

## A PHYLOGENETIC ANALYSIS OF THE CHANGE IN GENOMIC LOCATION OF THE SEX DETERMINING REGION IN THE GENUS *CHIRONOMUS* (DIPTERA: CHIRONOMIDAE)

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Sex determination in the genus *Chironomus* is a dominant male determining (MD) system, based on a single gene or a small group of genes at a particular chromosomal location. However, that location may change between, or even within, species. The MD location of the 24 species in which it is known has been plotted on a cytological phylogeny to determine whether there are phylogenetically significant trends in the locations observed. A site on arm G is observed in the basal southern hemisphere branches of the tree, then there is a marked difference in the frequency of sites between the species in the southern hemisphere and those of the northern hemisphere. The Australasian members of the pseudothummi-cytocomplex show a predominance of a site near the arm C centromere, while northern hemisphere members of this and the other cyto-complexes seem largely to have a site on arm F. A site on arm A is found in some species in both hemispheres, some of which can be explained by incomplete lineage sorting, but others are most likely due to independent origin. A similar possibility exists in relation to the other more sporadically occurring site on arm B, while the presence of an arm G site in *C. bernensis* of the lacunarius-cyto-complex is most likely an independent occurrence. Further studies, particularly of the species of other southern hemisphere areas, and of North America, are required to strengthen these initial tentative conclusions about the phylogenetic relationships of MD location.

**Key words:** *Chironomus*, polytene chromosomes, sex determination, aberrations, phylogeny.

### Introduction

Sex determination in the genus *Chironomus* is a dominant male determining (MD) system, based on a single gene or a small group of genes at a single chromosomal location. There is a report of female heterogamety in some populations of *Chironomus dilutus* (Thompson, 1971), but this is most likely due to a misinterpretation of the data, due to linked lethal factors confusing the results (Martin, Lee, 1984a), and is not considered here. The nature of the sex determining system was first demonstrated by Beerman (1955) in *C. tentans*, with the unusual feature that the MD was on different chromosome arms in different populations. Subsequently it has been shown that there are a number of different locations that may differ between or within species (e.g. Martin *et al.*, 1980, Table 1), and that the fixation at alternative sites may actually drive speciation, due to assortative mating (Martin *et al.*,

1980; Martin, Lee, 1988) or perhaps by sex ratio distorters (Kozielska *et al.*, 2010).

Two alternative hypotheses have been proposed to account for the change of location of the effective MD region. One is that the MD is associated with a transposable element (Green, 1980) that can insert into a limited number of sites in the genome (Martin, Lee, 2000). The other hypothesis is that the different MD locations are due to mutations of different genes in a sex-determining cascade (Kraemer, Schmidt, 1993). Martin and Lee (2000) postulated that each succeeding mutation should be at an earlier step in the pathway with the consequence that mutation of a gene at a particular site could not reappear later in phylogeny, as its phenotype would be masked by the mutation at the earlier step in the cascade. If the MD is associated with a transposable element, no such phylogenetic restriction need apply. However the recent report by Hediger *et al.* (2010), that the various autosomal

