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Phylogeny of *Cucumis* based on isozyme variability and its comparison with plastome phylogeny *

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Summary. An electrophoretic comparison of 29 nuclear-coded enzymes was carried out for 21 *Cucumis* species, and a phylogeny based on pairwise measurements of the respective genetic distances was computed. This phylogeny was compared to the one based on chlDNA cariation (Perl-Treves and Galun 1985). The two phylogenies were found to share the main dendrogram features; they also agree well with most taxonomic data available on *Cucumis*. Accordingly, most of the African *Cucumis* species form a close group ("Anguria group" – "Group A"), which is distant from the melon (*C. melo*), and from a few other distinct species, all of which are far apart from each other. The cucumber (*C. sativus*) is the most distant species within the genus. Some specific taxonomic implications as well as some general evolutionary problems related to such a parallel investigation of the nuclear genome and the plastome are evaluated.

Key words: Isozymes – Phylogeny – *Cucumis* – Chloroplast DNA – Dendrogram

Introduction

Traditional approaches in taxonomy and evolution are based on morphological traits, sexual crossability and cytogenetic considerations. These approaches are now being complemented by molecular methods in order to trace genetic diversification in a more direct manner. Such biochemical studies mainly compare proteins and DNAs to infer phylogenetic relationships between organisms. Biochemical techniques used to measure differences between taxons are (Thorpe 1982): protein sequencing, DNA sequencing, reannealing of DNAs from different species, immunological response to analogous proteins between different species, and electrophoresis of isozymes. Enzyme-electrophoresis has the advantage of sampling many loci and detecting different alleles; it is suitable for determining distances within and between species and has became an important taxonomic tool – in addition to its application in population genetics and breeding (Gottlieb 1981).

We previously presented a plastome phylogeny of the genus *Cucumis* based on the study of chloroplast DNA (chlDNA) variation (Perl-Treves and Galun 1985). In the present article, we based the phylogeny of *Cucumis* on the study of nuclear-coded traits: isozymes representing at least 29 loci. Furthermore, the phylogenies of these two genomes are compared and evolutionary implications of such parallel studies are evaluated.

Materials and methods

Plant material

Table 1 lists the cultivated varieties and wild *Cucumis* species used in this study. Additional information and photographs of the wild species fruits are provided in Table 1 and Fig. 1, respectively, of Perl-Preves and Galun (1985). Isozyme analysis was performed on the same 21 *Cucumis* species and 6 *C. melo* varieties analyzed for chlDNA, except for species 14, 24 and 28. A second accession from species 27, 40, 4 and 41 (Table 1) was included, hence this study comprises a total of 27 "populations" (species, accessions and varieties).

Isozyme extraction

Plants were grown in the greenhouse and small (2 cm^2) leaf samples were taken from young individual plants. Crude

* This publication is dedicated to Dr. T. W. Whitaker, with appreciation to his many contributions to genetics and taxonomy of *Cucurbitaceae*

Table 1. List of *Cucumis* species and varieties used or referred to, in this study

A Wild species

Name	Assigned code	Name	Assigned code
C. leptodermis (1)	41	*C. pustulatus*	24
C. leptodermis (2)	21	*C. dinteri*	28
C. dipsaceus (Ecuador)	27	*C. meeusei*	7
C. dipsaceus (Davir)	2	*C. africanus*	14
C. prophetarum	11	*C. figarei*	9
C. ficifolius	6	*C. anguria* (1)	4
C. melo var 'agrestis'	8	*C. anguria* (2)	5
C. metuliferus	3	*C. myriocarpus*	10
C. sagittatus	35	*C. zeyheri* (1)	12
C. heptadactylus	34	*C. zeyheri* (2)	40
C. humifructus	32	*C. hookeri*	43
C. longipes	33	*C. hardwickii*	CH

