AN APPROACH TO THE APPLICATION OF MOLECULAR BIOLOGICAL METHODS TO SOLVE TAXONOMICAL AND PHYLOGENETIC PROBLEMS OF THE EPHEMEROPTERA

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ABSTRACT

DNA restriction fragment analysis (restriction fragment length polymorphism, RFLP) has been tested for Ephemeroptera. Single specimens of *Ephemerella ignita* (Poda) of a given population present an identical ribosomal DNA (rDNA) fragment pattern. The rDNA cistron length amounts to about 14,4 kb (kilo basepairs). Comparisons of the DNA restriction fragment pattern originating from DNA of geographically isolated populations present a relationship between the geographic distance and genetic variation. The rDNA cistron length of *Ecdyonurus venosus* (F.) and *Ecdyonurus forcipula* (Pict.) have different sizes of 16.6 kb and 14.4 kb, respectively. These two species have been distinguished by their different ribosomal and mitochondrial DNA restriction fragment pattern.

INTRODUCTION

An important application of the molecular biological techniques is molecular diagnostics, which use the detection and qualification of specific DNA sequences by nucleic acid hybridization procedures. These techniques are very sensitive, and specific DNA hybridization probes can be applied for detecting and distinguishing different target organisms. Problems of inheritance have been solved by using the so-called DNA-fingerprinting (Jeffrey *et al.* 1985, Burke *et al.* 1989). Variation among populations has been demonstrated (Ferris *et al.* 1982, Hale and Singh 1986, Hall 1986, 1988, Wetton *et al.* 1987, Rand and Harrisson, 1989); complexes of species have been analyzed (Avise and Nelson, 1987, Finnerty and Collins, 1988); investigation at the species level have been executed (Powers *et al.* 1986, Piessens and McReynolds 1987, Burke *et al.* 1989) and phyogenic relationships on the DNA-level have been established (Ferris *et al.* 1981, Diamond 1989). Restriction fragment length polymorphism (RFLP) analysis will contribute further information supplementary to morphological and ecological data.

We focused our attention, in this preliminary study, on ribosomal DNA (rDNA) gene sequences which are present in several copies in the nuclear genome. We also have investigated mitochondrial DNA sequences (mDNA), which are situated on a relatively small extranuclear genome, usually highly amplified in eukaryotic cells. The purpose of the current study was to compare the pattern of genetic differentiation in the rDNA multigene family and mDNA sequences in individuals, in different populations as well as at the species level.

MATERIALS AND METHODS

Biological material

We collected the different Ephemeroptera throughout Europe in flight or reared the larvae to the adult stage. The specimens were frozen and stored at -70° C.

DNA isolation

Whole specimens were homogenized in a buffer comprised of 10mM Tris-HCl, pH 7.5, 10mM EDTA, 50mM NaCl (100ul buffer solution/specimen). The homogenate was brought to 1% sodium dodecyl sulfate (SDS) and was incubated first for about 10 minutes at 65° C followed by 2 hours at 37° C with 100mg/ml protease K. Sodium acetate (pH 6.6) was added to a final concentration of 300mM and the lysate was extracted, first with phenol then with chlorophorm/isoamylalcohol (24:1). The DNA then was concentrated by ethanol precipitation. After washing of the DNA pellet twice with cold 70% ethanol, the DNA was dissolved in a buffer of 10mM Tris-HCl, pH 7.6, 1mM EDTA. Treatment with RNase (10mg/ml) and α-amylase (50mg/ml) was carried out for 1 hour at 37°C. After incubation with protease K (10mg/ml), the DNA was extracted as described above.

