CHAPTER II

HIGH RESOLUTION THERMAL DENATURATION ANALYSIS OF DNAs
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SUMMARY

Thermal denaturation studies of DNAs of four Cucurbitaceae plant species have been carried out to determine the base composition, hyperchromicity and transition width of melting. The melting temperatures of the total, unsheared DNAs fall in the range of 83.2 to 85.7°C. The G+C contents calculated from the melting temperatures range from 34 to 40%, the lowest being in ivy gourd and the highest being in ridge gourd. The transition widths of 6 to 8°C represent a moderate sequence divergence in the four species. The high resolution thermal denaturation profiles have shown species-specific differences. About 12 out of 17 to 22 melting components are common to all the species, when a range of 1.5% G+C is considered for comparison. The two Luffa species show very similar first derivative melting profiles and hence may have a significant DNA sequence homology. The profiles of Luffa species are, however, very distinct from those of ash gourd and ivy gourd.

INTRODUCTION

Melting of DNA indicates the separation of the two component strands which are initially held together by hydrogen bonds and hydrophobic interactions. The DNA melting
profile in general is a two dimensional display obtained by plotting a change in some measurable property of DNA like alterations in viscosity, optical rotation, buoyant density, light scattering and extinction coefficient, against an external variable responsible for melting of DNA, such as temperature, pH, ionic strength, organic solvents and concentration of ligand molecules. The DNA melting profile is primarily characterized by the following parameters: (i) The melting temperature (Tm), which is defined as the temperature corresponding to the midpoint of the melting curve (1); (ii) the sharpness or breadth of melting, ΔT


or δ 2/3, defined as the difference between the temperatures of 'a' (17%) and 'b' (83%) melting (2,3); (iii) hyperchromicity which is expressed in terms of percent increase in absorbance after complete denaturation. Under constant conditions of ionic strength and pH, a linear relationship exists between the melting temperature and the base composition of DNA expressed in terms of G+C.

The most popular method for studying the melting of DNA is to monitor the change in ultra violet absorbance caused by elevating the temperature of DNA solution. This method is simple and reliable, and environmental conditions can be set with precision. The UV absorbance by nucleotide bases in the wavelength region around 260 nm is due to the π-π* electronic transition in purine and pyrimidine bases.
The intensity of absorbance increases by about 40% when the hydrogen bonds between the bases are disrupted. A linear relationship exists between this hyperchromicity and the extent of disruption of base pairs.

If a given DNA molecule contains regions within it which are markedly different in G+C content from the average G+C content of the whole molecule, then such regions will have different Tms from the rest of the molecule resulting in a heterogeneous denaturation behaviour. Such heterogeneity is seldom visible in the simple denaturation profiles, but can be readily detected by plotting "derived" melting curves, where the ratio of hyperchromicity over a small rise in temperature (e.g. 0.1°C) to the total hyperchromicity is plotted against temperature. Such studies have revealed an ordered and non-random distribution of base sequences in most organisms. Three kinds of derivative melting profiles are generally recognized: (i) Polyphasic melting of short, homogeneous viral DNAs consisting of unique sequences (4-8); (ii) smooth, monophasic melting of bacterial and large viral DNAs (8,9) and (iii) broad, polyphasic melting of higher eukaryotes (8-12). The peaks which appear distinctly in the melting of DNAs of higher organisms are not the products of internal base sequence heterogeneity, but may come from some special families such as satellite DNA sequences or some other
regions specific to functions of higher organisms, and at least in part from repeated sequences (8-13). The fine melting technique can be applied to establish a way in which the DNA double helix is constructed and to relate it with its biological function to find the genetic and evolutionary origin of this nonrandom sequence arrangement and also in recombination and gene manipulation technology (14).

Since the melting profiles provide a histogram of the local G+C content arranged over several hundred base pairs, it provides a convenient method to examine the homology or to explore the taxonomic relationships among DNAs, like deletion mutants of lambda phage (15), kinetoplast DNAs of hemoflagellates (16), and a series of related bacteriophages of *Bacillus subtilis* (4). Species- and family-specific melting curves have been obtained in some higher plants (12). This technique has been shown to have advantages over the other techniques of genetic distance evaluation, like restriction mapping and DNA-DNA hybridization (4). In addition, fine melting has apparently a greater resolution compared to techniques like density gradient centrifugation and electron microscopy employed for studying distribution in base composition (9,14).