B Cultivated varieties

Name	Assigned code
C. melo cv 'Yokneam'	MY
C. melo cv 'Tzahov-Yetzu' (Cassaba)	MC
C. melo cv 'Hales-Best' (Cantaloupe)	MH
C. melo var 'Flexuosus'	MF
C. melo cv 'Monoecious Rondo'	MR
C. sativus cv 'Shimshon'	CS

Note: For the source of seeds, see Table 1 of Perl-Treves and Galun (1985). In addition, accessions 2, 5 and 12 are from source 3, and accession 21 is from source 2

extraction was performed on ice by grinding the leaf samples with 120 µl of the following extraction buffers:

1) 0.1 M Tris-HCl (pH=7.5), 1.5% reduced glutathione, 12% PVP-40 – for systems 1 and 2.
2) 0.2 M KH_2PO_4, 14 mM 2-mercaptoethanol and 6% PVP-40, pH=7.5 (adjusted with KOH) for system 3.

A wick of Whatman 3 filter paper was absorbed with the extract and loaded on the gel (25 wicks/gel).

Electrophoretic systems

Starch gels were prepared and run by using one of the following three systems, which differed in their gel-buffer and running-buffer.

1) Tris Citrate pH=8.1 according to Tanksley (1979). The following enzymes were run in this system: Glutamic-oxaloacetic transaminase (GOT), Catalase (CAT), Glutamate dehydrogenase (GDH), Fructose 1, 6 diphosphatase (FDP).

2) Tris Citrate pH=7.7. This system is equal to system (1) but for its pH. The enzymes run in this system were: Diaphorase (DIA), Superoxide dismutase (SOD) Acid phosphatase (APS), Peroxidase (PRX), Esterase (EST), Triosephosphate isomerase (TPI).

3) Histidine HCl ("system 2" in Zamir and Ladizinsky 1984). Enzymes run in this system were: Phosphoglucoisomerase (PGI), Malate dehydrogenase (MDH), Phosphoglucomutase (PGM), Isocitrate dehydrogenase (IDH), 6-phosphoglucanate dehydrogenase (6PGD), Aconitase (ACO), Shikimic dehydrogenase (SKDH), Leucine aminopeptidase (LAP).

Gels were run at 3 °C for 6 h at 200 V for systems (1) and (2), and at 100 V for system (3). After the run, each gel was sliced horizontally into four slices, enabling each one to be stained for a different enzyme system. Staining methods were essentially those of Vallejos (1983).

Procedure of analysis

As a preparative stage, one representative sample of each species/accession was run on a "master gel" for a preliminary investigation of the amount of variability between species, and to properly choose the electrophoretic system. At a later stage, 1 to 12 individuals were sampled from each accession and run along with a "master gel" for comparison. Finally, "master gels" with two representatives of each accession were run for a final verification of the isozyme patterns. Only those isozymes that could be scored unequivocally were included in the present analysis. Loci are designated as 1 – the farther-most migrating (most anodal locus), 2, 3 etc. Allele (+) was assigned to the one present in *C. melo* cv 'Yokneam' (MY) and other alleles received numeral designations. The assays for PRX, EST and TPI produced complex zymograms which were scored for their overall banding pattern only.

The genetic distance (D) between any two populations and its standard error were computed according to Nei (1972, 1974), using a Fortran program provided by him. Cluster analysis was performed by a BMDP program, P1M, using the D values obtained previously (Hartigan 1979). Three clustering procedures, differing in the order ("rule") used to form clusters were tried, to evaluate some of the errors contributed by the computer procedure.

Results

Isozyme patterns in Cucumis

Eighteen enzyme systems, representing at least 29 loci were assayed, only one of them – Catalase – was monomorphic; all others had 2 to 8 alleles (Table 2). Figures 1 and 2 show examples of zymograms obtained for PGI and ACO, respectively. Very little variation was found within a given population, (whether species, accession or variety). Only in 12 out of 783 cases (loci × populations) was there polymorphism.