DNA digestion, blotting and hybridization

All restriction enzyme (DNA endonucleases) digestions were performed according to manufacturer instructions (Boehringer Mannheim). The DNA restriction fragments were separated on 1% agarose gels (3 to 10 µg DNA per lane). Electrophoresis was conducted in 89mM Tris-borate, 89mM boric acid, 2mM EDTA buffer solution, pH 8.0 and run for 12 hours at 35V. DNA was stained by ethidium bromide (0,5µg/ml), visualized and photographed on an ultraviolet transilluminator. The DNA fragments separated in agarose gels were transferred to nitrocellulose filters following standard protocols (Southern, 1975). Cloned mitochondrial and ribosomal DNA sequences were labelled with ^{32}P in vitro by nick translation according to Rigby *et al.* (1977). After a prehybridization of the filters for at least 2 hours at 37° C in 43% formamide as described in Maniatis *et al.* (1982), the DNA-DNA hybridization

with heat-denatured labelled DNA probe was carried out in the same solution at 37° C for 24 hours. The hybridized filters were washed three times in 2xSSC (300mM NaCl, 30mM sodium citrate), 0,1% SDS for 5 minutes at room temperature, twice for 20 minutes at 50° C and then exposed to X-ray films for 24 to 72 hours at -70° C, using an intensifying screen. The same filter can be used for hybridization with another labelled DNA probe after washing the filter 10 minutes at 100° C in distilled water (dehybridization).

RESULTS

A general representation of the method is given in Fig. 1. A DNA restriction enzyme cuts the isolated DNA molecules at specific sites (e.g. recognition sequence of BamH1 are the six nucleotides GGATCC) in fragments of various lengths. The restriction enzymes recognizing only four nucleotides (*e.g.* AluI: AGCT) cut the DNA much more frequently and therefore the mean DNA fragment length decreases. The size of DNA fragments can be estimated by comparison with known marker DNA fragments (*e.g.* λ^+/HindIII-, pBR322/HinfI-fragments). Repetitive DNA seqences in the genome which are cut by a DNA restriction enzyme produce a lot of fragments of the same length. They move at the same place on the gel during the electrophoresis forming a visible band. *Ephemerella ignita* DNA restricted with AluI shows, apart from the smear, a strong band corresponding to a 320 bp (base pairs) long DNA fragment (Fig. 1), indicated by the arrow. This DNA sequence is present in a high copy number (several thousands) in the genome. The fractionated DNA fragments on the gels are transferred on a filter membrane for the immobilization of the DNA molecules. Radio-active labelled DNA sequences associate with homologous DNA sequences on the filter (hybridization). The signals on the X-ray photographs show the presence of the tested DNA sequences and the resulting DNA fragment pattern.

Hybridization with ribosomal DNA sequences (rDNA) and mitochondrial DNA sequences (mDNA)

E. ignita DNA isolated from a population of the Gérine river (CH) was digested with different restriction enzymes and hybridized with a labelled rDNA probe. Fig. 2 shows the fragments of the rDNA containing DNA fragments. The BamHI enzyme cut once in the rDNA cistron and created two equal DNA fragments of about 7.2 kb (kilo basepairs) in length.

The HindIII restriction enzyme generated hybridizing fragments of 4.1, 3.7, 2.5, 2.45 and 1.5 kb in length. The sizes of the EcoRI fragments are about 5.2, 3.0 and 3.1 kb, those of the ClaI fragments 9.4 and two fragments near 2.5 kb. The estimated length of the rDNA cistron in *E. ignita* calculated by these data amounts to about 14,4 kb.

Fig. 3 presents a southern blot of E. ignita DNA digested with HindIII and hybridized with a rDNA probe. The DNA put in lane **a** to **h** came from single individuals. One *E. ignita* male (body weigth 2,94mg) provides about 4,75µg

DNA. The hybridization pattern of HindIII fragments clearly demonstrate an identical fragment pattern for all individuals. The DNA from the whole population (pop 2) shows the same pattern as the individuals as well as another population (pop 1) of the same river. The hybridization of the same filter with a mitochondrial DNA probe produced an identical pattern for the individuals as well as for the whole population (data not shown).