This chapter describes the studies carried out on denaturation characteristics and high resolution melting studies of DNAs of sponge gourd, ridge gourd, ash gourd
and ivy gourd, to determine the heterogeneity in base composition and also to assess the extent of similarities and differences among these four species of Cucurbitaceae.

MATERIALS AND METHODS

DNA sources and seed materials

Seeds of Luffa cylindrica (sponge gourd), Luffa acutangula (ridge gourd), Benincasa hispida (ash gourd) and Triticum aestivum (wheat) and fresh fruits of Coccinia indica (ivy gourd) were obtained locally. The source of seeds and the variety were kept constant in order to avoid qualitative differences, if any. The seeds were washed thoroughly with distilled water and treated with 0.001% mercuric chloride for 10 min. to reduce the bacterial contamination (17). Seeds were grown on wet cotton in dark under controlled conditions of temperature and humidity. About 6-8" long shoots (grown for 8-10 days) were cut and stored at -20°C till further use. In case of ivy gourd, fresh fruits were thoroughly washed and used for DNA extraction.

E. coli B NCIM 2089 was used for DNA extraction. Calf thymus DNA was obtained commercially.

Chemicals

All the chemicals used throughout the work were of Analytical Reagent (AR) or Guaranteed Reagent (GR) grade.
They were obtained from British Drug House (BDH), Sarabhai Chemicals or E. Merck. Fine chemicals/reagents such as RNase A, Tris (Tris-hydroxy methyl aminomethane) and agarose were obtained from Sigma Chemical Co., MO, USA. Molecular weight markers (λ DNA digested with Hind III and φ X 174 RF DNA digested with Hae III) and restriction enzymes were obtained from Boehringer Mannheim, West Germany or Bethesda Research Laboratories, USA.

**DNA isolation**

DNAs were extracted by a combination of procedures of Marmur (18) and Ranjekar et al. (19). Frozen plant tissue or fresh fruits cut to smaller pieces were homogenized in sucrose buffer (0.5 M sucrose, 0.05 M Tris, 0.05 M maleic acid, 0.003 M CaCl₂, pH 6.0, containing 0.1% Triton X-100) in a Remi blender at maximum speed for 1.5 min. The homogenate was filtered through two layers of cheese cloth or muslin cloth and the filtrate obtained was centrifuged at 1000 x g for 30 min. The crude nuclear pellet was resuspended in sucrose buffer and centrifuged again at 1000 x g for 20 min. This procedure was repeated twice. The washed 'nuclear' pellet was found to be composed of nuclei as well as a lot of cell debris, and was free of chloroplasts. The pellet was resuspended in saline EDTA (0.15 M NaCl, 0.1 M sodium salt of EDTA, pH 8.0) and the nuclei were lysed by the addition of sodium dodecyl
sulphate to a final concentration of 2% followed by incubation at 62°C for 30 min. with intermittent gentle shaking. The suspension was then cooled to 10°C and the proteins were denatured by adding sodium perchlorate to an effective concentration of 1M, followed by deproteinization with chloroform-isooamyl alcohol (24:1 v/v). The latter step was repeated till the interphase was totally absent. The aqueous layer was chilled and the nucleic acids were precipitated with 1.5 volumes of ethanol. The fibres were spooled out, dried, dissolved in 1 x SSC (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) and then incubated with RNase A (50 μg/ml) at 37°C for 1 h to remove the contaminating RNA. Prior to use, RNase was heated at 80°C for 10 min. to make it free from any DNase activity. The solution was then deproteinized with chloroform-isooamyl alcohol (24:1 v/v) and the DNA was precipitated with 1.5 volumes of ethanol and dissolved in 1 x SSC. About 5-8 mg of DNA was obtained from 1 kg tissue. Wheat DNA was also isolated according to this procedure. E. coli DNA was isolated according to Marmur's procedure (18).