Intra- and interspecific D values

The large number of loci assayed and the low intra-population variability account for the small standard errors (SE) in the genetic distance (D) even when only a small number of plants were sampled. Only with *C. figarei* (9) did a combination of small sample and variability in 3 loci make the SE rather large, otherwise it was between 0.03 and 0.10.

The distances calculated (data not shown) between every possible pair of the populations ranged between zero and 1.97. CS and CH are the closest, (D=0), not even one difference was found between them. Pairs of conspecific accessions did show some differences:

Table 2. Distribution of alleles of 29 enzyme loci among *Cucumis* species. Full enzyme names appear in "Materials and methods". Accession name" is the code/number of species, variety or accession. The + allele is the one found in species MY. "No" is the number of individuals assayed from a given "population"; "p" stands for "polymorphic" in the cases where different individuals had different alleles, and the allele frequencies are given below. When all tested plants were heterozygous, it was indicated in the table as 2/3, 3/1, 1/2, or +/4, (and the allele frequencies were 0.5 for both alleles)

Acc. Name	PGI 2	MDH 1	MDH 2	PGM 1	PGM 2	IDH 1	PGD 1	PGD 2	ACO 1	ACO 2	SKD 1	SKD 2	LAP	DIA 1	DIA 2	DIA 4	TPI 1	SOD 1	SOD 2	PRX	APS 2	EST	FDP	GDH 1	GOT 1	GOT 2	GOT 3	GOT 4	CAT	No
CS	1	+	1	1	2	1	p	+	1	2	5	4	2	5	1	1	1	+	1	1	+	1	2	1	2	1	3	+	+	6
CH	1	+	1	1	2	1	p	+	1	2	5	4	2	5	1	1	1	+	1	1	+	1	2	1	2	1	3	+	+	6
MY	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	6
MC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1	+	+	+	+	+	+	6
MR	+	+	+	3	+	+	+	+	+	+	+	+	1	+	+	+	+	+	+	+	+	+	1	+	+	+	+	+	+	1
MH	+	+	+	3	+	+	+	+	+	1	2	2	+	+	+	+	+	+	+	1	+	+	1	+	+	+	+	+	+	6
MF	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	2	+	+	+	+	+	+	6
8	+	+	+	+	+	+	+	+	+	1	+	+	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	6
3	1	1	1	+	3	2	1	1	+	5	3	3	2	3	+	2	2	2	+	2	1	2	3	+	+	+	3	+	+	6
32	2	+	+	7	1	+	+	2	2	3	3	1	+	1	+	+	+	1	+	13	+	16	4	+	+	2	3	1	+	2
35	5	+	+	5	3	+	1	4	2	4	3	3	2	1	+	+	+	2	+	14	2	15	2	+	+	+	+	+	+	2
11	4	+	2	6	3	+	2	2	2	4	4	3	2	4	2	3	+	1	+	12	+	14	3	+	1	1	3	+	+	5
33	2	+	+	3	3	+	2	2	2	3	4	3	1	+	2	3	+	1	+	4	3	13	3	+	1	1	3	+	+	12
4	3	+	+	+	3	+	2	2	2	3	4	3	1	+	2	3	+	1	+	6	1	7	3	+	1	1	1	+	+	7
5	3	+	+	+	3	+	2	2	2	3	4	3	1	+	2	3	+	1	+	6	3	8	3	+	1	1	1	+	+	7
41	3	+	+	5	3	+	2	2	2	2	5	3	3	2	2	3	+	1	+	7	2	6	3	+	1	1	3	+	+	7
21	3	+	+	5	3	+	2	2	2	3	5	3	3	2	2	3	+	1	+	10	2	6	3	+	1	1	3	+	+	8
2	3	+	+	4	3	+	2	2	2	4	3	3	2	5	2	3	+	1	+	6	+	5	3	+	1	1	3	+	+	11
27	3	+	+	5	3	+	2	2	2	4	5	3	2	4	2	3	+	1	+	11	2	5	3	+	1	1	2	+	+	10
40	3	+	+	2	3	+	2	2	p	3	3	3	2	+	2	3	+	1	+	4	2	5	3	+	1	1	3	+	+	7
12	3	+	+	2	3	+	2	2	2	3	4	3	3	+	2	3	+	1	+	4	2	4	3	+	1	1	3	+	+	4
9	2/3	+	+	4	4	+	2	2	1/2	2	4	3	2	+/4	2	3	+	1	+	3	1	3	3	+	1	1	2	+	+	3
10	3	+	+	5	3	+	2	2	2	3	5	3	2	2	2	3	+	1	+	4	2	6	3	+	1	1	3	+	+	3
43	+	+	+	+	p	+	2	2	2	3	5	3	3	2	2	3	+	1	+	5	2	9	3	+	1	1	3	+	+	7
7	3	+	+	+	3/1	+	2	2	3	4	3	3	2	+	2	3	+	1	+	8	2	10	3	+	1	1	2	+	+	6
6	2	+	+	4	p	+	2	p	2/3	2	3	3	2	4	2	3	+	1	+	8	1	11	3	+	1	1	2	+	+	10
34	p	+	+	4	3	+	2	2	2	3	3	3	2	+	2	3	+	2	+	9	2	12	3	+	1	1	1	+	+	10