Table 1

List of the currently known sex determiner locations in *Chironomus* species

| Species                          | Arm              | Reference                                     |
|----------------------------------|------------------|---|
| pseudothummi-cytocomplex         |                  |   |
| <i>C. australis</i>              | CD               | Martin, 1971                                  |
| <i>C. cloacalis</i>              | G                | Martin, Lee, 1984b                            |
| <i>C. duplex</i>                 | B                | Martin, 1971; Martin <i>et al.</i> , 1980     |
| <i>C. 'februarius'</i>           | G near nucleolus | Martin, 2010, present study                   |
| <i>C. 'jacksoni'</i>             | CD               | Martin, Lee, 1984b                            |
| <i>C. maddenii</i>               | CD               | Martin, 2010, present study                   |
| <i>C. magnivalva</i>             | BF or CD?        | Martin, 2010, present study                   |
| <i>C. nepeanensis</i>            | G                | Martin, Lee, 1984b                            |
| <i>C. novae-zelandiae</i>        | not on A         | Martin, 2010, present study                   |
| <i>C. occidentalis</i>           | A                | Martin <i>et al.</i> , 1980                   |
| <i>C. oppositus f. connori</i>   | A                | Martin, Lee, 1984b                            |
| <i>C. oppositus f. oppositus</i> | CD               | Martin, Lee, 1984b                            |
| <i>C. oppositus f. tyleri</i>    | G                | Martin <i>et al.</i> , 1980                   |
| <i>C. oppositus f. whitei</i>    | A, CD, F, G      | Martin <i>et al.</i> , 1980                   |
| <i>C. 'pseudoppositus'</i>       | CD               | Martin <i>et al.</i> , 1980                   |
| <i>C. samoensis</i>              | not on CD        | Martin, 2010, present study                   |
| <i>C. tepperi</i>                | A                | Martin, 1981; Martin, Lee, 1984b              |
| <i>C. zealandicus</i>            | C                | Martin, Lee, 1984b                            |
| thummi-cytocomplex               |                  |   |
| <i>C. annularius</i>             | F                | Beermann, 1955; Keyl, 1962                    |
| <i>C. crassicaudatus</i>         | A                | Wuelker, Martin, 1971                         |
| <i>C. nuditarsis</i>             | AB, G            | Rosin, Fischer, 1972                          |
| <i>C. obtusidens</i>             | F                | Keyl, 1961; Keyl, 1962                        |
| <i>C. plumosus</i>               | A, C             | Acton, 1957; Keyl, 1962; Rosin, Fischer, 1972 |
| <i>C. rempeli (anthracinus)*</i> | F                | Rempel <i>et al.</i> , 1962                   |
| <i>C. riparius</i>               | F                | Haegele, 1985                                 |
| <i>C. stigmaterus</i>            | F                | Martin, Wuelker, 1974                         |
| lacunarius-cytocomplex           |                  |   |
| <i>C. bernensis</i>              | F                | Rosin, Fischer, 1972; Wuelker, Kloetzli, 1973 |
| camptochironomus-cytocomplex     |                  |   |
| <i>C. dilutus</i>                | C                | Beermann, 1955; Kiknadze <i>et al.</i> , 1998 |
| <i>C. pallidivittatus</i>        | B, F(?)          | Beermann, 1955; Kiknadze <i>et al.</i> , 1998 |
| <i>C. tentans</i>                | B, F             | Beermann, 1955                                |

\* The MD location was determined from a North American population with the nomen nudum *C. 'rempeli'*. Kiknadze *et al.* (2005) subsequently placed this in *C. anthracinus*, although they have no data on the MD location in any other population.

MD locations in the housefly are regulatory genes that interact to control the expression of the *transformer (tra)* gene, leaves open the possibility that the situation may be a regulatory network rather than a simple cascade. They propose that this may represent the ancestral mode of sex determination in Diptera. In such a regulatory network the phylogenetic restriction could also be removed as different mutations of the same gene may have different dominance relationships with the other genes in the network in regard to their regulation of the *tra* gene. As such, it is not expected that this phylogenetic investigation of MD location will shed any further light on the nature of the sex determination system of *Chironomus*. Rather it will look at the distribution of MD locations in different cytochromes and previous phylogenetic analyses of the genus (e.g. Guryev *et al.*, 2001; Gunderina *et al.*, 2005; Kiknadze *et al.*, 2008), particularly the clusters recognized by Guryev *et al.* (2001).

### Materials and Methods

**MD locations:** Much of the data comes from previously published works, as indicated in Table 1. However, some new data are presented. These come from the examination of the inheritance of rearrangements induced by about 10 Gy gamma radiation (Martin, 1981), or of polymorphisms present in larvae reared from egg masses. Some negative results are also presented where these eliminate certain MD locations. The species examined were *C. 'februarius'* (manuscript name), *C. maddeni*, *C. magnivalva* and *C. novae-zelandiae*.