Criteria of purity of DNA preparations

(i) All the DNA preparations were analysed routinely for RNA, protein and polysaccharide contamination by orcinol test (20), Lowry's assay (21) and paper chromatography of sugars obtained by hydrolysis of polysaccharides (22)
respectively. The DNA preparations had less than 1% contamination of RNA and proteins.

Procedure for polysaccharide estimation: 10-15 μg of DNA precipitate was dissolved in 1 N HCl and hydrolysed in a boiling water bath for 30-35 min. All operations were carried out in a 1.5 ml capacity Eppendorf microfuge vial. After hydrolysis, a pinch of animal charcoal was added and the vial was shaken. The solution was then centrifuged and the supernatent was discarded. Only sugars get adsorbed on charcoal. The adsorbed sugars were eluted from charcoal by suspending the charcoal pellet in ethanol and collecting the supernatent by centrifugation. The ethanol containing the sugars was allowed to evaporate at room temperature to reduce the volume to 5-10 μl. It was then chromatographed on Whatman 1 (ascending) in N-propanol:acetic acid:water (7:2:1) system. The chamber and the paper were saturated before the run. The chromatograph was air-dried and developed in the following sequence: i) dipping in saturated solution of silver nitrate in acetone and drying, ii) dipping in 0.5% NaOH in 90% ethanol and drying, iii) finally dipping in 0.5% sodium thiosulphate solution.

Calf thymus DNA treated similarly, and the sugars like glucose, ribose and deoxyribose were used as standards. The standard sugars gave dark brown spots, while the DNA preparations showed only deoxyribose sugar (Figure 2.1).
Fig. 2.1: Determination of polysaccharide contamination of DNAs by paper chromatography.

Lane a : D-glucose
Lane b : DNA of sponge gourd
Lane c : DNA of ash gourd
Lane d : D-ribose
(II) The wavelength scanning of the DNAs was carried out in the range of 220 to 300 nm. All the DNA preparations exhibited an optical density ratio $A_{280}/A_{260} = 0.55$ and $A_{230}/A_{260} = 0.45$ and had absorbance less than 0.100 at 300 nm. Figure 2.2 represents a typical absorption spectrum for the DNA preparations.

(III) The DNAs showed approximately 25% hyperchromicity after denaturation. The presence of sharp melting profiles and absence of a 'foot' or shallow absorbance rise before the actual start of melting is indicative of absence of single-stranded DNA and RNA (Figure 2.4) (23).

**DNA sizing**

Average size of the DNA preparations was determined by agarose slab gel electrophoresis using 1% agarose (Sigma) gels in TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.1). Electrophoresis was carried out at 50 mV. The gels were stained in ethidium bromide (0.5 μg/ml) in dark for 10 min., visualized on ultraviolet transilluminater and photographed using a Pentax ES II camera with a 50 mm lens. Hind III digest of λ DNA and Hae III digest of φ X 174 RF DNA were used as molecular weight markers. The DNA preparations showed a molecular weight greater than 10 kilo base pairs (Figure 2.3).
Figure 2.2: Absorption spectrum of sponge gourd DNA. DNA was dissolved in 1 x SSC and scanned in the wavelength range of 220 to 300 nm.
Fig. 2.3: Agarose slab gel electrophoresis of sonicated ivy gourd (lane a), sponge gourd (lane c) DNAs and unsonicated ash gourd (lane d) and ridge gourd (lane f) DNAs. ϕ X 174 RF DNA digested with Hae III (lane b, 1342, 1078, 872, 606, 310 base pairs) and λ DNA digested with Hind III (lane e, 23300, 9500, 6400, 4200, 2200, 1800 base pairs) were used as molecular weight markers.
Thermal denaturation