The comparison of geographically separated populations of *E. ignita* is represented in Fig. 4. Populations coming from close geographic areas (Corsica: Fango and Tavignano (F)/ Central Europe: Gérine (CH), Rhine (FRG), Biber (FRG), Wilfin Beck (GB)/ Sweden: Storan and Vindelalven) have a similar hybridization pattern within the representatives of each of those regions. DNA of populations localised in distant geographic areas (Corsica/ Central Europe/ Sweden/ Iberian Peninsula: Eresma (E)/ Finnland near Russia: Oulankajokki) show a slightly different hybridization pattern, but all the divers populations possess at least one rDNA fragment of the same length demonstrating a degree of homology between the populations.

Ecdyonurus venosus (F.) and *Ecdyonurus forcipula* (PICT.) have no clearly distinguishable morphological characters. DNA of these closely related species were hybridized with a rDNA probe (Fig 5A). The estimated length of the *E. venosus* rDNA cistron amounts to about 16.6 kb (HindIII fragments of about 13.5, 1.8 and 1.25 kb, EcoRI fragments are 7.5, 6.2 and 3.0 kb in size). The rDNA cistron of *E. forcipula* is shorter, 14,4 kb in length. The lengths of the hybridized HindIII fragments are 8.2, 3.1, 1.8, 1.3 and the EcoRI fragments 7.2, 3.75, 3.05 and 0.4 kb (not visible on this blot), respectively. These species have , in addition to the different rDNA cistron lengths, rDNA fragment patterns for various endonucleases which also are very distinct (Fig. 5A). These results confirm that these specimens belong to different species. After dehybridization, the same filter was hybridized with mDNA sequences (Fig. 5B). The approximate length of the mDNA, estimated on the basis of DNA restriction fragment analysis, amounts to about 21 kb for both species. The comparison of the mDNA fragment pattern for these species revealed only a few common bands (DNA fragments of the same length) for the DNA restricted with the same endonuclease (only one in the EcoRI and MspI lanes, respectively). These results also show that these specimens belong to different taxa.

DISCUSSION

We present, in this study, information on the isolation technique and restriction fragment length analysis of Ephemeropteran DNA.

To facilitate the detection of DNA fragments differentiable from a single specimen we used rDNA and mDNA sequences which are reiterated in the genome. There are other repetitive DNA sequences in the genome such as the 320 bp long DNA fragment in *E. ignita* generated by AluI digestion (Fig. 1). Such a DNA fragment can be cloned and used as a DNA hybridization probe (actually carried out in our laboratories).

The sizes of ribosomal gene cistrons in different Ephemeroptera species, as estimated with endonuclease restriction products, are approximately 14 to 17 kb. These data agree well with the size range found for other insects (Beckingham, 1982). The estimated lengths of the mitochondrial DNA (21 kb) is also in the range found for other species (Moritz *et al.* 1987).

The constant restriction enzyme pattern within an *E. ignita* population, tested with rDNA and mDNA probes, indicates genetic homogenity between the members of a population. The investigation of rDNA sequences on different populations of *E. ignita* shows a relationship between geographic distance and genetic variation. The divers populations possess at least one common DNA fragment demonstrating a degree of homology between populations of a species. The differencies are probably provided by point mutations in the nontranscribed spacer region of the rDNA cistron, the DNA sequences of which present a higher genetic variation (Rae *et al.* 1982, Beckingham, 1982). These observations demonstrate the high sensitivity of the RFLP-analysis. Similar observations have been recorded for populations of *Aedes albopictus* (Skuse) by Black *et al.* (1989).