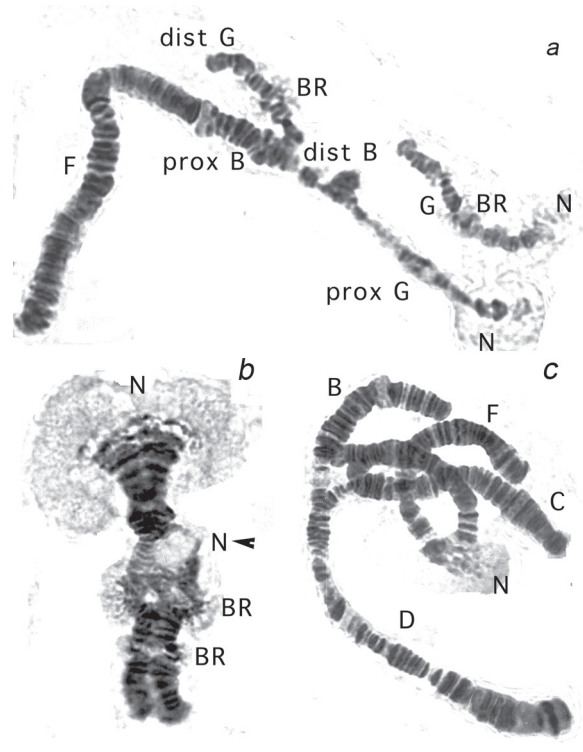
**Phylogenetic analysis:** Ideally DNA sequence would be used for construction of the phylogenetic trees (e.g. Guryev *et al.*, 2001). However, no such data exist for some of the critical species required for this analysis. Therefore a cytological phylogeny is used instead. Even here there is a similar problem in using the more recent '5 arm' analyses (i.e. based on data from five of the seven chromosome arms), such as those of Gunderina *et al.* (2005), since banding sequences for arms C and D are not available for many species. Therefore a 'three arm' tree based on arms A, E and F, as in the cytological analysis of Guryev *et al.* (2001), has been used. In addition to including all those species for which MD location was known, other species are also included to ensure that the tree

also contained representatives from the six clusters recognized by Guryev *et al.* (2001). The forms of *C. oppositus* are entered separately, as the change in MD location is often accompanied by a change of inversion sequence(s). Phylogenetic trees were constructed using Camin-Sokal parsimony (which assumes ancestral states are known) in MIX from the package Phylip 3.68 (Felsenstein 2008). Where necessary, cross-reference to other phylogenetic trees based on DNA sequence or cytology is used to clarify relationships, particularly at the base of the tree.

## Results

### Determination of MD locations

*C. 'februarius'*: Based on irradiation experiments, where sex linkage was observed for translocations involving arms B and G (Fig. 1, a); F and G; and a three-break event creating translocations between A and D, and between A and the distal part



**Fig. 1.** Chromosomal aberrations of *C. 'februarius'* (a and b) and *C. magnivalva* (c), that are inherited in an apparently sex linked manner. a – heterozygous BG translocation, b – heterozygous extra nucleolus in arm G (arrowed), c – heterozygous BD translocation. N – nucleolus, BR – Balbiani Ring.

of G. This latter rearrangement resulted in many aneuploid larvae and showed no clear evidence of sex linkage, thereby indicating that the MD is not in the distal part of arm G. There was also a small pericentric inversion of chromosome BF which showed no evidence of sex linkage, indicating an MD on B would have to be distal, and therefore not compatible with a translocation of F showing sex linkage due to the presence of an MD near the centromere of arm B. Separate evidence comes from larvae reared from an egg mass collected at Kilcoy, Queensland, which carried a small additional nucleolus just proximal of the median Balbiani ring of arm G (Fig. 1, b). This was present in 12 of 13 male larvae, but in only two of the 29 female larvae, suggesting about seven percent recombination between this nucleolus and the MD. All these results are most consistent with an MD near the subterminal nucleolus on arm G.

*C. maddeni*: This species will hybridize with the related species *C. 'pseudoppositus'*, *C. 'jacksoni'* (manuscript name) or *C. oppositus* (Martin, in press). When F1 males from hybridization crosses of *C. maddeni* males to females of the other species were backcrossed to either parental stock, madC1 and madD1 were passed only to the F2 backcross males, while other arms were inherited by both sexes.

*C. magnivalva*: Specimens from Nadi, Fiji were used in irradiation experiments, and a translocation involving the distal half of arm B and the distal two thirds of arm D (Fig. 1, c) appears to show sex linkage. However, due to the high level of infertility (84 %) and abnormal development apparent in many eggs, there were insufficient larvae available for the data to be conclusive.

*C. novae-zelandiae*: Larvae reared from an egg mass where both parents must have been heterozygous for novA1.2, showed clear autosomal inheritance of the alternative sequences. This inversion covers about the middle third of the arm and it is unlikely that an MD anywhere on the arm could appear unlinked to it.