Thermal denaturation was carried out using a Gilford 250 spectrophotometer equipped with thermoprogrammer (Model 2527), analog multiplexer (Model 6046) and automatic reference compensator (19). Thermal cuvettes were filled with 0.3 ml DNA solution (25-50 µg/ml) in 0.12 M sodium phosphate buffer, pH 6.8, and the absorbance was recorded at room temperature. Cuvettes were filled in such a way that the air-space was less than 10% of the solution volume and evaporation was prevented using teflon stoppers. The temperature was then raised to 98°C at the heat rate of 1°C/min. and the absorbance change occurring during the heating process from 62°C to 98°C was monitored, either with the recorder (Model 6051) or programmed printer. The hyperchromicity of the DNA samples was calculated using the formula

\[ H = \frac{A_{260}(98°C) - A_{260}(62°C)}{A_{260}(98°C)} \]  

(24, 25)

where \( H \) is the hyperchromicity and \( A_{260} \) is the absorbance at 260 nm. The total hyperchromicity was normalized to 100% and the graph of percent hyperchromicity versus temperature was plotted. The melting temperature (Tm) of the DNAs was calculated as the temperature at which half of the total increase in absorbance is obtained. The G+C
content of DNAs was determined according to the formula

\[ G+C = (Tm - 69.3)^2.44 \]  

(1,26).

The transition interval (\( \Delta T \)) was calculated as

\[ T_b \degree C \ (83\% \ hyperchromicity) - T_a \degree C \ (17\% \ hyperchromicity) \]

(2,3).

DNAs from E.coli, calf thymus and wheat were used as standards for comparison in these studies.

**High resolution thermal denaturation analysis**

For such analysis, DNAs were denatured at a heat rate of 0.25\( \degree \)C/min. and absorbance values were recorded at 0.1\( \degree \)C increment, from 62\( \degree \)C to 98\( \degree \)C. Thus the experimental curve consists of 300-400 experimental points depending on the melting range of DNA. Noise levels were reduced with a large slit width (1 mm) together with a high initial DNA concentration (50 \( \mu \)g/ml). Data smoothing and differentiation were performed by fitting ten contiguous data points at a time to a simple polynomial by the least squares method. A linear function was used as a fitting polynomial (14).

The differential curves were obtained by plotting the temperature derivative of the absorbance of DNA against temperature (dA/dT vs T). To check the reproducibility of the procedure, at least five DNA preparations, each in duplicate, were melted and the resulting curves were analyzed separately. Fitting 10 points at a time, each indicating 0.1\( \degree \)C increment, represents a temperature rise of 1 \( \degree \)C, •
which is expected to resolve individual regions melting below and above the average Tm of the DNA. The limit of resolution of this analysis is 0.001 absorbance, and peaks separated by an interval as narrow as 0.4°C (approx. 1% G+C) could be detected reproducibly.

RESULTS

The melting data obtained by denaturing all the DNAs at 1.0°C increments are depicted in Figure 2.4 and summarized in Table 2.1. All the melting curves are smooth, sharp and monophasic. The Tm values range from 83.2 to 85.7°C. The G+C contents as calculated from the Tm values fall in the range of 34 to 40% as in most other higher plant species (12). The G+C contents of E. coli, calf thymus and wheat DNAs compare well with the reported values (3,8,9). The AT (transition interval), which is an indication of base composition heterogeneity in DNA, ranges from 6 to 8°C. This range is narrow to moderate as compared to that in some higher plants (3). The hyperchromaticities fall in the range of 25 to 27.5%.

The first derivative melting profiles reveal several details about the heterogeneity in distribution of components differing in base composition (Figure 2.5). The derivative profiles of DNAs of E. coli, calf thymus and wheat were also obtained under identical conditions and found to compare well with the reported data (3,8,9). All the four Cucurbitaceae plant species show 17-22 melting components
Figure 2.4: Thermal denaturation profiles of Cucurbitaceae DNAs. High molecular weight DNAs (>10 kilo base pairs) in 0.12 M sodium phosphate buffer (pH 6.8) were melted in a Gilford 250 spectrophotometer at a heat rate of 1°C/min, and absorbance values were recorded at an interval of 1°C.
Table 2.1 Melting data of native unsheared DNAs