Cases of polymorphism

Locus	Sample	Frequencies (allele)
PGI-2	34	0.3 (2), 0.7 (3)
PGM-2	43	0.21 (1), 0.79 (3)
	6	0.14 (1), 0.86 (4)
PGD-1	CS	0.17 (+), 0.83 (1)
	CH	0.17 (+), 0.83 (1)
PGD-2	6	0.6 (2), 0.4 (3)
ACO-1	40	0,57 (2), 0.43 (3)

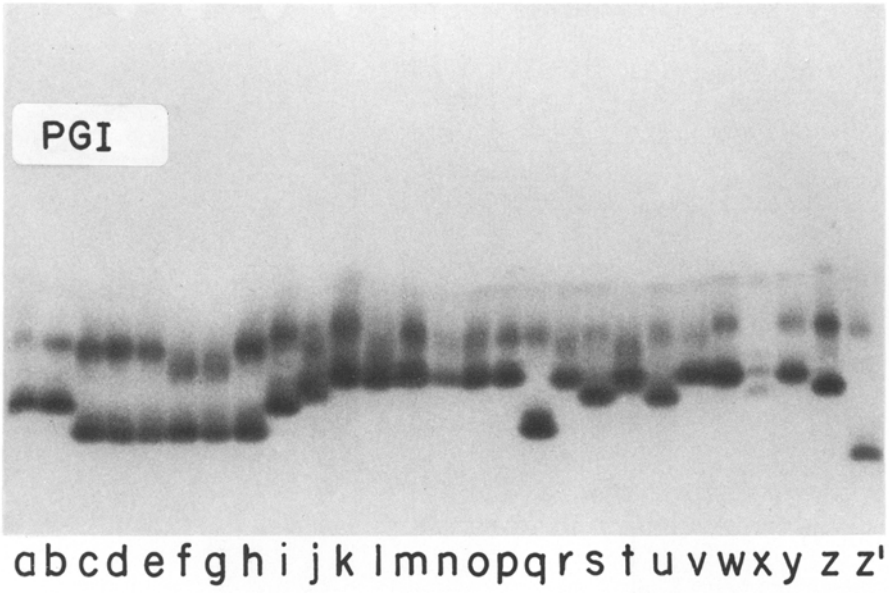

Fig. 1. Isozyme patterns of phosphoglucose isomerase (PGI). A representative of a different population was run in every slot. These are (from *left to right*): CS, CH, 8, MY, MC, MR, MH, MF, 3, 9, 12, 40, 10, 4, 5, 41, 43, 7, 6, 34, 33, 21, 27, 11, 2, 32, 35. Gels were run towards the anode (*upwards*). Only the lower locus was read. Allele +: lanes *c-h, q*; allele 1: lanes *a,b,i*; allele 2: lanes *s,u,z*; allele 3: *k-p, r,v, w,y*; heterozygous 2/3; *j,t*; allele 4: lane *x*; allele 5: *z'*

