Population genetics of plaice (pleuronectes platessa L.) in Northern Europe
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Chapter 7

HETEROPLASMY AND EVIDENCE FOR RECOMBINATION IN THE MITOCHONDRIAL CONTROL REGION OF THE FLATFISH Platichthys flesus

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Abstract
The general assumption that mitochondrial DNA (mtDNA) does not undergo recombination has been challenged recently in invertebrates. Here we present the first direct evidence for recombination in the mtDNA of a vertebrate, the flounder Platichthys flesus. The control region in the mtDNA of this flatfish is characterized by the presence of a VNTR and a high level of heteroplasmy. Two types of repeats were recognized, differing by two C/T point mutations. Most individuals carry a pure “C” or a pure “T” array, but one individual showed a compound “CT” array. Such a compound array is evidence for recombination in the mtDNA control region from the flounder.
Introduction

It has generally been assumed that animal mitochondrial DNA (mtDNA) does not undergo recombination. This assumption has come from indirect evidence such as failure to detect excision repair activity and crossover products (Clayton, Doda and Friedberg 1975), failure to detect mtDNA recombination in interspecific somatic cell hybrids (Zuckerman et al. 1984), clustering of mtDNA molecules preventing physical contact (Satoh and Kuriowa 1991), and high mutation rate (Howell 1997). However, two recent studies have directly demonstrated mtDNA recombination in two invertebrates, a nematode *Meloidogyne javanica* (Lunt and Hyman 1997) and the mussel *Mytilus galloprovincialis* (Ladoukakis and Zouros 2001). With respect to vertebrates, only indirect evidence for mtDNA recombination has been found in the form of high frequencies of homoplasies (Eyre-Walker, Smith and Maynard Smith 1999) and lower levels of linkage disequilibrium between more distance polymorphic sites (Awadalla, Eyre-Walker and Maynard Smith 1999).

Here, we provide the first direct evidence of recombination in the mtDNA of a vertebrate, the flounder *Platichthys flesus* (Teleostei : Pleuronectiformes [flatfish]). In flounder, as in many other fishes (Nesbo, Arab and Jakobsen 1998, Ludwig et al. 2000), the 5’-end of the mitochondrial control region is characterized by the presence of a Variable Number of Tandem Repeats (VNTRs) and a high level of length heteroplasmy for this region. The cause of variation in the repeat number is not fully understood but is variously attributed to slipped strand mispairing, illegitimate elongation and termination associated sequence (TAS) -based replication (Ludwig et al. 2000). It may also be caused by mtDNA recombination, although this is more difficult to demonstrate when VNTRs consist of perfect repeats.

Material and Methods

A total of 168 individuals of flounder collected from 5 locations in the northern Atlantic and the Mediterranean were used to analyze the variation of the VNTRs and the extent of heteroplasmy (Table 1). Total genomic DNA was extracted using a CTAB protocol (Hoarau et al 2002). PCR amplification of the 5’-end of the mitochondrial control region was carried out in 10 µl volumes containing 1 µl of
1/10 diluted DNA, 1X reaction buffer, 0.2 mM of each dNTP, 2 mM MgCl₂, 0.25 U Taq DNA polymerase (Promega) and 0.5 µM of each of the following primers, DLF (5’-CCA CCT CTA ACT CCC AAA GC-3’) (modified from A, Lee et al. 1995) and DLR3 (5’-GGT TAT TAT ACA TGA ATG TCC T-3’). For fragment analysis, primer DLF was fluorescently labeled with the 6-FAM (Carboxyfluorescein) dye label (Applied Biosystem). Amplifications were performed in either a PTC100 (MJ Research) or a Mastercycler gradient cycler (Eppendorf). The reaction profile was as follows: initial denaturation at 94 °C for 3 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Length variation was visualized on an ABI Prism-377 automatic sequencer (Applied Biosystems) using a 4.5 % denaturing polyacrylamide gel with an internal lane standard and Genescan™ software for determination of the fragment size. PCR products from 18 individuals were ligated into pGEM-T Easy plasmid (Promega) and cloned in Escherichia coli JM109 (Promega) according to the manufacturer’s instructions. PCR amplification of 6 to 48 clones per individual (288 clones in total) was performed as described above and 3-6 clones per individual (84 clones in total) were sequenced using a BigDye Cycle Sequencing kit (Applied Biosystem) and an ABI Prism-377 automatic sequencer (Applied Biosystems). Negative controls were used at every step of the experiment.