<table>
<thead>
<tr>
<th>Species</th>
<th>T_m (°C)</th>
<th>G+C content (%)</th>
<th>Hypochromicity (%)</th>
<th>ΔT °C (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>90.83±0.96</td>
<td>52.54±2.34</td>
<td>21.30±0.81</td>
<td>4.60±0.23</td>
</tr>
<tr>
<td>(6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calf thymus</td>
<td>86.18±0.41</td>
<td>41.19±1.00</td>
<td>24.89±1.68</td>
<td>7.36±0.33</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>85.56±0.12</td>
<td>39.69±0.28</td>
<td>24.34±0.29</td>
<td>10.67±0.23</td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sponge gourd</td>
<td>84.39±0.23</td>
<td>36.83±0.56</td>
<td>28.82±0.78</td>
<td>7.16±0.60</td>
</tr>
<tr>
<td>(7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ridge gourd</td>
<td>85.67±0.23</td>
<td>39.94±0.56</td>
<td>27.56±0.41</td>
<td>7.53±0.61</td>
</tr>
<tr>
<td>(6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ash gourd</td>
<td>84.21±0.46</td>
<td>36.39±1.13</td>
<td>25.27±1.07</td>
<td>6.39±0.44</td>
</tr>
<tr>
<td>(7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ivy gourd</td>
<td>83.24±0.46</td>
<td>34.01±1.12</td>
<td>26.57±1.02</td>
<td>7.96±0.39</td>
</tr>
<tr>
<td>(8)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Figures in parentheses indicate the number of experiments carried out in each case.
Fig. 2.5: First derivative melting profiles of unsheared DNAs of four Cucurbitaceae species and of *E. coli*, calf thymus and wheat. All the DNA melting experiments were carried out in 0.12 M sodium phosphate buffer (pH 6.8). The DNA concentrations used were 50-60 μg/ml. All the DNAs were denatured at a heat rate of 0.25°C/min. and the absorbance values were recorded at 0.1°C increments.
Figure 2.5

The diagram illustrates the temperature sensitivity of various samples:

- **E. coli**
- **Calf thymus**
- **Wheat**
- **Sponge gourd**
- **Ridge gourd**
- **Ash gourd**
- **Ivy gourd**

The x-axis represents temperature in °C, ranging from 72 to 96.
which can be arbitrarily divided into three groups. The first group includes maxima peaks at the Tm and peaks that are in the range of ± 1°C on either side of the Tm. The second group comprises early melting, A+T rich components, upto the melting maxima, while the third group represents late melting, G+C rich components melting at temperatures higher than the melting maxima.

In all the species, two to three maxima peaks which coincide with the mean melting temperature are observed. While the melting starts from 74-76°C in sponge gourd, ridge gourd and ash gourd, in ivy gourd it starts much earlier (72°C), and two very early melting components (72.5°C, 73.0°C) are observed. All the species show 7 to 9 A+T rich components and 7 to 8 G+C rich components. Thermal denaturation is complete by 95°C in sponge gourd, ash gourd and ivy gourd but continues upto 97°C in ridge gourd.

The derivative melting profiles of sponge gourd and ridge gourd are very similar and are skewed on G+C rich side, with distinct peaks between 87.5 and 88.0°C. The melting profile of ridge gourd, however, can be distinguished by its high melting component at 96.6°C. The fine melting profiles of ash gourd and ivy gourd are symmetrical, with apparently equal distribution of A+T rich and G+C rich components on either side of the melting maxima. The profile of ivy gourd, however, is distinguished by the presence of the two early melting components.
In order to determine the number of components that are shared among the four species, the positions of all the components are indicated in Figure 2.6. A temperature range of 0 to 0.6°C was considered to estimate the number of shared components and these data are summarized in Table 2.2. In the temperature range of 0.6°C (1.5% G+C), 12 melting components are shared by all the four species, six each on the A+T rich and G+C rich sides of the melting profiles. The number of common melting peaks is reduced to 11, 10, 7, 5 and 0 as the range for comparison is reduced to 0.5°C, 0.4°C, 0.3°C, 0.2°C and 0.1°C temperature difference (1.25%, 1.00%, 0.75%, 0.5% and 0.25% G+C, respectively). In the two Luffa species, fourteen out of seventeen components are common at a temperature difference of 0.6°C. Out of these, as many as eight are common even at 0.1°C difference and three appear at identical positions.