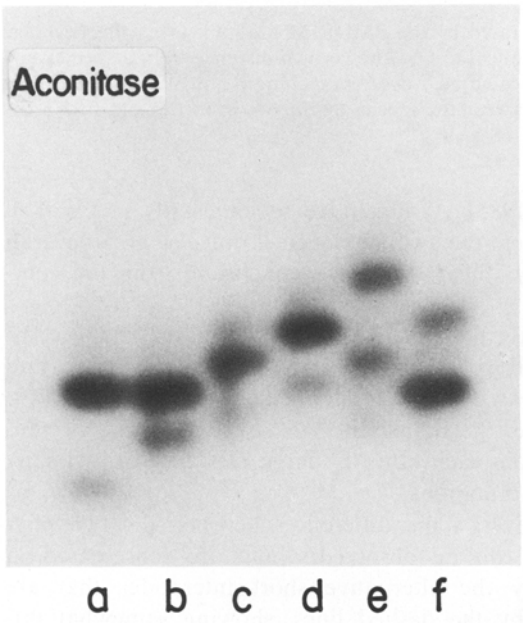

Fig. 2. Different isozymes of Aconitase in *Cucumis*. A representative of every pattern was loaded on the gel. Two loci were read: a dark staining (ACO-1) and a lighter-staining one (ACO-2). Except for lane *f*, ACO-1 is the anodal isozyme. ACO-1: Allele +: lanes *a,b,f*; allele 1: lane *c*; allele 2: *d*; allele 3: *e*. ACO-2: Allele +: lane *a*; allele 1: *b*; allele 2: *c*; allele 3: *d*; allele 4: *e*; allele 5: *f*

40/12, D=0.12; 27/2, D=0.23; 4/5 D=0.07; 41/21, D=0.07. There are, however, within "Group A" smaller values – between different species: e.g. 21/10, D=0.003; 2/10 and 27/10, D=0.16. "Group A" includes the following species: 4, 5, 33, 11, 41, 21, 10, 27, 2, 6, 34, 43, 40, 12, 24, 7, 14, 9.

Distances between different *C. melo* varieties ranged between 0.03 and 0.23, the cantaloupe type (MH) having the largest D values. Distances within the "Group A" ranged between zero and 0.50. All other distances were larger.

Phylogenetic tree

Figure 3 shows the tree that results from the clustering procedure. When trying three clustering rules (see "Materials and methods") the basic features of the tree remained the same: "Group A" formed a distinct cluster, all distances to other species are notably bigger than those within it. All *C. melo* varieties formed a tight cluster. CS (representing both cucumber and *C. hardwickii* in the scheme) is the most distant group, having large D values with all the other species.

The other three species – *C. sagittatus* (35), *C. humifructus* (32) and *C. metuliferus* (3) do not cluster tightly with any other species. Their position on the tree varied according to the procedure used (showing that the alternatives are not very different). Although 32 and 35 have a somewhat short D (0.73), distances with other groups are not much larger. These two species should, therefore, be taken as separate branches, considerably closer to "Group A" and MY and to each other, than to CS.

The branching order within "Group A" is also, in part, subject to changes depending on the clustering rule, as some of the D values between inner clusters are very similar to each other. Rooting this tree was unambiguous because cucumber behaved clearly as an outgroup, thus allowing the root to be placed on the branch between it and the rest of the species.

