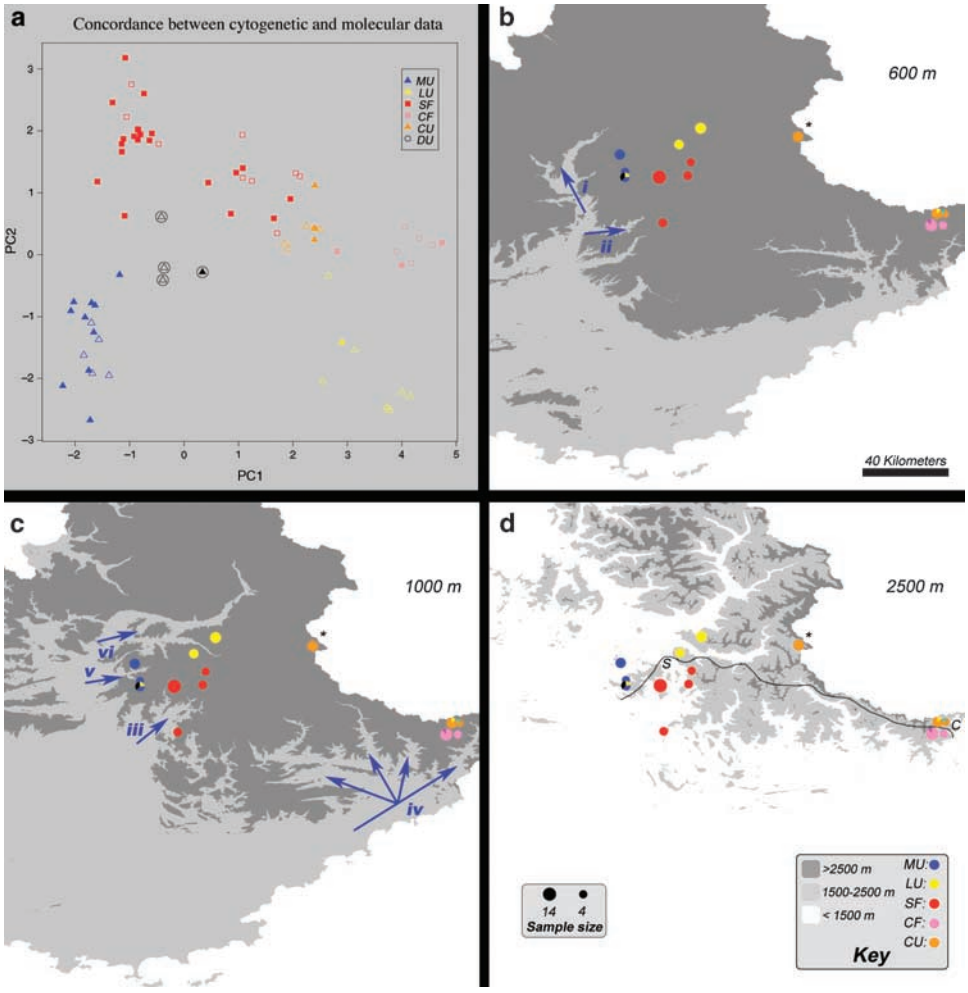

Figure 2 Comparison of the FISH and sequencing data. Panel (**a**) is a principal component plot based on the frequency of rDNA variants within each individual (redrawn from Keller *et al*., 2008). Each point represents an individual grasshopper (triangles, unfused; squares, fused). Filled shapes represent samples for which the new FISH data are also available; their colours indicate the cytogenetic group from Table 1. Circled individuals (in panel a) were collected at the site marked by an asterisk on the maps (Colle della Maddalena), see Discussion. The pie charts on the maps show the proportion of each cytogenetic group. The light grey area represents the habitable altitudinal range. The upper altitudinal limit of the light grey area in each of (**b**–**d**) is given in its top right corner. Panel d shows the current course of the hybrid zone (as determined by Hewitt, 1975) and the towns of Seyne and Casterino (marked S and C). The numbered arrows indicate valleys, which may have been routes of colonisation.

same south-western refugium. If these populations are not derived from the same ancestral population, they must have been closely adjacent. That logic would imply that the dramatic differences between Mélèzes unfused and Lac Noir unfused group either arose during the ascent (in the last 10 000 years) or at least some time in the last climatic cycle, which in turn suggests that rDNA is particularly mobile.

Possible molecular mechanisms causing rapid change

Why should rDNA be mobile, compared with the rest of the genome? Repeated DNA motifs in rDNA units may produce sequence matches to other genomic regions and could promote non-homologous recombination (Castro *et al*., 2001). Such sequence similarities may be particularly frequent in *P. pedestris*, whose large genome (Westerman *et al*., 1987) will include a high proportion of repeated elements and other rapidly evolving sequences, as exemplified by mtDNA pseudogenes (Vaughan *et al*., 1999; Bensasson *et al*., 2000, 2001). For example, the transposition of mobile elements (Sánchez-Gea *et al*., 2000) present in rDNA to new locations could produce matches around the genome. However, phylogenetic analysis of rDNA loci location in 49 grasshopper species argues against this type of explanation; Cabrero and Camacho (2008) argue that a recombination-mediated mechanism would produce greater variation among distal loci than proximal loci, whereas they observe the opposite.

Movement of sequences to novel chromosomal locations most likely by some form of transposition, followed by rapid expansion and contraction of rDNA arrays (Dubcovsky and Dvorak, 1995), could produce the variation observed in *P. pedestris*. Unequal crossing-over (because of mis-alignment of homologous chromosomes in regions of tandem repeats), which has been implicated in concerted evolution of rDNA, would also cause changes in the number of rDNA units within arrays (Eickbush and Eickbush, 2007). However, it is unclear

whether the rate of such changes would be sufficient to explain the variation observed in *P. pedestris*.

Evidence of additional processes that rapidly increase the number of rDNA units has been obtained from model organisms (*Saccharomyces cerevisiae*, *D. melanogaster*). A mechanism may be triggered when the number of rDNA genes falls sufficiently to impair fitness (Procunier and Tartof, 1978; Kobayashi *et al*., 1998; Kobayashi and Ganley, 2005). As well as low-copy number, DNA damage might initiate some form of repair mechanism; Russel and Zomerdijk (2005) have speculated that, as the rDNA polymerase (pol I) is so consistently active in the cell cycle, it might be under close molecular surveillance for stalled transcription. Damage to rDNA might therefore be particularly prone to set off a repair cascade. Whatever the proximate trigger, different genetic elements have been identified which seem to facilitate rapid amplification either in *cis* (Borisjuk *et al*., 2000) or in *trans* (Lohe and Roberts, 1990). This sort of expansion could potentially lead to very rapid changes in rDNA sequence composition within populations. In fact, we have earlier found rare individuals with completely different frequency of rDNA sequence variants from the rest of their cytogenetic group, yet, with no differences in their rDNA loci (Keller *et al*., 2008). As yet, the molecular mechanisms responsible for any of these observations remain unknown.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)