Results and Discussion

Nearly all (165/168) individuals were heteroplasmic (Table 1), containing 3 to 9 length variants per individual.

Table 1. Heteroplasmy in Platichthys flesus. N: number of individuals screened for length polymorphism, H: % of individuals showing heteroplasmy, N₁C: number of individuals cloned and sequenced, N₂C: total number of clones sequenced.

<table>
<thead>
<tr>
<th>Location</th>
<th>N</th>
<th>H</th>
<th>N₁C</th>
<th>N₂C</th>
<th>Array type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacares (France)</td>
<td>2</td>
<td>50</td>
<td>2</td>
<td>6</td>
<td>C</td>
</tr>
<tr>
<td>Bay of Biscay (France)</td>
<td>10</td>
<td>100</td>
<td>4</td>
<td>14</td>
<td>C</td>
</tr>
<tr>
<td>Terschellinger Bank (The Netherlands)</td>
<td>49</td>
<td>100</td>
<td>4</td>
<td>32</td>
<td>C, T, CT</td>
</tr>
<tr>
<td>Dollard Estuary (The Netherlands)</td>
<td>73</td>
<td>100</td>
<td>4</td>
<td>16</td>
<td>C, T</td>
</tr>
<tr>
<td>Lofoten (Norway)</td>
<td>34</td>
<td>97</td>
<td>4</td>
<td>16</td>
<td>C, T</td>
</tr>
</tbody>
</table>
The basic repeat unit is 19 bp long and the number of repeats ranges from 1 to 10. From the total set of 168 individuals, 18 individuals were chosen for further characterization. First, PCR products were cloned from these individuals and between 6 and 48 clones/individual (total of 288) were then reamplified and run on a gel to confirm that only one band was present as expected from individual clones. This was an important check because PCR artifacts (resulting in multiple bands) associated with these types of repeat motifs have been reported by Campbell et al. (2001). We found single sharp bands in 274 clones, whereas 14 amplifications produced double bands. Products involving more than two bands were never observed. Thus, although artifacts accounted for 4.8% of the observations, the high level of heteroplasmy observed is real though perhaps slightly overestimated.

Again from the same set of 18 individuals, 3-6 clones per individual were sequenced (total of 84) in order to characterize the array motifs themselves and their number. Two types of core repeats were detected, a “C” type and a “T” type, which differed by two point mutations (Fig. 1). Among the 18 individuals, 13 contained only the pure “C” type array, 4 only the pure “T” type array and one individual a compound array of “C” and “T” (Fig. 1, A, B and C respectively). The compound array is indicative of mtDNA recombination. It is highly unlikely that the compound array arose by mutation as the differences between the “C” type and the “T” type involve two independent mutations and neither seems to be associated with secondary structure such as hairpin (no palindrome in the sequence of the repeat unit). Moreover these two positions are highly conserved in the closely related species, Pleuronectes platessa (Hoarau G. unpublished data).

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**Fig. 1.** Alignment of the VNTR of mtDNA control region in *Platichthys flesus.* □ C type repeat, □ T type repeat. The two point mutations are boldface. Ind1 shows a pure “C” type array, Ind2 a pure “T” type array and Ind3 a “CT” compound array. GenBank AY143162 to AY143164.
Once again, however, the issue of possible PCR artifacts arises because Taq polymerase can produce chimeric DNA molecules (i.e., PCR jumping *sensu* Paabo, Irvin and Wilson 1990) when damaged fragments of a mixed template of DNAs are joined during the amplification process. Mixed templates of undamaged fragments do not result in PCR jumping (Ladoukakis and Zouros 2001). In order to check for this possibility, equal amounts of DNA from pure “C” and pure “T” individuals were mixed in the same PCR reaction in an effort to create a chimeric product. The PCR product was cloned and 16 clones were sequenced. Ten were pure “C” and 6 were pure “T”. There were no “CT” arrays detected. Thus a PCR artifact is unlikely.