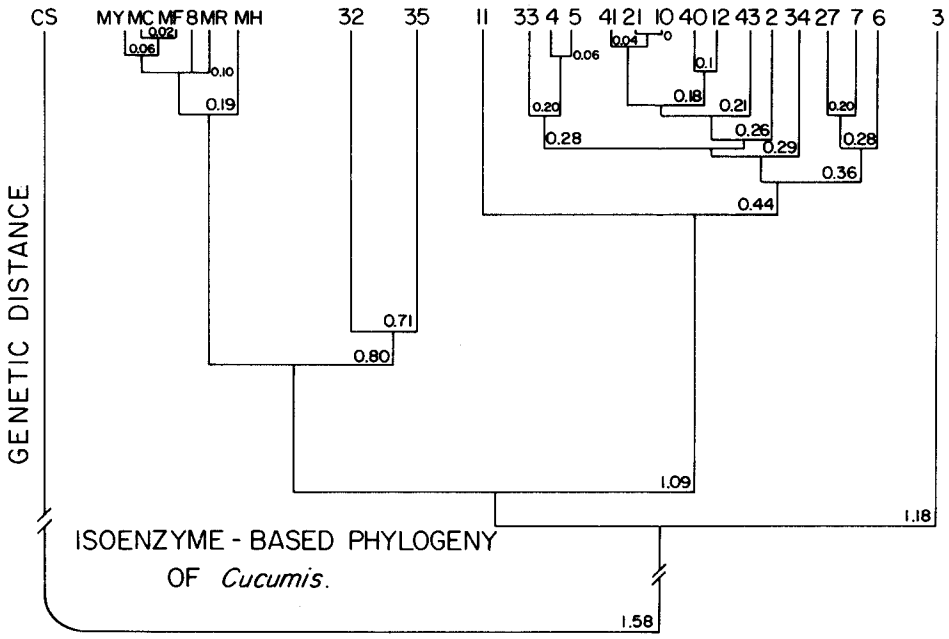

Fig. 3. *Cucumis* phylogenetic tree based on isozyme data. The tree was generated by the BMDP1M program using the "average distance" procedure. Species are denoted by their code/number; CH was identical to CS. The vertical distance from a species to a branching point is proportional to D, the Genetic Distance. D values are indicated near every branching point. When a branching point joins more than two species, D is represented as the average of all D's between the species on the two branches, e.g. 0.18 is the average of the D values of 40 and 10, 40–21, 40–41, 12–10, 12–21 and 12–41

Discussion

Isozyme phylogeny: sources of error, and comparison with other studies in Cucumis

Dane (1983) reported on a study of Esquinas-Alcazar of six enzyme systems (all present in our study) in a few wild *Cucumis* species. His interpretations as to the number of loci per enzyme are similar to ours. As in our study, differences between accessions of the same species were found, but populations were uniform. His D values and phylogenetic conclusions agree with ours.

Other workers (Puchalski 1978; Staub et al. 1984) presented data based on either few enzymes or few species. Some of the latter's specific results do not agree with ours with respect to polymorphism encountered in certain enzyme systems between species of "Group A". Some of the discrepancies between the different results can be explained by the intraspecific variation encountered between accessions of the same species. This is probably a major source of error in an isozyme study unless many accessions are checked for each species. The sampling of one accession may well account for having sometimes a larger D between conspecific accessions than between two close species.

Another source of error is in the choice, or sampling, of enzymes for the analysis. Different proteins evolve with different rates, and if more variable or more conservative proteins are sampled the D values will be different. Including many loci in the analysis reduces the problem.

Comparison of isozyme and chlDNA phylogenies

The rootless tree resulting from chlDNA restriction pattern analysis is shown in Fig. 8 of Perl-Treves and Galun (1985). As illustrated schematically in Fig. 4 of this paper, the two phylogenies have a great overall similarity; this is the main conclusion from the comparison.

Exactly the same species are included in the compact "Group A" in both phylogenies; cucumber and *C. hardwickii* are also identical in both phylogenies; otherwise, the branches of the trees are well differentiated from each other by large D values or by many chlDNA mutations.