**DISCUSSION**

Since fine melting analysis is a very sensitive method, several precautions were taken to avoid artifacts. High molecular weight DNA preparations (molecular weight > 10 kilo base pairs), free from detectable levels of polysaccharides, RNA and single stranded DNA were used. Experimental conditions such as salt concentration (0.18M Na+), and DNA concentrations (50-60 μg/ml) were
Figure 2.6: The positions of melting components observed in the derivative profiles of four Cucurbitaceae species, shown as light lines. The bold lines indicate the components that are shared by all the four species within a temperature range of 0.6°C.
Table 2.2 The number of melting components shared by the four Cucurbitaceae species

<table>
<thead>
<tr>
<th>Temperature range (°C)</th>
<th>0.6</th>
<th>0.5</th>
<th>0.4</th>
<th>0.3</th>
<th>0.2</th>
<th>0.1</th>
<th>0.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>G+C content range (%)</td>
<td>1.46</td>
<td>1.22</td>
<td>0.98</td>
<td>0.73</td>
<td>0.49</td>
<td>0.24</td>
<td>0.00</td>
</tr>
<tr>
<td>Number of components shared by all the four species</td>
<td>12</td>
<td>11</td>
<td>10</td>
<td>7</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of components shared by two <em>Luffa</em> species</td>
<td>14</td>
<td>13</td>
<td>13</td>
<td>11</td>
<td>10</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>
kept constant. The number and position of all the components in every DNA preparation were reproducible in the case of all the DNAs.

From Figure 2.5, it is clear that each species shows a polyphasic derivative melting profile, and distinct species-specific differences are observed. The appearance of polyphasic melting profiles in eukaryotic DNAs is considered to be the effect of satellite and repetitive families. The bacteriophage DNAs are made of unique sequences and show the presence of 'thermalities' melting in very narrow intervals (9). However, as the genome size increases, the overlap between the neighbouring sequences increases and this results in smooth, monophasic melting observed in the bacterial DNAs, which are also composed of only unique sequences (8). This should also be true for the higher plants and animals, if their genomes are to consist of only unique sequences. Thus the multiphasic melting profiles of higher organisms could be arising from the different repetitive sequences in their genomes. Each repetitive family in general should have a distinct base composition, and behave as a 'block' in the DNA melting (9). The technique thus allows identification of major blocks of repetitive DNA, even if they are interspersed with other DNA sequences, and therefore not detectable in cesium chloride gradients. The satellite sequences studied so far
in Cucurbitaceae are G+C rich (27,28). The present four species do not show any such distinct high melting component. However, our work on other species of Cucurbitaceae has shown that satellite sequences can be resolved very effectively (29). Considering that the present technique allows the qualitative determination of repetitive families (by their melting temperatures), it appears that the repeated sequences of the two Luffa species, sponge gourd and ridge gourd, are very similar. On the other hand, they are distinctly different from those of ash gourd and ivy gourd.

It is necessary to further complement this qualitative analysis by quantitative studies. Attempts were made to resolve these derivative profiles into a number of Gaussian components, to compare the areas under each, and to determine the proportion of different repetitive families in the genomes. However, the extremely high number of melting maxima, and the tremendous overlap of their areas has made it impossible to develop a suitable programme for such resolution. Personal correspondence with Dr. R.D. Blake (Department of Biochemistry, University of Maine, Orono), Dr. C.E. Cuellar (Carnegie Institution of Washington, Stanford, California) and Dr. A. Wada (Department of Physics, University of Tokyo, Japan) has indicated that it is possible to resolve derivative
profiles with 5-9 melting maxima fairly well; however, it is not possible to resolve the profiles of higher organisms characterized by numerous peaks satisfactorily. Attempts are being made at present to overlap the profiles and calculate the area under the curves that is shared and not shared by the species, and thus derive the genetic distance between them.
REFERENCES