The similarities in morphology make species differentiation between *Ecdyonurus venosus* and *E. forcipula* difficult. Based on two different alleles in isoenzyme analysis, Zurrwerra *et al.* (1987) showed that these two species are genetically divergent. The different length of the rDNA cistron as well as the different rDNA and mDNA fragment pattern recorded in this study indicate the existence of two species. The DNA restriction fragment length polymorphism method confirms earlier observations cited above. Upholt (1977) as well as Nei and Li (1979) published a mathematical approach to express the inheritance of species comparing common and non-common DNA restriction fragments. The banding pattern data in this preliminary study do not allow a statistical calculation of the relationship of these two species. The number of DNA fragments to be compared on hybridization patterns should be increased for a statistical treatment by variation of the endonucleases as well as by the application of additional DNA hybridization probes. The choice of the DNA probe defines the sensitivity of the applied method. The analysis of mDNA sequences reflects a higher variation in the DNA fragment pattern compared to enzyme coding or ribosomal gene sequences (Brown *et al.* 1979). We will use conservative gene DNA sequences to test the relationship on a higher taxonomic level. This technique will yield data additional to morphological and ecological characters to clarify systematic positions.

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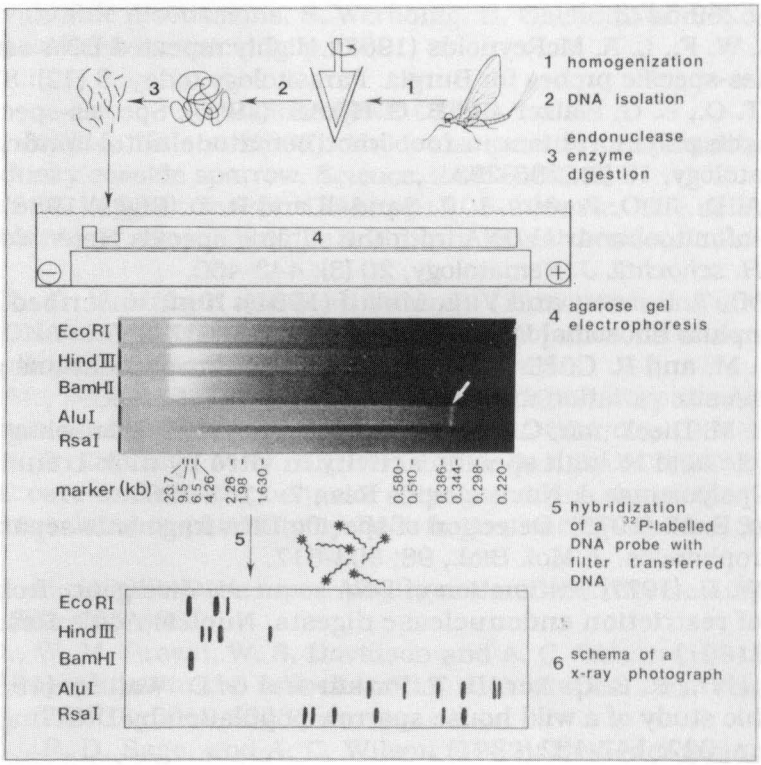

Fig.1: Schematic representation of DNA restriction fragment analysis. Specimens were homogenized (1) and the DNA extracted (2) as described in Materials and Methods. After fragmentation of the DNA by endonuclease enzymes (3), the generated DNA fragments were separated according to their length by electrophoresis on agarose gel (4). The length size was estimated by comparison with length marker fragments (λ^+/HindIII, pBR322/HinfI). The arrow in lane AluI (E. ignita DNA digested with AluI) at 0.320 kb (kilo basepairs) indicate a visible band containing many DNA fragments of the same length. The DNA transferred on a nitrocellulose filter (Southern blot) was hybridized with a labelled DNA probe (5). The homologous DNA sequences on the filter and the labelled DNA probe sequences associate. The X-ray photograph show the presence and localization of the probe DNA sequences (6).