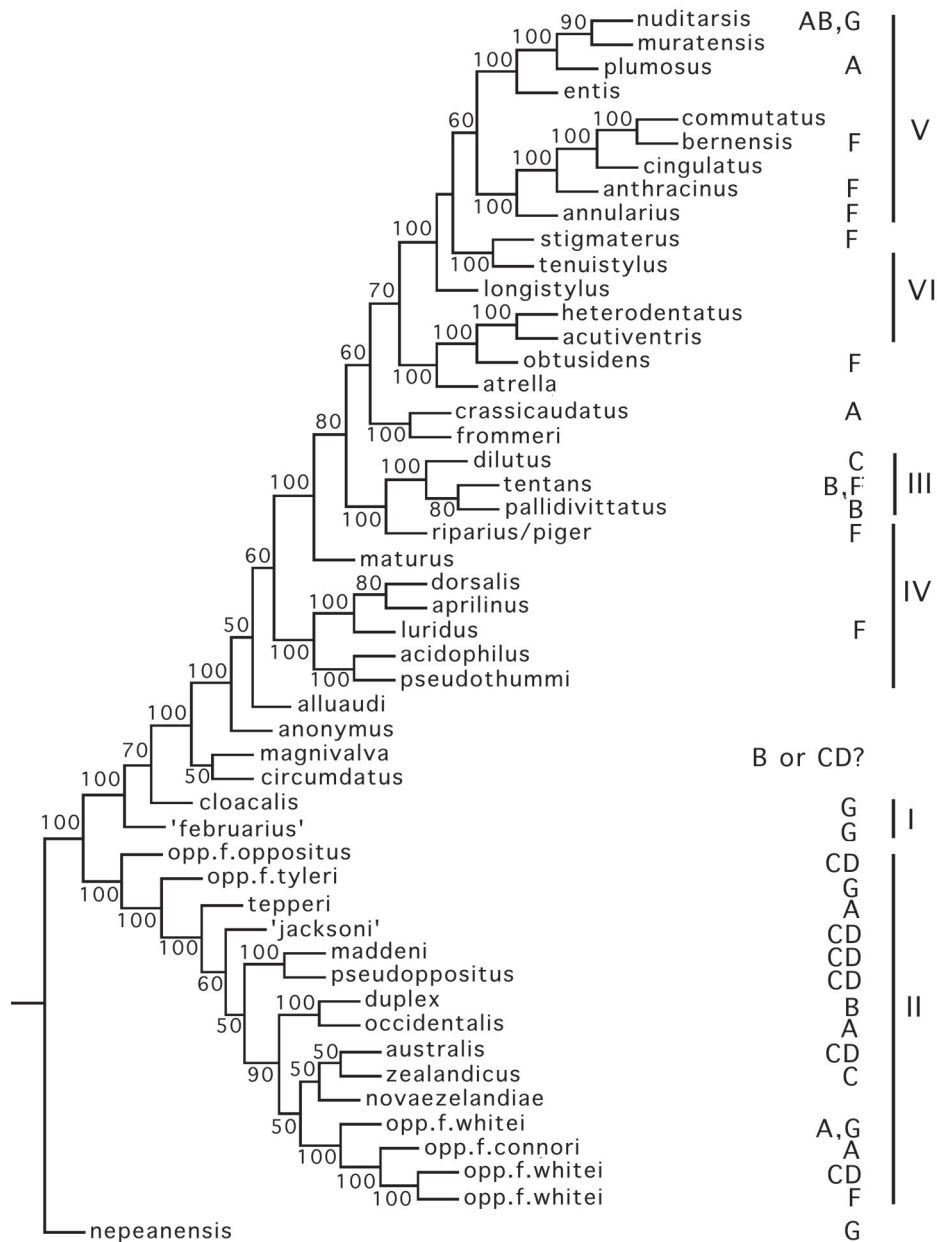
### Phylogeny

Trees were constructed from consensus data of a single MIX analysis (100 trees) and from 200 bootstrapped data sets. The two consensus trees obtained were very similar, but only that from the single run