However, some differences between the two phylogenies can be observed; these are represented in Fig. 4 by the alternative short internodes that are formed by the dashed lines, showing somewhat different relationships between main branches.

In the isozyme tree, cucumber (CS) is relatively more distant from all other *Cucumis* species than in the chlDNA tree, where it has two mutations in common with the melon, MY (indeed also in the D values, CS is

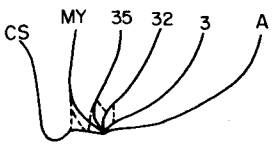

Fig. 4. Main features common to both chlDNA and isozyme phylogenetic trees

somewhat closer to MY than to "Group A"). Whether the two mutations define a common ancestor to MY and CS plastomes in contrast to 35, 32, 3 and A depends on the root chosen. However, the two phylogenies will agree better if rooted with CS as outgroup, taking into account the many less-characterized mutations specific to it. Another difference is the position of species 3 (*C. metuliferus*) and 32 (*C. humifructus*). They share at least 3 chlDNA mutations, suggesting a closer relationship between them than with any other species; the isozymic D value between them is, however, very large (D = 1.4), 32 being closer to any other branch (except CS) than to 3.

Other differences between the trees can be seen when the shorter branches within Group A and the melon cluster are compared. The isozyme method is more sensitive to differences over short phylogenetic distances. It differentiated between accessions of the same wild species, and between melon cultivars, while their chlDNA, when compared, was equal.

As for the branching order, the chlDNA tree groups the five cultivated melon types apart from 8, the wild variety. In the isozyme phylogeny, MH is the most distal and 8 in the middle of the group.

Similar differences in branching order can be observed in "Group A", but no firm conclusions can be drawn from them because both too few chlDNA mutations were found and the D values did not differ enough as to suggest a consistent branching order. If one wants to resolve such a group, more restriction sites, preferably having 4 base pairs-sites, should be assayed.

Whether all those differences should be ragarded as biologically significant phenomena is debatable. The differences between the phylogenies derived by us could be due to the different sensitivities and different errors involved in the two methods, while the true evolutionary tree, i.e. the sequence of branching events, was the same for plastome and nucleus. It could be, however, that the trees differ in some features because the plastome and nuclear genome did evolve differently in some cases. Two reasons may account for such a difference: 1. Introgression, or outcross, in some stage of the evolution (see Avise and Saunders 1984 for mitochondrial and nuclear DNA in sunfish hybrids): because of the different mode of inheritance of the plastome (maternal; complete linkage) the effect of the outcross on the plastome and on the nuclear genome may be different: two species with distinct nuclear genomes could thus share similar plastomes, or vice versa. If such an event occurred in the remote past only part of the resemblance in the plastone would be recognized today. 2. Different rates of evolution for different lineages on either tree: this would result in the two trees (derived by assuming equal rates) being different or "twisted" with respect to each other. In our case the differences between the two trees are not large enough to support either of these specific interpretations.

Comparison with existent phylogenetic studies in Cucumis

Cucurbit breeders have attempted to cross different *Cucumis* species in order to introgress new genetic variability into melon and cucumber. Some of the species in "Group A" are intercrossable and the resulting F1 progenies are fully fertile. On this basis *C. anguria,* 4, semi-domestic in the West-Indies, is considered conspecific with the african *C. longipes,* 33, presumably being its wild progenitor (Dane et al. 1980), in spite of their distinct morphologies. They share, in our study, identical chlDNA patterns and relatively small D values, showing that morphological criteria should be regarded with caution in this variable genus.

C. myriocarpus, 10, and *C. leptodermis,* 41, produce fully fertile F1 progenies (Dane et al. 1980; Deakin et al. 1971; Kroon et al. 1979; Kho et al. 1980). They share some chlDNA resemblance and look quite similar. On the isozyme level they appear as the same species. Dane et al. (1980) reported a successful cross between *C. africanus* and *C. myriocarpus* (fertile F1), but seedling death of F1 was described after a similar cross by Kho et al. (1980). Other crosses within "Group A" were done, but with reduced or no fertility in the F1. *C. figarei,* 9, a hexaploid, is cross-compatible with many species of "Group A". Its chlDNA appeared in our study (Perl-Treves and Galun 1985) as being identical to that of diploid *C. ficifolius,* 6, which could therefore be its maternal progenitor.