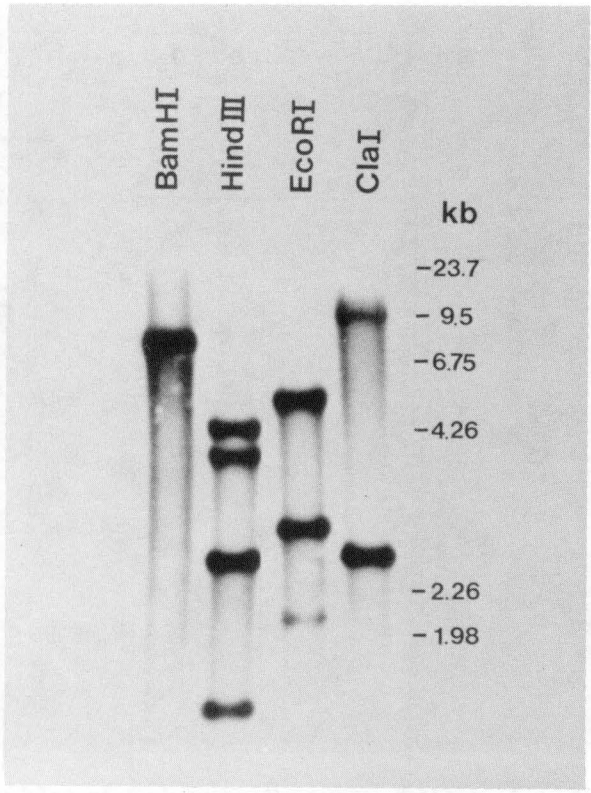

Fig. 2: Southern blot of *Ephemerella ignita* DNA digested with four different restriction endonucleases and hybridized to a ribosomal DNA probe. λ^+/HindIII fragments were used as length standards (kb = kilo basepairs).

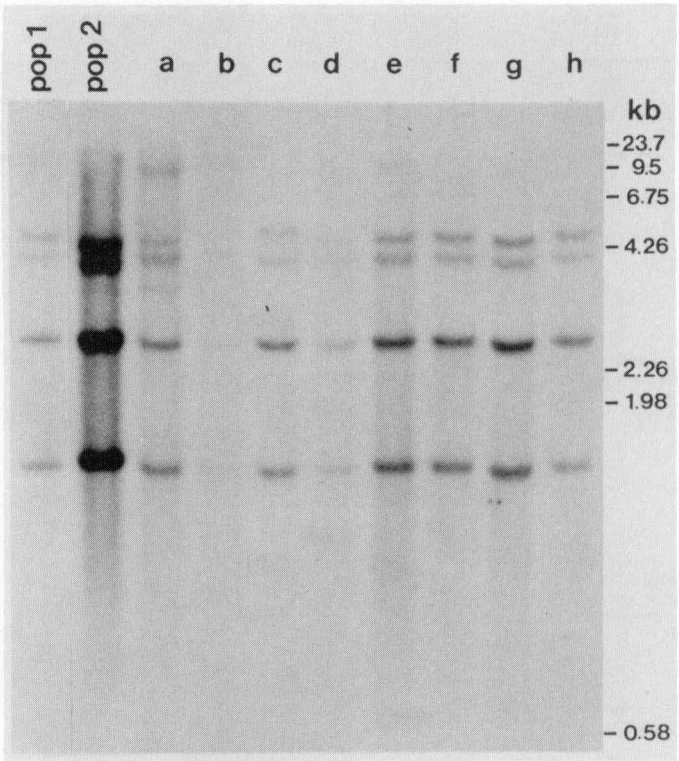

Fig. 3: Southern blot of *Ephemerella ignita* DNA digested with HindIII and hybridized to a rDNA probe. The samples in each lane were the following: lanes pop1, pop2 represented DNA of two populations. Lane **a** to **h** were samples of single individuals. λ^+/HindIII fragments were used as length standards.