is presented because its placement of cluster 1 more closely reflects the DNA phylogenies (Fig. 2). The six clusters recognized by Guryev *et al.* (2001) are conserved, and some of the additional species included in the analysis can be placed into those clusters. Thus *C. occidentalis* and *C. novae-zelandiae* fit into cluster II, *C. acidophilus* into cluster IV, *C. nuditarsis* into cluster V, and *C. stigmaterus* and *C. longiventris* into cluster VI. The root species, *C. nepeanensis*, and the two species in cluster I have the MD on arm G. An arm G MD is also found in two of the forms of *C. oppositus* in cluster II, and in *C. bernensis* from cluster V. In cluster II, the most common site is on the CD chromosome (6 species), probably near the centromere of arm C (Martin, Lee, 1984a), then arm A in 3 species, arm B in one species and arm F in one species (assuming that MDs in the different forms of *C. oppositus* are of common origin (see Discussion)). In cluster III, the camp-tochironomus-cytocomplex species, there are three sites represented, arm B in both *C. tentans* and *C. pallidivittatus*, arm F in *C. tentans*, and arm C in *C. dilutus*. The only two species from cluster IV for which the MD location is known, *C. riparius* and *C. luridus*, both have it on arm F. Cluster V appears to be able to be split into two groups, the *C. plumosus*-group species with the MD on either arm A or arm G, and the other species, including the lacunarius-cytocomplex species, where all known cases are on arm F. No MD locations are known for previously recognized members of cluster VI, but *C. obtusidens*, which is included with the cluster VI species in the 'five arm' cytological tree of Gunderina *et al.* (2005), and *C. stigmaterus*, which also appears to belong in this cluster, both have the MD on arm F.

### Discussion

One problem that arises with the cytological phylogeny based on just arms A, E and F that is used here, is that these three arms, more so than arms C, D and probably B, are those upon which Wuelker (1980) based his formulation of basic patterns within *Chironomus*, i.e. those banding sequences that are found in many species across several cyto-complexes. Often the same species carry the basic patterns in all of these three arms and, as the term 'basic' implies, these species tend to be



**Fig. 2.** Consensus mixed parsimony (MIX) tree, of 100 trees found, based on the cytological relationships of arms A, E, and F, and rooted on *C. nepeanensis* to conform to the mitochondrial and globin DNA sequence trees of Guryev *et al.* (2001). MD location is indicated to the right of the tree; I – VI are the groupings recognized by Guryev *et al.* (2001).

near the base of the tree. Consequently the relationships between clusters I to IV tend to be obscured. For example cluster III appears derived from cluster IV in Fig. 2, while in the DNA tree (Guryev *et al.*, 2001), as in the 'five arm cytological tree (allowing for the fact that this is an unrooted tree) (Gunderina *et al.*, 2005), it is more basal and closer to cluster

II, with cluster IV in a more terminal position. The following discussion will therefore use the latter relationships in deriving phylogenetic inferences of MD locations. Wuelker's (1980) basic pattern hypothesis also carries the implication that the basic patterns were spread across the cytochromes in an evolutionary short period, leading to a strong

likelihood that incomplete lineage sorting may play a role in the distribution of MD locations between species and cytochromes.

The results clearly suggest that the MD location in arm G is ancestral, since it is found in the root species, *C. nepeanensis*, and the two representatives of cluster I, the most basal in the DNA phylogenies of Guryev *et al.* (2001). However, there is no solid evidence to suggest that it is ancestral in the wider Chironomidae, largely due to a lack of information on the sex chromosomes of other genera. Sex linkage associated with arm G has been reported for *Polypedilum nubifer*, but this is a female heterogametic species (Martin, 1966; Porter, Martin, 1977), and may therefore be a different gene, or it may be a different mutation of the same gene. In the housefly, the female determinant F has been shown to be the homolog of the *transformer* gene (Hediger *et al.*, 2010), so it will be interesting to see whether this arm G-located gene in chironomids also proves to be the *transformer* homolog. In *Kiefferulus interinctus*, a sister genus to *Chironomus*, the MD is on one of the metacentric chromosomes, not on arm G (Martin, 1962), but it cannot be concluded that this site is ancestral in that genus. In the most primitive chironomid in which sex chromosomes have been identified, *Telmatogeton hirtus* (subfamily Telmatogetoninae), the small heterochromatic chromosome is involved in a complex sex chromosome (Newman, 1977), but it cannot be assumed that this chromosome is homologous to the small chromosome of *Chironomini*, which is not heterochromatic.