The morphologically similar *C. dinteri,* 28, and *C. sagittatus,* 35, are interfertile and considered conspecific (Dane et al. 1980; Deakin et al. 1971). We compared them only at the ChlDNA level and they were identical.

C. sativus, CS, and *C. hardwickii,* CH, are known to be interfertile. In our study they shared not only identical chlDNA patterns, but isozyme patterns as well (being the closest pair compared). They should be regarded as varieties; the second may be a wild escape.

No successful crosses were reported by the above (or other) authors between Group A and *C. metuliferus,* 3, *C. sagittatus* (35) or *C. dinteri* (28), *C. humifructus,* 32, (all of them being morphologically distinct entities), the melon and the cucumber in any combination, despite continuous efforts. Our studies thus support the view that *C. melo* and *C. sativus* are isolated species, not having close relatives among the wild species checked. Our phylogenies are in good agreement with the crossing data.

Evolutionary implications of the study

The "molecular clock" hypothesis (Thorpe 1982; Wilson et al. 1977) claims that sequence mutations occur, on the average, at fixed rates. This provides the basis for the derivation, from biochemical data, or the absolute or relative time of the branching events that form the tree. The clock theory, although very popular, is still criticized by a few researchers (e.g. Radinski 1978; see also Wilson et al. 1977).

The fact that the two independently derived phylogenies of *Cucumis* share the same main features seems to support the clock hypothesis – in the sense that, if there was no constant rate at all, the trees would most likely be different. It is still possible that the rates were variable between species and periods but the

same pattern of rate variation occurred both in respect to chlDNA and isozymes.

Another test, often employed to show similar rates, is to compare relative distances between different branches in the same tree. When comparing the chlDNA distances between different members in "Group A" and CS, MY or 35, (for 32 and especially 3 more non-identified mutations may exist), no strong deviation from similar distances is encountered (such as a species in "Group A" having 4–5 specific mutations, or a lonely branch like 35 having only 1–2). As for isozyme D values, comparison over different branches gives usually consistent results except for some particular cases. Hence, species 32 and 35 are considerably closer to each other and to A and MY groups (D around 0.8) than A and MY to each other (D = 1.2). No parsimonious tree, under constant-rate assumption, can account for such a phenomenon. But, if "Group A" and MY had faster evolution than the 35 and 32 lineages, such D values would be explained. Assuming slow or faster rates for any branch should yield, however, consistent results: e.g., the D value between 3 and 32 should again be smaller than 3/MY and 3/A, which is not the case. A second explanation could be some outcrossings between 32 and 35 to each other and to MY and "Group A", but not between "Group A" and MY. Such phenomena (and a few others observed) are possibly the compound result of errors in measuring D, of variation in the evolution rate, and of outcrossing events.

A second question regards the meaning and the relatedness of biochemical evolution to "real" organismal evolution. In our case the data gathered from crosses, cytology and even morphology fits well with the biochemical phylogenies. When this is true, the tree reflects the actual amount of divergence on the organismal level. Experimental results do not confirm this as a general rule; cases are known of slow morphological evolution (e.g. some amphibians) or extremely fast (most birds), but they nevertheless have ordinary protein evolution rates (Wilson et al. 1977; Thorpe 1982). In such cases the biochemical data would be meaningful only if attributed a clock property.

The agreement between biochemical and taxonomic data does not necessarily imply that such mutations were the cause of the divergence between species. The events leading to speciation could be completely different; e.g. geographical isolation or chromosome rearrangement resulting in accumulation of biochemical mutations.

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