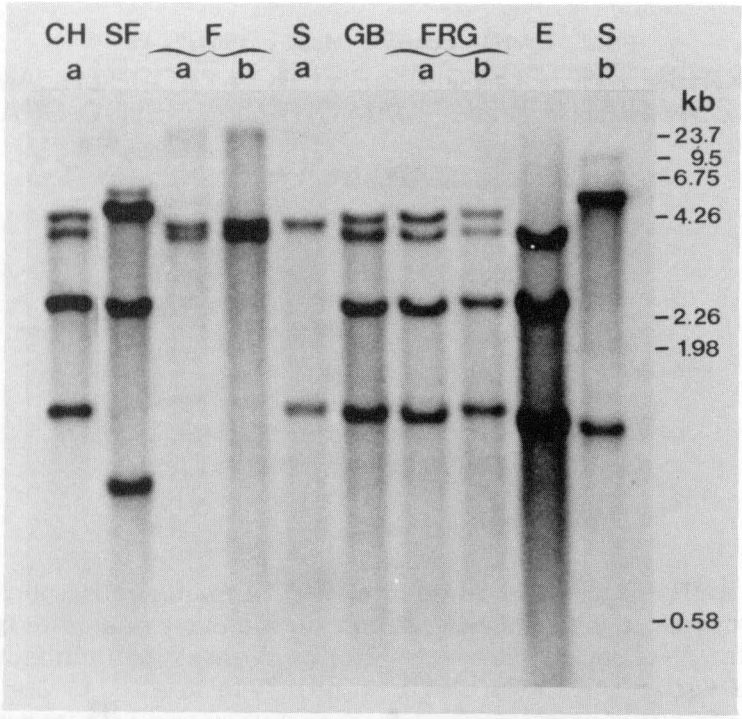

Fig. 4: Southern blot of *Ephemerella ignita* DNA isolated from different populations digested with HindIII and hybridized to a rDNA probe. The tested samples were: CH a: Gérine, Marly/ SF: Oulankajokki, Kuusamo/ F a: Fango, Galeria; F b: Tavignano, Corte/ S a: Storan, Idre/ GB: Wilfin Beck, Windermere/ FRG a: Biber, Tiengen; b: Rhine, Rheinweiler/ E: Eresma, Valsain/ S b: Vindelalven, Vindeln. λ^+/HindIII fragments were used as length standards.

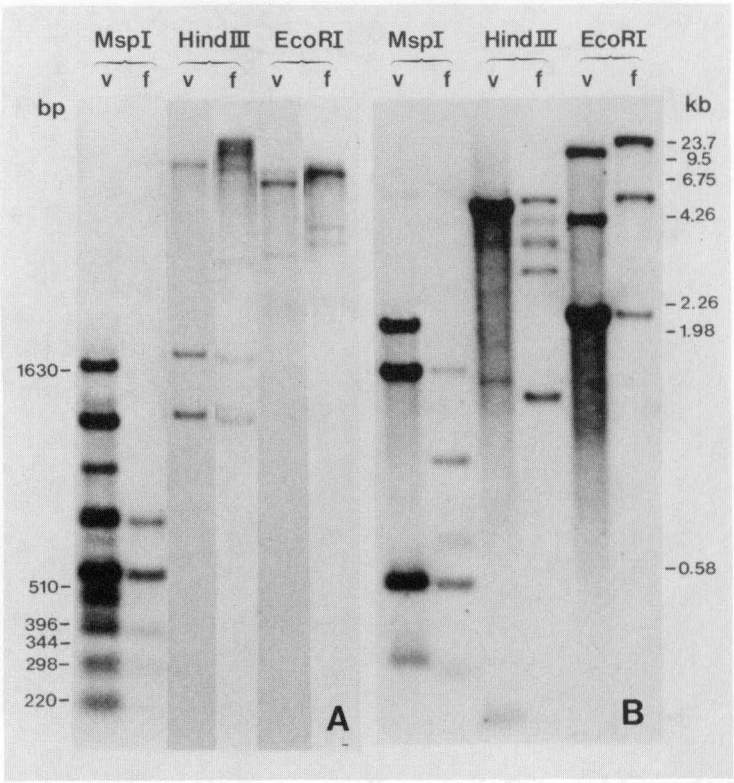

Fig. 5: Southern blot of *Ecdyonurus venosus* (v) and *Ecdyonurus forcipula* (f) DNA digested with three different endonucleases were subsequently hybridized to a rDNA probe (5A) and a mDNA probe (5B). λ^+/HindIII fragments were used as length standards (in kb) and pBR322/HinfI (in bp).