It is then parsimonious to assume that the MD on arm G was carried through into cluster II, perhaps due to incomplete lineage sorting, since *C. oppositus* is not the most basal species of the cluster (at least in the DNA phylogenies). The presence of this MD in a small number of populations of form *whitei* can probably be attributed to introgression, since natural hybridization is known to occur between forms (Martin, in press). A similar explanation may also apply to the presence of an arm A location in at least two forms. However, the occurrence of a MD on arm A of two other species of this cluster cannot necessarily be attributed to a single event, as that of *C. occidentalis* is close to the centromere in band group 19 (Martin *et al.*, 1980), while that of *C. tepperi*, which has an identical banding sequences in the region, shows about

eight percent recombination with the centromere and is therefore probably distal of group 19. The arm A MD of *C. oppositus*, however, may be from a common origin with that of *C. tepperi*. The arm B location found in *C. duplex* would appear to be a unique origin within the cluster, although it is not impossible that it has a common origin with that found in cluster III (or in *C. magnivalva* if that species proves to have the MD on arm B, rather than on CD).

More commonly, the MD location of species in cluster II is on the CD chromosome, with all cases compatible with a location on arm C near the centromere. Therefore only a single origin needs to be postulated. The only other proven case of an arm C location, in *C. dilutus* of cluster III, could be from the same event, particularly if *C. magnivalva*, which is intermediate between these two clusters has its MD on this chromosome.

The most noteworthy point about the remaining clusters (as well as some populations of *C. tentans*) is the apparently widespread occurrence of an MD on arm F. This may be a single origin for all these groups, but possibly independent of the single known case in cluster II. The most accurately identified location in clusters III to VI is that of *C. riparius* (= *thummi*), which Haegele (1985) and Kraemer and Schmidt (1993) placed at the distal end near the junction of band groups 1 and 2. In *C. oppositus* f. *whitei*, the arm F MD is associated with a more proximal inversion involving band groups 11–15, but also includes band 2c, which is close to the region identified by Kraemer and Schmidt (1993) in *C. riparius*, i.e. the MD in all cases could potentially be at band 2c.

The remaining MD locations are essentially in the *C. plumosus*-group of cluster V, since little can be concluded for the arm A location in the North American *C. crassicaudatus*, since no DNA sequence is available to confirm its relationships to the other species considered here. Unless further information becomes available to suggest otherwise, it is simplest to assume that the AB location in *C. nuditarsis* is on arm A and homologous to that of *C. plumosus*. These species are phylogenetically removed from those species with an arm A MD in cluster I, so it seems unlikely that incomplete lineage sorting can be the explanation, and a similar situation applies to the arm G location in *C. nuditarsis*. As noted in the introduction, an independent

origin of an arm G location in this terminal branch does not contribute to an understanding of the nature of the MD genes, since it can be explained by either an independent transposition event or a regulatory mutation with different dominance relationships.

It has been noted that the cytologically based phylogeny is biased due to the presence of 'basic' sequences that occur in many species and in different cytochromes. It is therefore pertinent to ask whether this cytological conservation is reflected in the distribution of MD locations. For the 'basic' sequences of arms A and F there is only a single species for each with a known MD, but for arm E there are sufficient to suggest that there is no strong correlation: species with In10b-3f have the MD on arm C (*C. australis*), arm A (*C. tepperi*) and arm F (*C. luridus*), and those with In5-10b have both arm A (*C. plumosus*) and arm F (*C. rempeli*) locations.

The questions raised by this phylogenetically based analysis, clearly indicate the need for further studies to determine the MD location in other *Chironomus* species. Presently there are limited data for North America in the northern hemisphere, and no data for any area of the southern hemisphere besides Australasia. As well, sequencing of the genome of *Chironomus* species will be important for the understanding of phylogenetic relationships. Are we seeing the results of transpositions to a limited number of sites, or to mutations in the genes that regulate the sex determination pathway? Are all sites on the same chromosome arm really the same site or gene? Since it has been suggested that fixation of a new MD location may depend on selective advantage of genes linked to the particular site (Martin *et al.*, 1980), it will be useful to know what genes occur nearby, and if the functions of these genes shed light on whether the different distributions of the arm C and arm F locations between the northern and southern hemisphere reflect adaptation to particular environmental conditions. For example, the much milder winter conditions in Australasia are one obvious difference.

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