Further support for true recombination comes from the fact that the recombinant “CT” array was found in each of 3 independent DNA extractions, PCR and sequencing reactions from the recombinant individual. In addition, the entire DNA extraction, amplification, cloning and sequencing procedure was conducted in an independent laboratory where no studies on fish have ever been conducted. Twelve clones were sequenced and recombinant “CT” arrays were found in 7 clones and a parental “T” was found in the 5 other clones (Table 2). Interestingly, only one of the “parental” types (“T”) is found together with recombinant arrays. This suggests that recombination took place at least a generation ago and that the other “parental” type (“C”) was lost by random drift during ontogenesis or gametogenesis (Chinnery *et al.* 2000). Variation in the number of “C” and/or “T” repeats in recombinant arrays further suggests that other mechanisms, such as slipped strand mispairing, besides (or in addition to) recombination are involved in the evolution of the number of repeats.

<table>
<thead>
<tr>
<th>Array</th>
<th>Number of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-T-T</td>
<td>5</td>
</tr>
<tr>
<td>C-T-T-T</td>
<td>1</td>
</tr>
<tr>
<td>C-C-T-T</td>
<td>2</td>
</tr>
<tr>
<td>C-C-T-T-T-T</td>
<td>2</td>
</tr>
<tr>
<td>C-C-C-T-T-T</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. Type of repeat arrays found in the recombinant individual.
Recombination can explain the evolution of mtDNA VNTRs, but it is usually difficult to trace when VNTRs exist as perfect arrays. Because flounder VNTRs are imperfect and the two different types of repeat unit are found, the demonstration of mtDNA recombination is straightforward.

In principle, an alternative hypothesis to recombination could be that the compound array is the ancestral form and that the derived type of array we have observed evolved by a series of duplication-and-loss events. In this scenario, the two mutations (i.e., switching between the C to T type and vice versa) occurred in a duplicated region, such that both it and the ancestral form would be retained in the same individual. The different number of basic repeats could then be accounted for by a history of duplication and loss. Given the range in the number of repeat regions found (1-10), such duplications and losses might occur fairly regularly. However, we do not favor this alternative hypothesis because: 1) If the “CT” arrays are indeed the ancestral form, then they should have been found more frequently in *P. flesus* and in closely related flatfish such as *Pleuronectes platessa* (=plaice) - plaice contains only one repeat, suggesting that the ancestral form was one repeat; and 2) this hypothesis requires that the two mutations be retained in the same individual, whereas the recombination scenario requires only that the two types be present in the same population.

The mtDNA recombination we found between “C” and “T” type arrays in flounder has several implications for vertebrate mtDNA in general. Before such recombination is possible, the “C” and “T” type mitochondria must be present in the same cell and their DNA must be coupled. This implies paternal leakage followed by fusion of the mitochondria. Paternal leakage has been reported for mice (Gyllensten *et al.* 1991) and anchovies (Magoulas and Zouros 1993), whereas fusion of mitochondria has been demonstrated in *Drosophila* (Yaffe 1999), and the enzymes necessary for recombination have been found in human mitochondria (Thyagarajan, Padua and Campbell 1996). It therefore appears that all of these properties are present in flounder and in vertebrates more generally.

The importance of recombination in vertebrate mitochondria has broad implications across several fields, ranging from human mitochondrial diseases (Schon 2000) to the compromise of phylogenetic and population studies that assume strict clonal inheritance of mtDNA (Schierup and Hein 2000). In the case of human mitochondrial diseases, mtDNA recombination will greatly change the mode and
patterns of inheritance, which in turn may affect current diagnostic methods. Recombination can also affect the accuracy of phylogenetic reconstruction (Posada and Crandall 2002), inferences related to demographic history, and the application of molecular clocks (Schierup and Hein 2000).

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References
